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In vitro and *in vivo* differential expression of rainbow trout (*Oncorhynchus mykiss*) Mx isoforms in response to viral haemorrhagic septicaemia virus (VHSV) G gene, poly I:C and VHSV

C. Tafalla [a], V. Chico [b], L. Pérez [b], J.M. Coll [c], A. Estepa [b,*]

[a] *CISA-INIA. Carretera de Algete a El Casar km. 8,1, Valdeolmos, 28130 Madrid, Spain*
[b] *IBMC, Universidad Miguel Hernández, 03202 Elche, Alicante, Spain*
[c] *SGIT, INIA, Biotecnología, Carretera de La Coruña km. 7, 28040 Madrid, Spain*

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Abstract

Three different Mx isoforms are known to be present in rainbow trout, however, to date, neither their mechanism of action nor their regulation have been established. Because most previous studies have focused only on one Mx isoform of the three present in rainbow trout, the expression of all isoforms was simultaneously studied in this work in response to the viral haemorrhagic septicaemia virus (VHSV) G gene, poly I:C or VHSV. Thus, RT–PCR assays were specifically designed to amplify each of the Mx1, Mx2 and Mx3 transcripts induced both *in vitro* (RTG-2 cell line and head kidney leucocytes) and *in vivo* (muscle, head kidney, spleen and liver). Regardless of the inducer used, *in vitro* results showed that while in RTG-2 cells Mx3 was predominantly induced, all three isoforms were similarly induced in head kidney leukocytes. *In vivo*, regardless of the inducer used a predominant expression of Mx3 transcripts was also observed in muscle but expression of all three Mx isoforms or predominantly Mx1 and Mx2 was found in head kidney and spleen. Mx expression in the liver was however more dependant on the inducer used. In conclusion, the results obtained demonstrated, for the first time, that both in vitro and in vivo the expression of the different Mx genes is differentially regulated. Moreover, this is also the first report showing Mx induction after cell transfection with a plasmid coding for the VHSV-G protein.

Keywords: Rainbow trout; Mx; Interferon; DNA vaccine; Viral haemorrhagic septicaemia virus (VHSV)

1. Introduction

Type I (α/β) interferon (IFN)-inducible Mx proteins are highly conserved large GTPases with homology to the dynamin superfamily [1,2]. They have been found in all vertebrate species examined including mammals, birds and fish [3]. In addition to a conserved N-terminal tripartite GTP-binding domain, Mx family members share

* Corresponding author: Tel.: +34 96 665 8436; fax: +34 96 665 8758.
E-mail address: aestepa@umh.es (A. Estepa).

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a central-interacting domain and a leucine zipper motif located in the C-terminal domain [2]. Interactions between all these domains are important for oligomerisation, GTPase activity and antiviral function.

In fish, the cDNAs of Mx proteins have been cloned and characterised in rainbow trout, *Oncorhynchus mykiss* [4,5], atlantic salmon, *Salmo salar* [6], japanese flounder, *paralichthys olivaceus* [7], atlantic halibut, *Hippoglossus hippoglossus* [8], pufferfish, *Takifugu rubripes*, gilthead sea bream, *Sparus aurata* [9], channel catfish, *Ictalurus punctatus* [10], crucian carp, *Carassius auratus* [11], zebrafish, *Danio rerio* [12], turbot, *Scophthalmus maximus* [13] and orange-spotted grouper, *Epinephelus coioides* [14] However, evidence for antiviral activity has only been established for Mx proteins from japanese flounder and atlantic salmon [15,16].

In rainbow trout, three different Mx genes, Mx1, Mx2 and Mx3, have been cloned and characterised [4,5]. Mx1 and Mx3 are very similar in sequence, but Mx2 has a differential cysteine at position 324 and lacks the cysteine at position 16, there is a 13–15 amino acid insert at positions around 550 (with high probability to form coiled coils) and a nuclear localisation signal NLS (^{506}KKRK^{510}) [17]. Moreover, previous results have shown that trout Mx1 and Mx3 were localised in the cytoplasm while Mx2 was localised in the nucleus [5]. On the other hand, the antiviral effect of their encoded proteins was assayed by transfection of salmon cells with plasmids coding for their genes, but no antiviral activity could be demonstrated for any of the isoforms [5]. To date, Mx1 is the only trout isoform known to be directly induced by IFN [18].

DNA vaccines using the G genes of IHNV (infectious hematopoietic necrosis virus) or VHSV (viral haemorrhagic septicaemia virus) are more effective than previously developed vaccines [19–22]. These vaccines are characterised by inducing a long-term specific immunity which is preceded by a strong early non-specific protective response [23–26] similar to that of natural infection induced in non-vaccinated fish [19,27]. This strong early antiviral response, mostly elicited by the endogenous expression of the G protein [19–22,28–30], is now considered the basis of the high efficacy of these vaccines [2,19,21,28].

Non-specific responses following administration of rhabdovirus G-gene DNA vaccines in fish have included early up-regulation and expression of different immune-related genes including type I IFN genes [21] and type I IFN-induced genes [21,23,28,31–35]. Among those genes, Mx genes are of importance because a correlation between early protection and Mx3 gene expression has been observed in DNA vaccinated rainbow trout [27]. In all these studies, however, only one Mx isoform has been studied, either the Mx1 [21,32] or Mx3 [21,27,31,33].

Since differential antiviral activity has been demonstrated for murine, rat and human [3,36–40] and suggested for carp [11] Mx proteins, we studied the expression pattern of the three trout Mx isoforms stimulated by different inducers. Thus, Mx expression was induced *in vitro* (RTG-2 cell line or head kidney leucocytes) and *in vivo* (muscle, head kidney, spleen and liver) by the VHSV-G gene, polyriboinosinic-polyribocytidylic acid (poly I:C) and VHSV and then analysed by using RT–PCR assays specifically designed to amplify each isoform. The results obtained demonstrated, for the fist time, that both in vitro and in vivo the expression of the different Mx genes is differentially regulated. Moreover, this is also the first report showing Mx induction after cell transfection with a plasmid coding for VHSV-G protein.

2. Materials and methods

2.1. Cell cultures and virus

The fish cell lines EPC (*epithelioma papulosum cyprinid*) [41], purchased from the European collection of cell cultures (ECACC no. 93120820), and RTG-2 (rainbow trout gonad) [42], purchased from the American Type Culture Collection (ATCC CCL 55), were used in this work.

EPC cells were maintained at 28 °C in a 5% CO_2 atmosphere with RPMI-1640 Dutch modified (Gibco, Invitrogen Corp., UK) cell culture medium containing 10% foetal calf serum (Sigma Chemical Co, St. Louis, MO), 1 mM pyruvate (Gibco), 2 mM glutamine (Gibco), 50 μg ml^{-1} gentamicin (Gibco) and 2 μg ml^{-1} fungizone. RTG-2 were maintained at 20 °C in a 5% CO_2 atmosphere with MEM (with Earle's salts) cell culture medium (Gibco) containing 10% foetal calf serum (Sigma), 2 mM glutamine (Gibco) and 50 μg ml^{-1} neomycin sulphonate (Sigma).

Viral haemorrhagic septicaemia virus (VHSV 07.71) isolated in France from rainbow trout, *Oncorhynchus mykiss* [43], was propagated in EPC cells at 14 °C as previously reported [44]. Supernatants from VHSV-07.71 infected EPC cell monolayers were clarified by centrifugation at 1000 × *g* for 20 min and kept in aliquots at −70 °C. Clarified supernatants were used for the experiments. The virus stock was titrated in 96-well plates according to Reed and Müench [45].

2.2. Fish

Rainbow trout (*Oncorhynchus mykiss*) of approximately 8–10 cm obtained from Lillogen (Leon, Spain) were maintained at the Centro de Investigaciones en Sanidad Animal (CISA-INIA) laboratory at 14 °C and fed daily with a commercial diet (Trouw, Leon, Spain). Prior to experiments, fish were acclimatised to laboratory conditions for 2 weeks.

2.3. Induction of Mx isoforms in RTG-2 cells

Mx expression was studied in RTG-2 cells transfected with the G gene of VHSV, incubated with poly I:C or infected with VHSV.

To express the G-protein from VHSV in RTG-2 cells, the pMCV1.4-G plasmid which expresses the glycoprotein G gene of VHSV under the control of the long cytomegalovirus (CMV) promoter [46] was used. For cell transfection, RTG-2 cells, grown in culture flasks of 75 cm^2, were detached using trypsin (Sigma), washed, resuspended in MEM supplemented with 10% FCS and dispensed into 24-well plates at a concentration of 6×10^4 cells per well in a final volume of 300 μl. Then 0.5 μg of pMCV 1.4-G complexed with 1 μl of FuGene 6 (Roche, Barcelona, Spain) were added to each well and the plates were further incubated at 20 °C for 24, 48, 72 and 96 h as previously described [47].

At 48 h post-transfection the number of transfected cells was estimated by immunofluorescence using a monoclonal anti-G protein of VHSV antibody (I10) [48] as previously described [49]. The efficiency of transfection was calculated by the formula: number of cells expressing VHSV-G protein per well/total number of cells per well × 100. Both the total number of cells and the number of cells expressing the VHSV-G protein were counted using an inverted fluorescence microscope (Leica Ltd., Cambridge, UK) provided with a digital camera.

For poly I:C stimulation, RTG-2 cells grown in 24-well plates were treated with 30 μg ml^{-1} poly I:C in MEM supplemented with 10% FCS. Non-treated controls were also included. Cells were then incubated for 24, 48, 72 and 96 h at 20 °C.

For VHSV infection, RTG-2 cells grown in 24-well plates were incubated with VHSV at a multiplicity of infection (m.o.i.) of 0.001 for 2 h at 14 °C in MEM supplemented with 2% FCS. Controls were treated with culture media with 2% FCS only. The infected RTG-2 cell monolayers were then washed and further incubated at 14 °C for 24, 48, 72 and 96 h.

In all cases, after the different incubation periods, the medium was removed, cells detached with Ca^{2+}- and Mg^{2+}-free phosphate buffered isotonic saline (PBS) and total RNA extracted.

2.4. Induction of Mx isoforms in head kidney leucocytes

Head kidney leukocytes were isolated following the method previously described [50]. Briefly, fish were sacrificed by over exposure to MS-222 and the anterior kidney removed aseptically and passed through a 100 μm nylon mesh using Leibovitz medium (L-15, Gibco) supplemented with penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}), heparin (10 units ml^{-1}) and 2% foetal calf serum (FCS). The resulting cell suspension was placed onto 51% Percoll density gradients and then centrifuged at 500 × *g* for 30 min at 4 °C. The interface cells were collected and washed twice at 500 × *g* for 5 min in L-15 containing 0.1% FCS. The viable cell concentration was determined by Trypan blue exclusion and the cells were resuspended in L-15 with 0.1% FCS. The cells were dispensed into 24-well plates at a concentration of 1×10^6 cells ml^{-1}, and after 3 h at 20 °C were either exposed to 30 μg ml^{-1} of poly I:C (Sigma) or infected with VHSV at an m.o.i. of 0.1. After a further 24 or 48 h of incubation at 20 °C, VHSV-infected leucocytes were harvested, cells collected by centrifugation (500 g × 5 min) and total RNA extracted from the cells.

2.5. Induction of Mx in rainbow trout organs

Mx induction in response to pMCV1.4-G, poly I:C or VHSV was evaluated in independent experiments. Prior to handling, the trout were anaesthetised by immersion in MS-222 and then divided into groups of 20 trout per group. Groups were injected with pMCV1.4-G (intramuscular injection of 100 μl of PBS containing 0.5 μg pMCV1.4-G plasmid DNA per fish), poly I:C (intramuscular injection of 100 μl of PBS containing 500 μg of poly I:C per fish)

or VHSV (intraperitoneal injection of 100 μl of 1×10^7 $TCID_{50}$ ml^{-1} per fish). In every experiment, a control group was injected either intramuscularly or intraperitoneally with PBS (100 μl per fish).

At days 1, 2, 3 and 7, three control fish and four injected fish from each group were sacrificed by overexposure to MS-222 and head kidney, spleen, liver and muscle extracted and pooled. Fish injected with pMCV1.4-G were only sampled at day 10, because preliminary experiments showed this was the time at which the highest Mx expression was detected.

2.6. RNA extraction and cDNA synthesis

The "Total RNA Isolation System" (Promega) was used for RNA extraction from RTG-2 cells and head kidney leucocytes, while Trizol (Invitrogen) was used for RNA extraction from the different rainbow trout organs. In both cases, manufacturer's instructions were followed. Isolated RNAs were resuspended in diethylpyrocarbonate (DEPC)-treated water and stored at −80 °C until used.

Two micrograms of RNA were used to obtain cDNA using the Superscript II reverse transcriptase (Invitrogen). Briefly, RNA was incubated with 1 μl of oligo $(dT)_{12-18}$ (0.5 μg/ml) and 1 μl 10 mM dinucleoside triphosphate (dNTP) mix for 5 min at 65 °C. After the incubation, 4 μl of 5× first-strand buffer and 2 μl 0.1 M dithiothreitol (DTT) were added, mixed and incubated for 2 min at 42 °C. Then, 1 μl of Superscript II reverse transcriptase was added and the mixture incubated at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min, and the resulting cDNA was diluted 1:5 with DEPC-treated water and stored at −20 °C.

2.7. PCR amplification of Mx1, Mx2 and Mx3

The primers previously designed by McLauchlan et al. [27] to amplify a 381 bp fragment in the Mx3 sequence were used. The forward primer (MxF primer 5′-ATGCCACCCTACAGGAGATGAT-3′) had a 100% homology with all Mx isoforms, and was also used for the amplification of Mx1 and Mx2. Reverse primers for the amplification of Mx1 and Mx2 isoforms (Mx1R 5′-TAACTTCTATTACATTTACTATGCAA-3′ and Mx2R 5′-GGAAGCATAGTAACTTTAT TATAAC-3′) were then designed to amplify 421 bp and 400 bp fragments, respectively.

All PCR amplification reactions were performed using 0.5 μl dNTP mix (10 mM each), 0.2 μl Taq polymerase (5 units μl^{-1}, Invitrogen), 2.5 μl Taq 10× buffer, 0.75 μl $MgCl_2$ 50 mM, 0.5 μl of each primer (50 μM) and 1 μl of cDNA in a final volume of 25 μl. A parallel PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed with all samples as a house-keeping gene to standardise the RT–PCR results using the conditions previously described [51].

All PCRs were carried out in a Perkin Elmer 2720 cycler. The amplification conditions consisted in a denaturing step of 94 °C for 5 min followed by 25–30 cycles of 94 °C 30 s, 52 °C 30 s and 72 °C 30 s, followed by a final extension step of 72 °C for 7 min. The PCR amplification of all three isoforms was performed simultaneously in the thermocycler, using the same conditions. All amplifications were repeated at least twice to verify results. PCR products (8 μl) were visualised on a 1% agarose gel stained with ethidium bromide. Samples that were to be compared were run in the same agarose gel. A 100 bp ladder was used as a size marker. The intensity of the amplification bands was estimated using Image Gauge v. 4.0 software (Fujifilm). A semi-quantitative analysis of mRNA transcription of the Mx isoforms was performed and expressed as relative to the GAPDH gene transcription (expression relative to GAPDH) estimated in the same sample by using the formula: intensity of the Mx band/intensity of the GAPDH band.

The PCR products from Mx1, Mx2 and Mx3 were sequenced from both ends by using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Mix (Perkin Elmer) according to the manufacturer's instructions to verify that with each reaction only the specific Mx isoform was being amplified.

3. Results

3.1. Expression of Mx1, Mx2 and Mx3 in RTG-2 cells

Prior to analysing the expression levels of trout Mx isoforms, the nucleotide sequences of PCR products obtained using the designed primer sets were determined to confirm their identities and relative locations within Mx1, Mx2 and Mx3 mRNA sequences previous reported in GenBank (GenBank accession numbers of trout Mx1, Mx2 and Mx3

mRNAs are U30253, U47945 and U47946 respectively). In all cases, a 100% identity with the sequence previously submitted was found, showing that only the targeted Mx isoform was being amplified in each specific PCR.

Only Mx3 transcripts were significantly induced in response to transfection with pMCV1.4-G at 24 h (5–6-fold) and 48 h post-transfection (2–3-fold) (Fig. 1A). No expression of Mx3 was detected after 72 h or 96 h post-transfection.

Mx3 transcripts were also induced (10–12-fold) in poly I:C stimulated cells but Mx1 and Mx2 inductions (3–4-fold) were also observed (Fig. 1B). After 48 h, the levels of all three isoforms began to decrease and after 72 h only the Mx3 was still detected.

The induction of Mx transcripts was delayed to 72 h post-infection in VHSV infected cells (Fig. 1C). Only Mx3 transcripts were induced (~4-fold) at 72 h, whereas the Mx1 and Mx2 genes were also induced at 96 h post-infection, although at 2–3-fold lower levels than those of Mx3.

3.2. Expression of Mx1, Mx2 and Mx3 in head kidney leucocytes

Because head kidney leucocytes could not be transfected with pMCV1.4-G, the expression of the different Mx transcripts was only assayed after induction with poly I:C or VHSV. Contrary to what occurred in RTG-2, head kidney

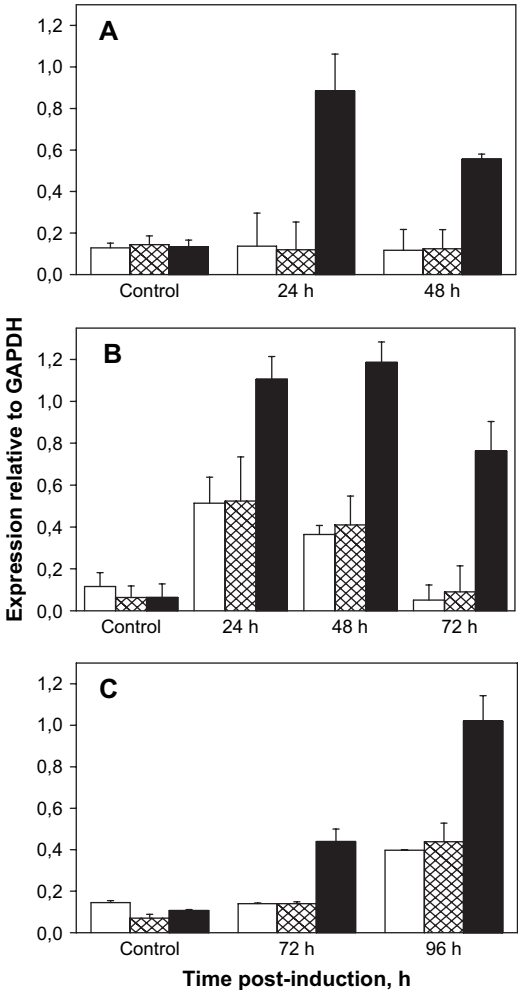

Fig. 1. Expression of Mx1, Mx2 and Mx3 isoforms in RTG-2 cells transfected with pMCV 1.4-G (A), treated with poly I:C (B) or infected with VHSV (C). After incubation for 24, 48, 72 or 96 h, total RNA was extracted and Mx transcripts were analysed by RT–PCR. Data are presented as mean relative expression ± SD from two different experiments each performed in triplicate. Control, untreated RTG-2 cells. White bars, Mx1. Hatched bars, Mx2. Black bars, Mx3.

leucocytes showed similar levels of induction (~10-fold) for the three Mx transcripts (Fig. 2A). Furthermore, a similar expression pattern and lower level of induction (~5-fold) of all three Mx isoforms was observed when head kidney leucocytes were infected with VHSV (Fig. 2B). In both cases, the expression pattern of the three Mx isoforms was not significantly different at 24 or 48 h post-stimulation.

3.3. Expression of Mx1, Mx2 and Mx3 in organs of rainbow trout induced by injection of pMCV1.4-G

To investigate whether the *in vivo* expression pattern of trout Mx isoforms shows any correlation with any of the *in vitro* situations, trout were intramuscularly injected with pMCV1.4-G. We first examined the primary response at the site of injection (skeletal muscle tissue) and then in secondary tissue, particularly in those which are targets of fish rhabdovirus replication, such as the haematopoietic tissues (head kidney and spleen) and liver.

At the site of injection, a 2–3-fold induction of the level of Mx3 transcripts was observed compared to the levels of Mx1 or Mx2 transcripts (Fig. 3). In contrast, all three Mx isoforms were 1.5–2-fold induced in the head kidney, spleen and liver (Fig. 3) and in all fish sampled, the level of induction of the different isoforms was equivalent.

3.4. Expression of Mx1, Mx2 and Mx3 in organs of rainbow trout induced by injection of poly I:C

For poly I:C, fish were sampled at different days post-treatment (1, 2, 3 and 7 days), since in previous experiments we observed a different timing for the maximum expression of each isoform. Due to the high number of samples that

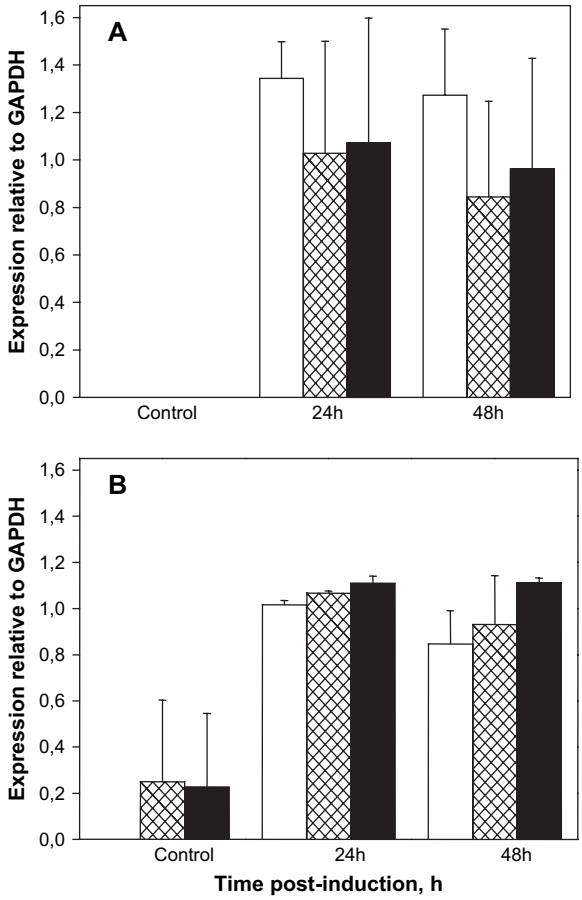

Fig. 2. Expression of Mx1, Mx2 and Mx3 isoforms in head kidney leucocytes in response to poly I:C (A) or infected with VHSV (B). After incubation for 24 or 48 h, total RNA was extracted and Mx transcripts were analysed by RT–PCR. Data are presented as mean relative expression ± SD ($n = 2$). Control, untreated head kidney leucocytes. White bars, Mx1. Hatched bars, Mx2. Black bars, Mx3.

were therefore analysed, data are presented as the expression of Mx isoforms in organ pools obtained at each time point (4 individuals per pool).

Although the three Mx transcripts were induced in muscle of fish intramuscularly injected with poly I:C, the Mx3 expression levels were higher than those of Mx1 or Mx2 (4–5-fold of Mx3 compared to 3-fold for Mx1 or 2–3-fold for Mx2). The differential expressions were maintained from day 1 to day 7 post-injection (Fig. 4). In the head kidney, transcripts from all the three Mx isoforms were equally induced like in the spleen at day 1 (Fig. 4). At days 2, 3 and 7, Mx1 transcripts were expressed at slightly higher levels than the Mx2 and Mx3 transcripts.

While in muscle the levels of expression were nearly maintained through day 1 to 7 post-treatment, in head kidney, spleen and liver, the Mx levels increased at days 1 (spleen and liver) or 3 (head kidney) and then decreased at day 7 to control values. Therefore, the *in vivo* results obtained by injection with poly I:C showed that although there were differences at the time of optimal induction of Mx among the studied organs, all three Mx isoforms were expressed with similar levels of induction (ranging from 6- to 12-fold). The lowest induction levels (1.5- to 3-fold lower) were obtained for Mx3 (Fig. 4).

3.5. Expression of Mx1, Mx2 and Mx3 in organs of rainbow trout induced by infection with VHSV

As in fish injected with poly I:C, the expression of Mx isoforms in trout intraperitoneally injected with VHSV was also analysed at different times post-infection using organ pools. Thus, in muscle Mx3 was again the isoform

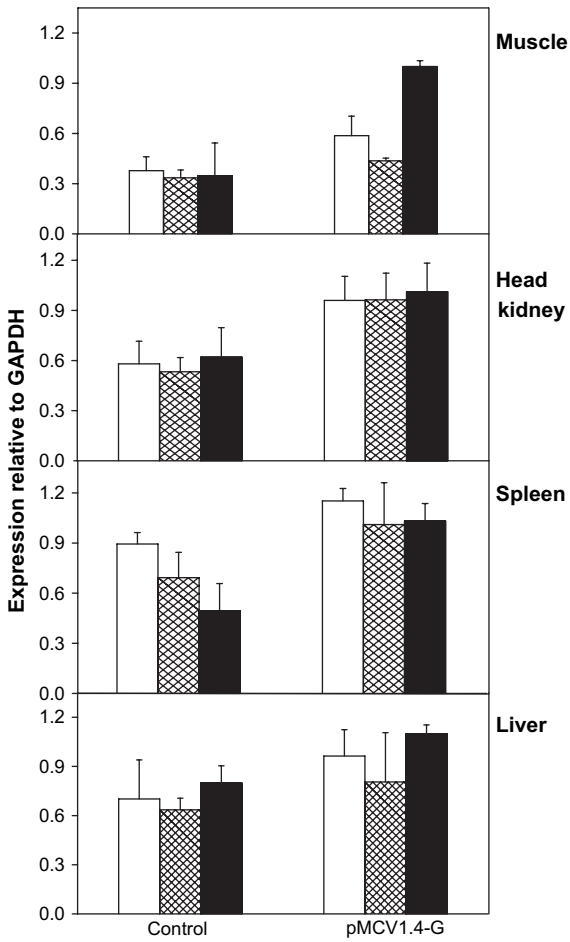

Fig. 3. Expression of Mx1, Mx2 and Mx3 isoforms in fish intramuscularly injected with pMCV1.4-G. Mx expression was analysed in the muscle, head kidney, spleen and liver of trout 10 days after injection. Data are presented as mean relative expression ± SD from four individuals from each group. White bars, Mx1. Hatched bars, Mx2. Black bars, Mx3.

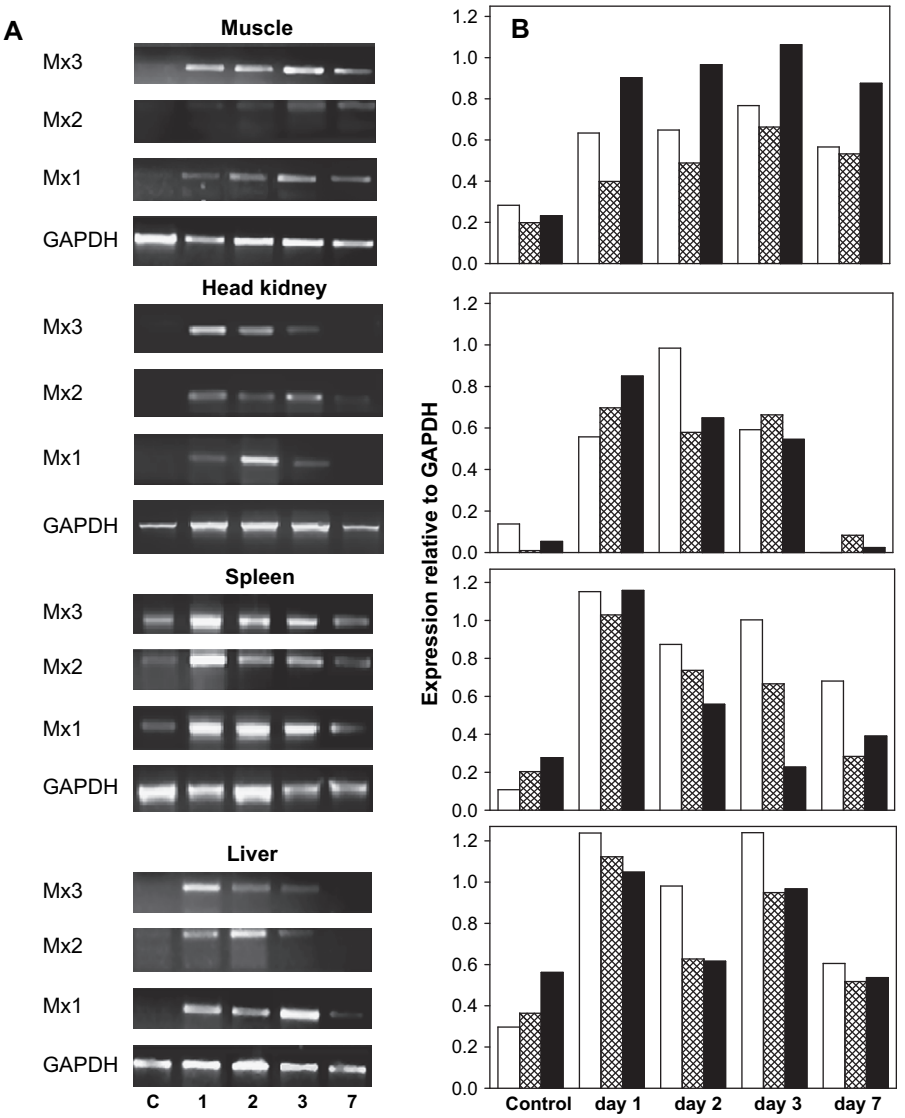

Fig. 4. Expression of Mx1, Mx2 and Mx3 isoforms in muscle, head kidney, spleen and liver of fish intramuscularly injected with poly I:C. The expression of the different isoforms was analysed in pooled tissues from 4 trout made at days 1, 2, 3 and 7 post-injection. (A) Stained gels showing the amplification bands of each isoform (B). Data presented as relative Mx expression to GAPDH. White bars, Mx1. Hatched bars, Mx2. Black bars, Mx3.

predominantly induced at all times (from 4- to 5-fold) while in the head kidney, spleen and liver, all three Mx isoforms were induced in response to VHSV from day 1 post-infection. In those organs, the lowest induction levels were always obtained for Mx3 (Fig. 5).

4. Discussion

To study possible differences in the expression of trout Mx isoforms, we have analysed *in vitro* and *in vivo* the expression of its three Mx genes in response to different well known IFN inducers such as the G gene of VHSV (pMCV1.4-G), poly I:C and VHSV. This is the first time all Mx isoforms are simultaneously studied in rainbow trout.

Only Mx3 transcripts were significantly induced in response to pMCV1.4-G in RTG-2 cells. Although the percentage of pMCV1.4-G transfected cells was only of 5–7% (data not shown), it was similar to a previous report in which this low transfection rate was enough for the induction of IFN both in transfected and neighbouring cells [30]. To

investigate whether the observed preferential expression of Mx3 transcripts was a unique property of pMCV1.4-G, the possible responses to poly I:C and VHSV infection (two other IFN inducers) were also studied. All the three Mx transcripts were expressed in RTG-2 cells when induced with poly I:C but Mx3 transcripts were predominantly expressed. This result confirms a previous report where the expression of the three isoforms of Mx protein was detected by using a polyclonal antibody [52] and suggests a good correlation between expression of Mx transcripts and Mx proteins. However, no reference to the relative expression of each isoform was indicated in this work. On the other hand, the same authors [52] reported that IHNV was not a good Mx inducer in RTG-2 cells, while we have found that VHSV infected RTG-2 cells efficiently express all Mx transcripts after 96 h post-infection, with Mx3 the predominantly expressed form. The similar time course and induction level profiles of the three Mx isoforms induced with pMCV1.4-G and poly I:C suggests a common induction mechanism while the relative delayed response for the Mx induction by VHSV infection might be due to a different mechanism of Mx induction or to viral anti-IFN mechanisms. In contrast, all three Mx isoforms were induced to equivalent levels in head kidney cells. This alternative pattern of expression of the Mx isoforms suggests that the relative expression of the different Mx isoforms is more

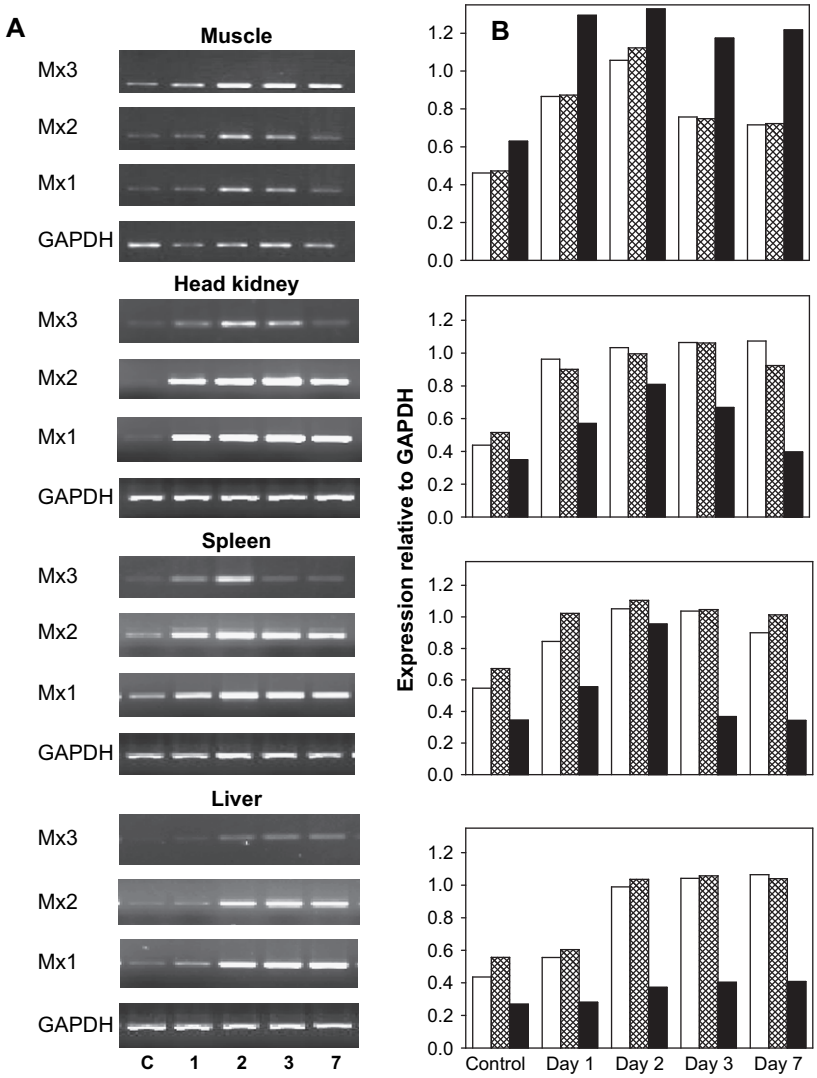

Fig. 5. Expression of Mx1, Mx2 and Mx3 isoforms in muscle, head kidney, spleen and liver of fish intraperitoneally injected with VHSV. The expression of the different isoforms was analysed in pooled tissues from 4 trout at 1, 2, 3 and 7 days post-infection. (A) Stained gels showing the amplification bands of each isoform (B). Data presented as relative Mx expression to GAPDH. White bars, Mx1. Hatched bars, Mx2. Black bars, Mx3.

dependent on the cell type rather than on the inducer used and may be due to the different functions of the Mx isoforms in each cell type and/or to the different roles in the immune response of each cell type.

Because the ability of fish rhabdovirus-G DNA vaccines to produce a systemic response is correlated with the magnitude of the early immune response at the intramuscular site of injection [20], we first analysed Mx expression in response to the injection of pMCV1.4-G in the muscle. The Mx3 expression was 2-fold higher than Mx1 and Mx2 expression, as observed in the muscle of fish injected with poly I:C. Surprisingly, an enhanced Mx3 level of expression was also observed in the muscle of trout intraperitoneally injected with VHSV, suggesting that in the muscle, as in RTG-2 cells (non-immune cells or tissues), Mx3 was predominantly expressed regardless of the inducer or the route of injection used.

In the haematopoietic organs studied (head kidney and spleen) and in the liver, the Mx response to pMCV1.4-G or poly I:C implicated all three Mx isoforms contrasting with what was observed in the muscle and was slightly different depending on the organ. Thus, in the head kidney, a similar level of induction in response to pMCV1.4-G was observed for the three Mx isoforms in all fish studied. Mx induction in response to the G gene of rhabdoviruses in head kidney has been previously described [21,23]. Similarly, all Mx isoforms were equivalently induced in head kidney of fish injected with poly I:C, even though there were differences in the time of maximal induction for the different isoforms. The similar Mx expression patterns observed in head kidney leucocytes both *in vitro* and *in vivo* suggests that this haematopoietic organ expresses all Mx isoforms regardless of the stimulant used. In the spleen, Mx1 was the isoform predominantly expressed in response to pMCV1.4-G and poly I:C, thus suggesting again a tissue specificity. In the liver, however, there were some small differences in the expression pattern of the Mx isoforms depending on the inducer used. Thus in pMCV1.4-G injected fish, Mx3 was predominantly expressed, while a more equivalent expression of the different isoforms was observed in response to poly I:C.

In response to VHSV, the expression of Mx1 and Mx2 isoforms was higher than the expression of Mx3 in spleen, head kidney and liver. Taking into account that all of these tissues are major targets for VHSV replication [21], this result might suggest that Mx1 and Mx2 play a more important role in the immune response to VHSV. Moreover, the induction of both Mx1 and Mx2 and to a lesser extent of Mx3 in the VHSV-target tissues could explain why no antiviral activity of trout Mx was found when salmon cells transfected with a single Mx isoform were infected with IHNV [5]. Nevertheless, antiviral activity has been demonstrated for other fish Mx isoforms through single transfection into homologous cell types [15,16] and therefore this interpretation requires further experimentation. On the other hand, as Mx2 is a nuclear isoform, it is surprising that it is induced in response to VHSV (a virus that replicates in the cytoplasm), since the antiviral specificity of mammalian Mx isoforms correlates with their subcellular location. Furthermore, the lack of induction of Mx3 in the liver of fish injected with VHSV is also surprising since it is in contrast with Mx3 expression observed in the liver after pMCV1.4-G injection and with a previous report in which the induction of liver Mx3 by DNA vaccines correlated with protection [27], thus suggesting other factors that might also be implicated.

In conclusion, we have shown that the three trout Mx genes were differentially induced depending on the targeted cell type/tissue. In non-immune cells (RTG-2) and tissues (muscle), Mx3 was predominantly expressed, while in hematopoietic organs such as the head kidney and the spleen, all three isoforms or predominantly Mx1 and Mx2 were expressed. Further work is needed to determine whether the differences observed in the expression pattern of trout Mx isoforms correlate with differences in functionality or antiviral specificity.

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