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In vitro inhibition of fish rhabdoviruses by Japanese flounder, *Paralichthys olivaceus* Mx

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Abstract

A homologous fish cell line stably expressing the recombinant Japanese flounder Mx (JFMx) was infected with hirame rhabdovirus (HIRRV) and viral hemorrhagic septicemia virus (VHSV), both of which are negative single-stranded RNA viruses belonging to the *Rhabdoviridae* family. Analysis of primary transcription of the two rhabdoviruses showed that there was lower expression level and copy number of the viral nucleoprotein transcript in the JFMx-transfected cell line than the infected, control cells, although no significant difference was observed. This suggests that JFMx may not be a potent inhibitor of rhabdoviral primary transcription. Kinetics of rhabdovirus expression by RT-PCR and quantitative real-time RT-PCR showed reduced levels of the rhabdoviral glycoprotein and nucleoprotein transcripts over time, indicating the possible role of JFMx in blocking rhabdoviral replication by interfering with the transcription of the viral subgenomic mRNAs. Significant inhibition in rhabdovirus replication consequently resulted in the synthesis of fewer viral particles. This may explain why JFMx-expressing cells are less susceptible to virus-induced cell lysis, and thus, why they would have a significantly higher survival than the infected, control cells. These results provide direct evidence that JFMx has an antiviral effect in vitro.

Keywords: Japanese flounder; *Paralichthys olivaceus*; Mx; Rhabdovirus; Transfection; Real-time PCR

Introduction

Fish rhabdoviruses are of particular interest since they affect diverse fish species living in a wide variety of habitats (Wolf, 1988). The hirame rhabdovirus (HIRRV) and the viral hemorrhagic septicemia virus (VHSV) are fish rhabdoviruses which belong to the genus *Novirhabdovirus* and possess a single molecule of linear, negative-sense ssRNA coding for six viral genes, namely, nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonviral protein (NV), and RNA polymerase (L) (Walker et al., 2000). They have been implicated as the cause of mass mortalities in both cultured and wild fish stocks in Japan. HIRRV, the causative agent of the HIRRV disease (HIRRVD), caused severe mortality in farmed Japanese flounder, *Paralichthys olivaceus*, during the 1980s (Kimura et al., 1986). The diseased fish showed signs of severe hemorrhage in the lateral musculature and visceral organs accompanied by marked necrotic lesions in the spleen and hematopoietic tissues (Isshiki et al., 2001). VHSV, on the other hand, is the causative agent of the most serious viral disease of farmed rainbow trout, *Oncorhynchus mykiss*, in continental Europe. Recently, however, this virus has been isolated from wild-caught (Takano et al., 2000) and farmed Japanese flounder (Isshiki et al., 2001). Clinical signs of infection include dark body coloration, an expanded abdomen due to ascites, congested liver, splenomegaly, and a swollen kidney (Isshiki et al., 2001).

As the host's first line of defense during viral infection, interferons (IFNs) are rapidly synthesized to combat the invading pathogens. Secretion of these cytokines triggers the up-regulation of other genes whose products are responsible for inhibiting viral replication (Landolfo et al., 1995) and in degrading viral components (Oritani et al., 2001). Among them, three have been extensively studied: double-

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strand RNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetases (OAS), and Mx proteins (Levy and Garcia-Sastre, 2001).

Mx proteins belong to the dynamin superfamily of high molecular weight GTPases (Staeheli et al., 1993; Arnheiter et al., 1996) and are found in a diverse number of organisms that include mammals, birds, and fish (Leong et al., 1998). Proteins belonging to this family are implicated in a wide number of cellular processes including membrane fission events, antiviral activity, plant cell plate formation, and chloroplast biogenesis (Hinshaw, 2000). Transfection studies have shown that Mx effectively inhibits the replication of enveloped single-stranded RNA viruses of negative polarity (Staeheli et al., 1993; Haller et al., 1998), although some positive-stranded viruses, such as Semliki Forest virus (Landis et al., 1998) and the coxsackievirus B (CVB) (Chieux et al., 2001), were inhibited in infected cells stably expressing the Mx.

The antiviral activity of Mx has been established in mammalian cells, specifically mice, rats, and humans (Leong et al., 1998). Murine Mx2, rat Mx2, and human MxA have significant antiviral activity against vesicular stomatitis virus (Pavlovic et al., 1990). Murine Mx1 inhibits influenza virus (Staeheli et al., 1986), Dhori virus (Thimme et al., 1995), and the Thogoto virus (Haller et al., 1995). Aside from the inhibition of vesicular stomatitis virus by human MxA, it also has antiviral activity against measles paramyxovirus (Schneider-Schaulies et al., 1994), bunyavirus, phlebovirus, hantavirus (Frese et al., 1996), and the hepatitis B virus (Gordien et al., 2001). Recently, chicken Mx isolated from different breeds showed antiviral activity against influenza and vesicular stomatitis virus (Ko et al., 2002). Moreover, transgenic animals expressing Mx have been shown to exhibit resistance to certain viruses (Arnheiter et al., 1990).

However, human MxB and rat Mx3 seemingly do not have antiviral activity (Haller et al., 1998). In addition, neither duck Mx (Bazzhiger et al., 1992) nor trout Mx (Trobridge et al., 1997) were found to have activity against avian influenza virus and a fish rhabdovirus, respectively.

Full-length Japanese flounder Mx (JFMx) cDNA had already been cloned and its expression patterns in the different organs and tissues of the fish were analyzed (Lee et al., 2000). It is composed of 2385 bp coding for 620 amino acids. The amino acid sequence shared approximately 51 and 78% identity with the human and trout Mx cDNAs, respectively. Experimental infection of the Japanese flounder with HIRRV showed elevated levels of Mx expression in the leukocytes starting at 48 h postinfection and reaching peak levels at 72 h.

In our previous study, we showed that HIRRV and VHSV had lower titers in Japanese flounder natural embryo (HINAE) cells stably expressing the JFMx compared with the nontransfected cells upon infection (Caipang et al., 2002). This led us to hypothesize that JFMx plays a major role in inhibiting fish rhabdoviruses.

The aim of the present study was to determine the degree of rhabdoviral inhibition in a homologous cell line stably expressing the JFMx. Although primary transcripts of rhabdoviruses were lower in JFMx-expressing cells than the infected, control cells, no significant difference was observed. This suggests that JFMx may not be a potent inhibitor of rhabdovirus primary transcription. Kinetics of the rhabdoviral expression demonstrated reduced levels of glycoprotein and nucleoprotein transcripts over time, leading us to speculate that JFMx blocks rhabdoviral replication by interfering with subsequent steps in transcription of viral subgenomic mRNAs. As a result of this inhibition mechanism, cells stably expressing JFMx are less susceptible to cell lysis associated with rhabdoviral infection.

Results

Expression of recombinant JFMx in transfected cells

JFMx expression was assayed in both transfected and normal HINAE, 1 month posttransfection by RT-PCR. CMV-driven JFMx was strongly expressed, although faint Mx signal was detected in normal HINAE (Fig. 1A). Quantitative real-time RT-PCR was conducted to determine the level of increase in JFMx copy number in the transfected cell over the control. Results showed that stably transfected homologous cells had a 1000-fold increase in Mx copy numbers relative to the control cells (Fig. 1B).

Effect of JFMx on rhabdovirus primary transcription

To test whether primary transcription of rhabdoviruses was indeed affected by JFMx, we measured this replication step in infected cells stably expressing JFMx and in nontransfected cells. Parallel cultures of JFMx-expressing cells and the nontransfected cells were infected with the rhabdoviruses in the presence of cycloheximide (CHX), a protein synthesis inhibitor. Total RNA was extracted 1 h after infection, and poly(A)$^+$ RNA was reversed transcribed to cDNA. Expression levels and the amounts of the rhabdoviral nucleoprotein transcripts were determined by RT-PCR and quantitative real-time RT-PCR, respectively. The infected control cells had relatively higher expressions of HIRRV and VHSV nucleoprotein transcripts than the JFMx-expressing HINAE (Fig. 2A–B). This was further supported by quantitative real-time RT-PCR in which the control cells had a higher amount of nucleoprotein transcripts than the JFMx-transfected HINAE (Fig. 2C–D), although the difference was not significant ($P > 0.05$).

Kinetics of rhabdoviral transcripts and JFMx expression by RT-PCR

JFMx-transfected and normal HINAE were infected with the two viruses at 10 $TCID_{50}$/ml and total RNA was ex-

tracted at 1, 2, and 4 days postinfection. RT-PCR was done to determine the expression level of the rhabdoviral transcripts.

Increased levels of HIRRV glycoprotein and nucleoprotein transcripts were observed in HIRRV-infected control HINAE, while the levels were low in the JFMx-transfected HINAE (Fig. 3A). Moreover, a constantly high level of JFMx expression was seen in the transfected HINAE, regardless of the duration of infection.

The same pattern of expression was also observed upon VHSV infection. There were increased glycoprotein and nucleoprotein transcript expression levels over time in the infected nontransfected HINAE, and a constantly high JFMx expression level in the transfected HINAE regardless of the duration of infection (Fig. 4A).

Quantification of the viral transcripts by quantitative real-time RT-PCR

Primers were constructed to amplify approximately 100-bp fragments of the viral genes. The copy numbers of the HIRRV and VHSV glycoprotein and nucleoprotein transcripts were determined using quantitative real-time RT-PCR.

The glycoprotein transcript copy numbers of the HIRRV-infected HINAE increased over time, with significantly higher ($P < 0.05$) copy numbers at the second and fourth day postinfection (Fig. 3B). In contrast to the JFMx-transfected HINAE, the levels of the HIRRV glycoprotein transcripts remained more or less uniform within the range of 4.6–5.4 $\times$ 10^4 copies regardless of the duration of infection.

The HIRRV nucleoprotein transcript copy number was also significantly higher ($P < 0.05$) in control HINAE than in the JFMx-transfected cells (Fig. 3B). A significant increase in the HIRRV nucleoprotein transcript copy number was observed in the control HINAE at the fourth day postinfection.

A similar trend was observed in VHSV-infected control HINAE, with increased levels of VHSV glycoprotein transcripts over time (Fig. 4B). A significant increase at the fourth day postinfection was observed. The trend of VHSV glycoprotein transcript copy number in JFMx-transfected HINAE infected with VHSV was similar to that of HIRRV infection. The copy number was in a range of 8.0 $\times$ 10^4–2.8 $\times$ 10^5 copies.

The VHSV nucleoprotein transcript also showed an increase in copy number over time in control HINAE, while the levels were constantly low in JFMx-transfected HINAE (Fig. 4B). Significantly higher ($P < 0.05$) copy number of the VHSV nucleoprotein transcript was obtained in control HINAE at the fourth day postinfection.

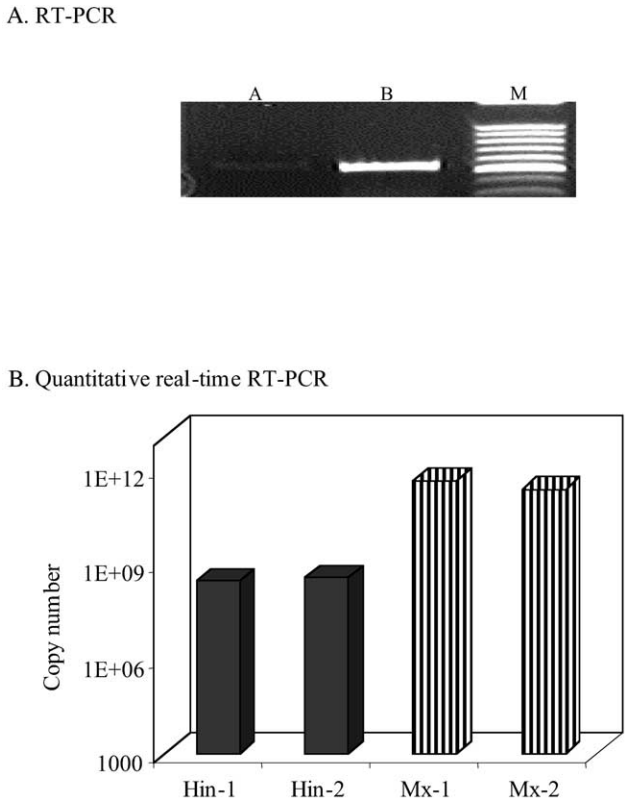

Fig. 1. Expression of the recombinant JFMx 1 month posttransfection. (A) Detection of JFMx expression by RT-PCR. Lane A, control HINAE; lane B, pCMV-JFMx-transfected HINAE; lane M, 100-bp ladder DNA marker. (B) Quantification of JFMx copy number using quantitative real-time RT-PCR. Five microliters of normalized cDNA samples (10 μg/ml) of nontransfected (Hin) and JF-Mx-transfected (Mx) HINAE cells were used in a 25-μl PCR reaction. Two clones of JFMx-expressing HINAE cells and two independent nontransfected cells were used for the analysis.

Effect of JFMx on rhabdovirus-induced cell lysis

To determine whether JFMx is able to protect cells from virus-induced lysis, transfected and control HINAE were infected with HIRRV and VHSV at a multiplicity of infection (m.o.i.) of 0.01. When cytopathic effect (CPE) was evident, the cells were fixed, stained with crystal violet, washed, and air-dried, and the stain was dissolved in sodium citrate and citric acid solution. The resulting solution was read using a microplate reader. After HIRRV infection, survival of JFMx-expressing HINAE (92.6 $\pm$ 5.1%) was significantly higher ($P < 0.05$) than the survival of control cells (75.4 $\pm$ 5.2%) (Fig. 5A). Similarly, after VHSV infection, survival of JFMx-expressing HINAE (91.6 $\pm$ 4.4%) was significantly higher ($P < 0.05$) than the survival of control cells (71.6 $\pm$ 4.7%) (Fig. 5B).

Discussion

This study examined the antiviral activities of JFMx in a homologous fish cell line. Conventional RT-PCR and quantitative real-time RT-PCR 1 month posttransfection showed

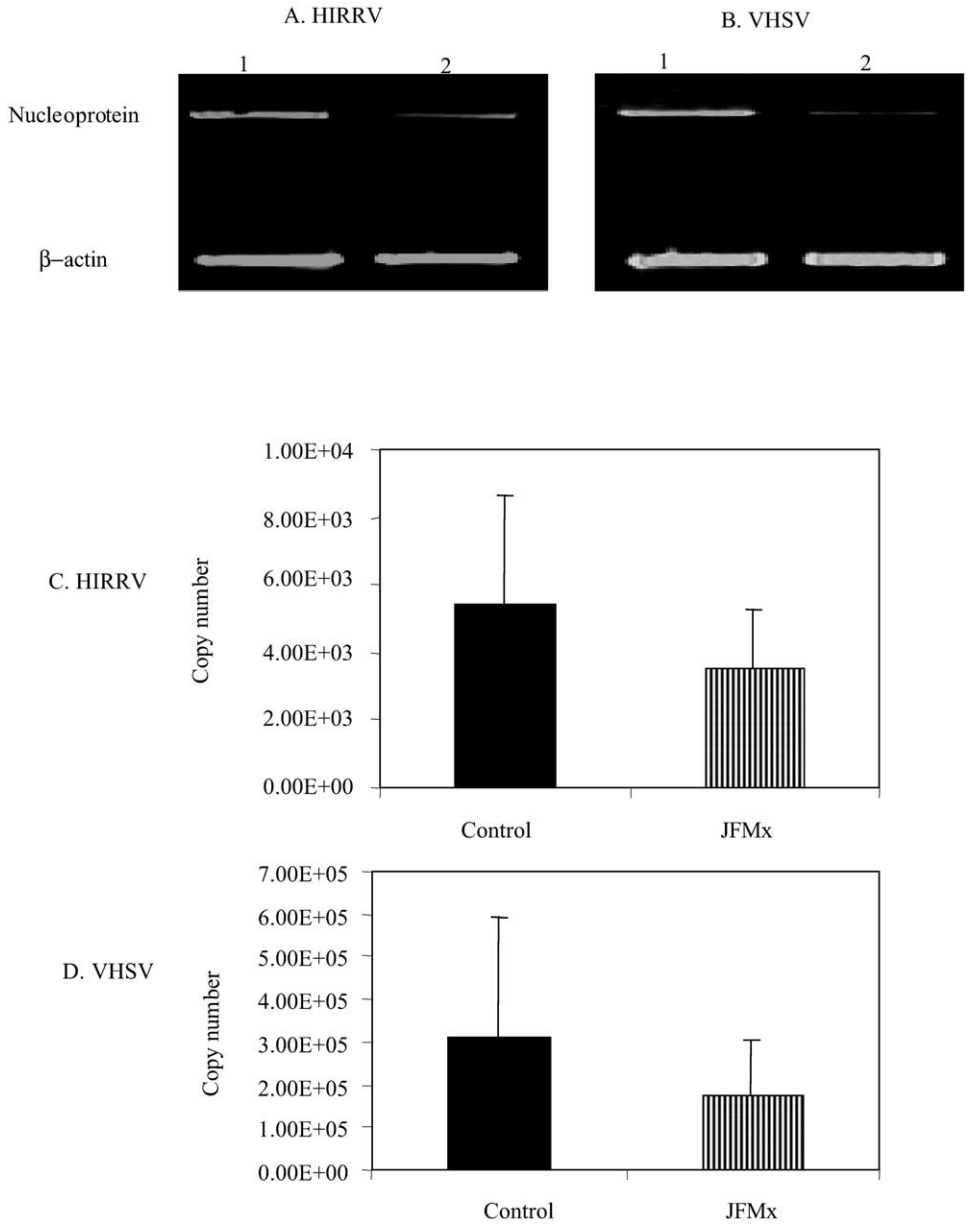

Fig. 2. Detection of primary rhabdovirus nucleoprotein transcripts. (A) Using RT-PCR. Parallel cultures of JFMX-expressing HINAE cells (lane 2) or control cells (lane 1) were infected with 1 m.o.i. of HIRRV or VHSV in the presence of 50 μg/ml CHX. Total RNA was extracted 1 h after infection and poly(A)$^+$ RNA was reverse-transcribed into cDNA. One microliter of the normalized cDNA sample (100 μg/ml) was used in a 25-μl PCR reaction. β-Actin was used as an internal control. (B) Determination of HIRRV and VHSV primary nucleoprotein transcripts by quantitative real-time RT-PCR. Five microliters of the reverse-transcribed samples (10 μg/ml DNA concentration) of both control and JFMx-expressing cells was used in a 25-μl reaction.

higher Mx expression in JFMx-transfected cells; however, a weak signal of Mx was observed in the nontransfected cells, suggesting constitutive expression of JFMx in fish cells. Haller et al. (1998) contend that even in the absence of viral infection, the cells still produce type I interferon, although at low levels. While the amount circulating within the system is insignificant, it might be enough to trigger the production of Mx. Moreover, there are no known established Mx-negative fish cells (Trobridge et al., 1997).

Both HIRRV and VHSV are negative single-stranded RNA viruses, and the ability of JFMx to inhibit these types of viruses is in agreement with the results of previous studies on other Mx proteins (Staeheli et al., 1986; Schnorr et al., 1993; Marschall et al., 2000; Jin et al., 2001).

Upon entry in the host cell, the viral genome undergoes primary transcription as a result of the catalytic action of the incoming virion polymerase. The transcription occurs in the absence of the host cell protein synthesis and the extent of primary transcription is determined by measuring the accumulation of viral mRNAs in infected cells that are treated with a protein synthesis inhibitor, CHX (Kochs and Haller, 1999b). Our RT-PCR and quantitative real-time RT-PCR

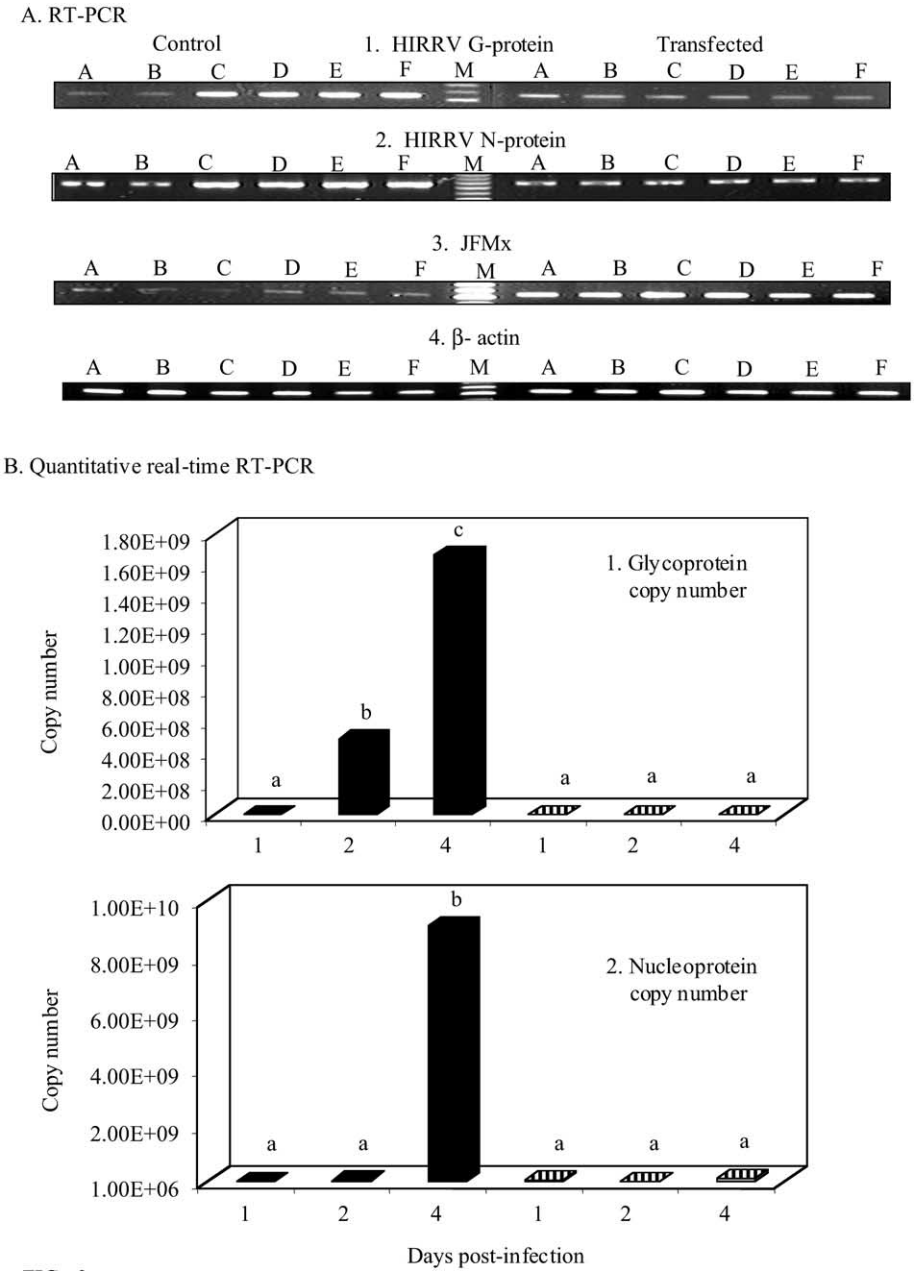

Fig. 3. Kinetics of HIRRV expression in infected cells. (A) Expression of HIRRV G-protein, N-protein, JFMx, and β-actin in infected cells at 1, 2, and 4 days postinfection (dpi) by RT-PCR. Lanes A, B, 1 dpi; lanes C, D, 2 dpi; and Lanes E, F, 4 dpi; lane M, 100-bp DNA marker. One microliter of the reverse-transcribed cDNA was used in a 25-μl PCR reaction. Number of cycles, 30. (B) HIRRV glycoprotein and nucleoprotein cDNA copy number in JFMx-transfected (▥) and nontransfected (■) HINAE at 1, 2, and 4 days postinfection (dpi). Column bars with the same superscripts are not significantly different at $P > 0.05$ ($N = 3$).

results show that cells stably expressing the JFMx were able to partly inhibit primary transcription of the rhabdoviral nucleoprotein, although they were not significantly different from the infected, control cells. This may indicate that JFMx is not a potent inhibitor of rhabdovirus primary transcription. Human MxA has been found to act on primary transcription of vesicular stomatitis virus (Pavlovic et al., 1990), measles virus (Schneider-Schaulies et al., 1994), and Thogoto virus (Kochs and Haller, 1999a; Weber et al., 2000). In the case of the Thogoto virus, human MxA may prevent the nucleocapsid from being transported to the cell nucleus, either by wrapping the incoming nucleocapsids, thereby covering the karyophilic signals, or by directing the nucleocapsids to alternative sites in the cytoplasm where they are immobilized and subsequently degraded (Kochs and Haller, 1999b). JFMx may be able to inhibit nucleocapsid transcription by a wrapping action because dynamins, of which JFMx is one (Staeheli et al., 1993), can self-assemble into long helical structures (Hinshaw and Schmid, 1995). These structures have an outer diameter of

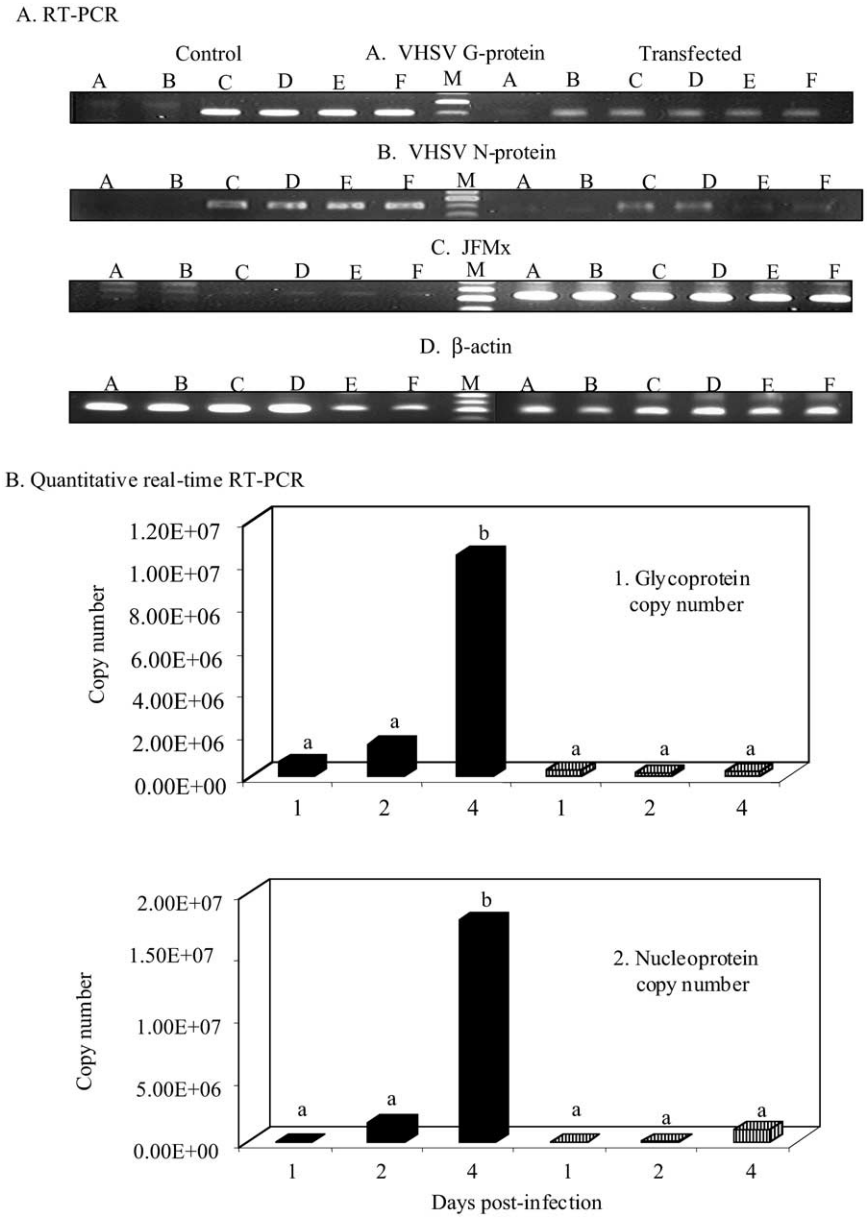

Fig. 4. Kinetics of VHSV expression in infected cells. (A) Expression of VHSV G-protein, N-protein, JFMx, and β-actin in infected cells at 1, 2, and 4 days postinfection (dpi) by RT-PCR. Lanes A, B, 1 dpi; lanes C, D, 2 dpi; lanes E, F, 4 dpi; and lane M, 100-bp DNA marker. One microliter of the reverse-transcribed cDNA was used in a 25-μl PCR reaction. Number of cycles, 30. (B) VHSV glycoprotein and nucleoprotein cDNA copy number in JFMx-transfected (▥) and nontransfected (■) HINAE at 1, 2, and 4 days postinfection (dpi). Column bars with the same superscripts are not significantly different at $P > 0.05$ ($N = 3$).

approximately 50 nm and an inner diameter of about 30 mm. However, the virion size of HIRRV is about 80 × 160–180 nm (Oseko, 1994), which is typical of rhabdoviruses (Wagner, 1988). We speculate that this size difference might allow some of the transcripts to escape, which could explain why JFMx only partially inhibits the viral nucleocapsid primary transcripts. Nucleocapsid transcripts that have leaked through or escaped the action of JFMx could be responsible in facilitating subsequent steps in viral replication.

JFMx, however, was able to inhibit rhabdoviral replication by blocking the transcription of viral subgenomic mRNAs as indicated by the low expression levels of HIRRV and VHSV glycoprotein and nucleoprotein transcripts in JFMx-expressing HINAE sampled at different time points postinfection. The inhibition of expression of rhabdoviral glycoprotein transcripts by JFMx is of particular significance in aquaculture because the glycoprotein is responsible for the antigenic characteristic of rhabdoviruses (Hill et al., 1975). Because it is the only protein present on the surface of the virus particle (Lorenzen et al., 1999), it is responsible for eliciting neutralizing antibodies in the infected organ-

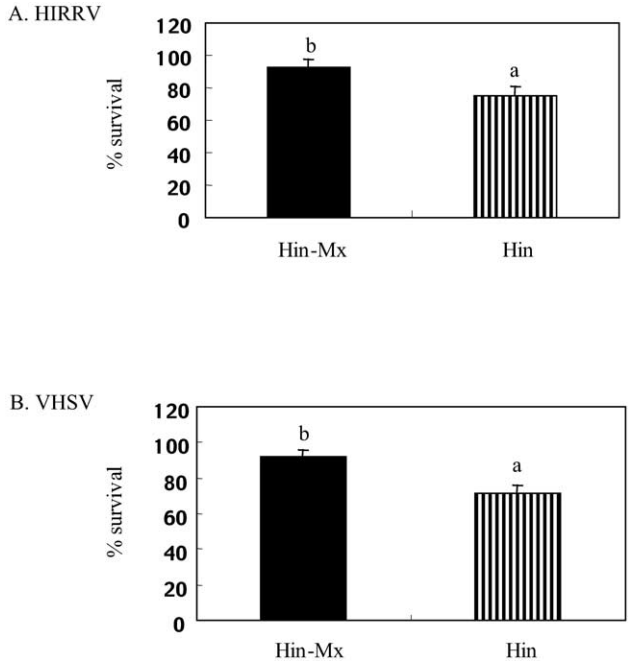

Fig. 5. Survival of cells upon virus infection as measured by cytotoxicity assay. JFMx-expressing HINAE (Hin-Mx) and control HINAE (Hin) were infected with 0.01 m.o.i. of (A) HIRRV and (B) VHSV. When cytopathic effect (CPE) was evident, the cells were fixed, stained, and dissolved in a 0.05 M citric acid and 0.05 M sodium citrate solution, and absorbance was read at 540 nm. Survival of the infected cells was measured based on the survival of the noninfected, control cells. Column bars with different letters are significantly different at $P < 0.05$ ($N = 8$).

ism. Inhibiting expression of the rhabdoviral glycoprotein transcripts might lead to a decrease in the antigenicity of the virus, rendering it less pathogenic to the infected host. In a related study, Schnorr et al. (1993) also observed that the human MxA seemed to interfere with the measles virus glycoprotein synthesis.

Our results confirm earlier findings that rhabdoviruses cause apoptosis in infected cells (Koyama, 1995; Bjorklund et al., 1997). Cell death associated with rhabdoviral infection is due to necrosis following cell membrane damage caused by the budding virions (Wolf, 1988). We previously showed that JFMx was able to reduce HIRRV and VHSV titers in vitro (Caipang et al., 2002). Here, we have further demonstrated that JFMx-expressing cells have a significantly higher survival than control cells upon infection, suggesting that JFMx has a role in protecting the cells from virus-induced cell lysis. The protection that was observed appears to be a consequence of the inhibition of rhabdovirus replication, which would lead to the synthesis of fewer viral particles during productive infection. However, we cannot rule out the possibility that JFMx confers protection to the cells either by inhibiting viral proteins that cause apoptosis or by interfering with cellular proteins that are required for viral replication. Landis et al. (1998) observed that the human MxA targets the viral components other than the structural proteins of the Semliki Forest virus. Whether JFMx also targets apoptosis-related viral proteins needs further study. Taken together, our results suggest that the antiviral activity of JFMx against fish rhabdoviruses is due to the interference with different steps in viral replication.

Our present results support our previous findings that Japanese flounder Mx possesses an antiviral activity in vitro, i.e., it has weak activity against rhabdoviral primary transcription but strong activity against rhabdovirus replication. In the future, we will examine the effect of JFMx in other cell lines derived from other fish species or in other cell types derived from Japanese flounder to determine whether its inhibitory effects are specific to certain cell types. The results of these in vitro studies will provide a better understanding of the function of JFMx in in vivo experiments.

Materials and methods

Cells and viruses

Japanese flounder or hirame natural embryo cell line (HINAE) (Kasai and Yoshimizu, 2001) was grown as a monolayer in Leibovitz's L-15 medium (Gibco-BRL, Grand Island, NY) supplemented with 20% fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 IU of penicillin G per milliliter, and 100 μg of streptomycin per milliliter (Gibco-BRL). Two negative single-stranded RNA viruses belonging to the *Rhabdoviridae* family were used: the HIRRV-8601 strain, and the VHSV-KRRV 9822 strain. For virus propagation, HINAE cells were infected with the viral suspensions and incubated at 20°C until apparent CPE was observed. At that time, the supernatant was harvested, centrifuged at 2500 *g* for 5 min, and then filtered through a 0.22-μm low protein-binding filter (Millipore, Bedford, MA). The resulting filtrate was dispensed in 1-ml aliquots and stored at −80°C until use.

Construction of JFMx expression vector

Full-length JFMx was PCR-amplified from a previously obtained JFMx cDNA (Lee et al., 2000). To facilitate subsequent cloning, an *Eco*RI restriction enzyme site was added to the sense primer (5′GCGAATTCACTCTCTG TCTCCATCAC 3′) and an *Xba*I site was added to the reverse primer (5′ ATTCTAGAGCACAGTAGAGTTAT-GCTC 3′).

The pCMV-JFMx expression vector was constructed by ligating purified enzyme digested-JFMx cDNA into an *Eco*RI-*Xba*I-digested pCIneo mammalian expression vector (Promega, Madison, WI), which contains the cytomegalovirus immediate/early enhancer promoter and the neomycin phosphotransferase gene.

Transfection of HINAE cells with JFMx expression plasmid

HINAE cells grown in 25-cm^2 flasks were transfected with the pCMV-JFMx using Effectene (Qiagen, Hilden, Germany) following the protocol of the manufacturer. The vector, pCIneo, was also transfected to serve as a control. Stable clones were selected following the procedures described by Caipang et al. (2002).

Analysis of JFMx expression in transfected cells

Transfected cells were analyzed for JFMx expression using RT-PCR 1 month after transfection. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) following the protocol of the manufacturer. The purified total RNA (10 μg) was reverse-transcribed into cDNA using the AMV reverse-transcriptase first-strand cDNA synthesis kit (Life Science Technologies, St. Petersburg, FL) in a 25-μl cDNA synthesis reaction.

Primers for the JFMx (sense: 5′-TGCCAAGAGG AAAAGGCAT-3′, and antisense: 5′-CTTCTTCAGGTG-GATGACCT-3′) were constructed to amplify a 550-bp fragment. Amplification conditions were the same as described previously. The reaction product was visualized in a 1.0% agarose gel using a densitometer (ATTO Co., Tokyo, Japan).

The reverse-transcribed cDNA samples from transfected and control cells were normalized to contain equal amounts of cDNA at 100 μg/ml by diluting the samples with 1× TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8.0). JFMx copy number in both cell types were subjected to quantitative real-time PCR analysis.

Analysis of primary transcription

To examine the effect of JFMx on the activity of the incoming viral polymerase complex, defined as primary transcription, cells were treated with CHX (Wako, Tokyo, Japan) throughout the experiment. Transfected and nontransfected HINAE cells grown in 25-cm^2 cell-culture flasks were mixed with 50 μg/ml CHX and incubated for 45 min before infection (Kochs and Haller, 1999b). Then, the cells were infected with HIRRV and VHSV at an m.o.i. of 1.0 and 0.1, respectively, for 1 h. Total RNA was extracted by Trizol, and poly(A)$^+$ RNA was reverse-transcribed using the AMV reverse transcriptase kit (Life Science Technologies).

The extent of inhibition of primary transcription in both transfected and nontransfected cells was assessed by determining the expression levels and copy numbers of the viral nucleoprotein transcripts by RT-PCR and quantitative real-time RT-PCR, respectively.

Table 1
Primers used in RT-PCR

Primer	Sequence
JFMx-F	5′CTGCCAAGAGGAAAAGGCAT 3′
JFMx-R	5′CTTCTTCAGGTGGATGACCT 3′
HIRRV glycoprotein-F	5′TGCCTACCCTGCTGTCATCAG 3′
HIRRV glycoprotein-R	5′TCCATGGTTTTCCACAGAAGG 3′
VHSV glycoprotein-F	5′TATCAGTCACC AGCGTCTCC 3′
VHSV glycoprotein-R	5′GTTTCCGACGTGGGGCAATG 3′
HIRRV nucleoprotein-F	5′ATGCTGACCCTGATAAGCAC 3′
HIRRV nucleoprotein-R	5′TATGCGGACAAGATCTGAGG 3′
VHSV nucleoprotein-R	5′TTCGTGCAGCTTTTTCAGGC 3′
VHSV nucleoprotein-R	5′CTTGGTCAAAAGGGAACACG 3′

Determination of viral gene kinetics

JFMx-transfected and nontransfected HINAE cells grown in 25-cm^2 conical flasks were infected with 1 ml of the viral suspension at a concentration of 10 $TCID_{50}$/ml units. Total RNA was extracted using Trizol (Gibco-BRL) and poly(A)$^+$ RNA was reverse-transcribed into cDNA using the AMV reverse-transcriptase first-strand cDNA synthesis kit (Life Science Technologies) at 1, 2, and 4 days postinfection.

VHSV glycoprotein and nucleoprotein genes and the HIRRV glycoprotein (GenBank/EMBL Accession No. U24073) and nucleoprotein (GenBank/EMBL Accession No. D45422) genes from the cDNAs of the infected cells were PCR-amplified using primers shown in Table 1. β-Actin was used as an internal control and was also amplified using primers described by Katagiri et al. (1997). Amplification conditions were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 1 min, and a final elongation step at 72°C for 5 min. The PCR products were electrophoresed and visualized in a 1% agarose gel.

Copy numbers of the HIRRV and VHSV glycoprotein and nucleoprotein transcripts in virus-infected cells were determined by quantitative real-time RT-PCR.

Quantitative real-time RT-PCR

The primers used and the corresponding annealing temperatures for the quantitative real-time RT-PCR analysis are listed in Table 2. Fifty microliters of the PCR reaction, which consisted of 5 μl template DNA (10 μg/ml), 5 μl of both forward and reverse primers (5 μM), 5.0 μl 10× SYBR PCR buffer, 6.0 μl 25 mM $MgCl_2$, 4.0 μl dNTP blend (2.5 mM dATP, 2.5 mM dCTP, 2.5. mM dGTP, 5.0 mM dUTP), 0.25 μl AmpliTaq Gold (5 U/μl), 0.5 μl AmpErase UNG (1 U/μl), and 19.25 μl distilled water, was prepared and subsequently used for quantitative real-time PCR. All reactions were done in triplicate.

Quantitative real-time RT-PCR was performed with a

Table 2
Primers used in quantitative real time PCR (qRT-PCR)

Primer	Sequence	Annealing temperature (°C)
JFMx-F	5′TAAAATGGCTGGGGTCGGTGTG 3′	61
JFMx-R	5′ACCCTGGTGATTCCAGGCAGGT 3′	
HIRRV glycoprotein-F	5′CATGCATGACGGGTCCATATACC 3′	61
HIRRV glycoprotein-R	5′TCTGGTGGGCACGATAAGTTCG 3′	
VHSV glycoprotein-F	5′GGCAAGGCACACTATCTTCTCG 3′	58
VHSV glycoprotein-R	5′GGTTTCCATGTGTTGTCCACCG 3′	
HIRRV nucleoprotein-F	5′ATAGGGCAAAGGCCCTTTGTGC 3′	62
HIRRV nucleoprotein-R	5′GGCAGAGATCTTCCAGCAGATC 3′	
VHSV nucleoprotein-F	5′AGGTGATCGTGGATGCACTTGC 3′	60
VHSV nucleoprotein-R	5′TGTTGACAGTCTGGGCCAGTTC 3′	

Gene Amp 5700 Sequence Detection System (PE Biosystems, Foster City, CA) using SYBR green detection following the protocol of the manufacturer. Copy numbers of each cDNA were determined from a standard curve of cDNA samples of known concentration using Avogadro's number. Separate standard curves were run for each PCR reaction.

Cell cytotoxicity assay

Measurement of antiviral activity in the transfected and nontransfected HINAE cells was conducted using a cytotoxicity assay adapted from the procedures described by Renault et al. (1991). This assay measures protection of cells against virus-induced lysis by calculation of the percentage of surviving cells after virus infection. Cells (approximately 10^5/ml) seeded in 96-well plates were infected with 100 μl HIRRV and VHSV diluted in cell-culture medium at an m.o.i. of 0.01, except for the 16 wells corresponding to the 100% protection control. When CPE was evident in the infected cells, the cells were fixed and stained for 10 min with staining solution (0.5% crystal violet, 10% formaldehyde, and 0.5 mM sodium citrate), rinsed with water, and air-dried. The stain was then dissolved by adding 100 μl 50% ethanol containing 0.05 M sodium citrate and 0.05 M citric acid to each well. Absorbance was read in a microplate reader at 540 nm. Results are presented as a percentage of surviving cells in which 100% represents the dye absorbance of noninfected cells treated the same way as infected cells.

Statistics

Statistical analyses were performed using SYSTAT 8.0 for Windows (SPSS, Chicago, IL). One-way ANOVA was used to determine significant differences in the cell survival and copy number of the virus transcripts in cells infected with the different viral suspensions. If significant, the least significant difference test was used to further determine differences among treatments. *P* values of less than 0.05 were assumed to be significant.

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