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The Mx protein family of rainbow trout (*Oncorhynchus mykiss*) was characterized at the molecular level. With primers derived from a partial sequence of Perch (Perca fluviatis) Mx genomic DNA, a partial rainbow trout Mx genomic DNA sequence was obtained by polymerase chain reaction (PCR) amplification. All salmonid fish investigated contained Mx homologous DNA sequences as determined by Southern blot. Northern blot analysis of Mx expression demonstrated that Mx mRNA was induced by poly inosinic cytidylic (IC) dsRNA both in vitro and in vivo and by infectious hematopoietic necrosis virus (IHNV) in vivo. RACE-PCR (rapid amplification of cDNA ends-polymerase chain reaction) was used to clone three Mx cDNA clones (designated RBTMx) from the rainbow trout gonad cell line, RTG-2, after induction by poly IC dsRNA. The deduced RBTMx1, 2, and 3 proteins are 621, 636, and 623 amino acids in length with molecular weights of 70.6, 72, and 70.8 kD respectively. The trout Mx proteins share approximately 50% identity at the amino acid level with mammalian and avian Mx proteins and contain the tripartite GTP binding domain and leucine zipper motifs common to all Mx proteins.

Polyclonal antisera generated to an *E. coli* expressed trout Mx protein fragment recognized all three trout Mx proteins. Poly IC dsRNA induced the expression of trout Mx protein in vitro. Mx protein was detected in the liver and kidney of rainbow trout challenged with IHNV. Immunohistochemical staining demonstrated that Mx protein was expressed in the kidney tubules of rainbow trout following exposure to IHNV.

Trout Mx cDNA clones were transiently expressed under the control of a eukaryotic viral promoter in a salmon embryo cell line by transfection. RBTMx1 was expressed in the cytoplasm in regions surrounding the nucleus. Staining for RBTMx2 produced a punctate pattern within the nucleus, characteristic of mammalian and rodent Mx proteins, and in the cytoplasm of some cells. RBTMx3 was expressed in a diffuse pattern, uniformly distributed throughout the cytoplasm, but not within the nucleus. Transient expression of the trout Mx proteins did not result in inhibition of IHNV replication as evidenced by nucleocapsid protein accumulation.

MOLECULAR CHARACTERIZATION OF THE MX GENES OF RAINBOW TROUT (ONCORHYNCHUS MYKISS)

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Grant David Trobridge, Author

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CONTRIBUTION OF AUTHORS

Dr. Jo-Ann Leong was involved in all aspects of this work. Pinwen Chiou constructed the CMV-beta-galactosidase control plasmid of Chapter 4 and 5 and conducted the *in vitro* transcription and translation experiment of chapter 4. Pinwen Chiou and Dr. Carol Kim were involved with the experimental design, conducting experiments, and interpretation of data for the *in vivo* induction of Mx in Chapter 5.

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CHAPTER 1
THESIS INTRODUCTION

Viral diseases of salmonid fish have severely constrained aquaculture. It has been estimated that one of every ten fish produced is lost to disease. However, during epizootics viral diseases can be particularly devastating. The salmonid rhabdovirus IHNV (Infectious Hematopoietic Necrosis Virus) can often cause mortality rates over 90% in fish up to two months of age. In 1991, 52% of the trout production of the United States (30.8 million trout) were lost to disease, the majority of these due to viral infection (Leong et al., 1995). The need to control viral diseases has lead to improved fish husbandry practices, development of vaccines and chemotherapeutics, and research on the pathogenesis of economically important viral pathogens. In addition to these approaches, an understanding of the basis of host immunity, both innate and specific, is needed to control fish viruses.

This thesis reports the molecular cloning and initial characterization of the Mx genes of rainbow trout (Oncorhynchus mykiss). In mammals the Mx proteins are a component of the interferon (IFN) system known to be intracellular mediators of innate viral resistance. The antiviral activity of specific Mx proteins against specific viruses occurs at distinct stages of the viral replication cycle. The molecular mechanism of Mx protein action is not yet understood, but in some cases the stage of viral replication that is inhibited is known. The work described here was conducted to provide tools to understand the role of Mx genes and IFN induction during infection of salmonid fish with viruses. In addition to basic research of host-virus interactions, the data presented here has the potential to lead to transgenic methods of increasing host resistance.

Mx proteins have been identified in all mammals tested (Horisberger and Gunst, 1991) and have been cloned from several species including humans, mice, rats, and chickens. Their common characteristics are inducibility by type I IFN (alpha and beta), a MW of 70,000 to 80,000 D, and antigenic cross reactivity. Several members of the Mx protein family have been shown to have specific intracellular antiviral properties. To date Mx proteins have been demonstrated to inhibit the replication of three orthomyxoviruses, the rhabdovirus vesicular stomatitis virus (VSV), and the measles paramyxovirus. All Mx proteins have a characteristic tripartite GTP binding motif in the amino terminal half

of the protein. This GTP binding motif is essential for GTP binding and antiviral activity of human MxA and murine Mx1 (Pitossi *et al.*, 1993; Melen and Julkunen, 1994).

A report of a partial Mx gene sequence from Perch (*Perca fluviatis*) (Staeheli *et al.*, 1989) led us to attempt cloning the Mx protein(s) of rainbow trout (Chapter 3). A partial Mx DNA sequence was polymerase chain reaction (PCR) amplified using oligonucleotide primers derived from the published perch sequence. This DNA probe was used to investigate the expression of Mx mRNA *in vitro* and *in vivo*. A complete cDNA nucleotide sequence of rainbow trout Mx1 was obtained and compared to mammalian Mx proteins.

In order to test the Mx proteins for antiviral activity full length clones for all three Mx proteins were obtained using high fidelity considerations for PCR in chapter 4. A polyclonal antibody specific for Mx was generated that recognized the recombinant Mx proteins. This antibody was used to determine the subcellular localization of the three trout Mx proteins and to determine if constitutive expression of the trout Mx proteins would inhibit expression of the IHNV N protein. Trout Mx protein expression is examined in chapter 5 using the polyclonal antibody developed in chapter 4.

CHAPTER 2
LITERATURE REVIEW

The IFN System of Vertebrates

Overview of IFNs

IFNs are inducible cytokines that stimulate antiviral activity in vertebrate cells. They were discovered by Aleck Isaacs and Jean Lindenmann in chicken embryo chorioallantoic membrane cultures treated with heat-inactivated influenza virus. These cultures released a soluble factor that inhibited replication of influenza virus when put on a secondary membrane culture and tested by a virus growth inhibition assay (Isaacs and Lindenmann, 1957). The critical importance of the IFN response as a first line of defense in viral infections has since been demonstrated; injection of anti-IFN antibodies in animals greatly exacerbates viral infection (Fauconnier, 1970; Gresser *et al.*, 1976). In addition to their antiviral activities it is now known that IFNs can regulate the control of cellular functions such as cell growth and differentiation, and the immune response (Stewart, 1976; Pestka *et al.*, 1987, Samuel, 1988).

There are many species of IFNs encoded by three families of genes. These are divided into two types, I and II, based primarily on antigenicity, see table 2.1. Type I IFNs are induced by viral infection and include the alpha (i) or leukocyte IFNs, the beta or fibroblast IFNs and the alpha (ii) or omega, trophoblast IFNs. Type II, or gamma IFN, is induced by mitogenic or antigenic stimulation of T lymphocytes and natural killer (NK) cells. The human IFN genes have been extensively studied (Weissman and Weber, 1986; Taniguchi, 1988; Roberts, 1989). There are more than 20 human IFN alpha genes but only one IFN beta gene. They lack introns and map to the short arm of chromosome 9. The single human IFN gamma gene has three introns and maps to the long arm of chromosome 12.

IFNs bind to cell surface receptors and activate IFN regulated proteins (IRPs) which are ultimately responsible for the antiviral state. IFN treatment of animal cells leads to increased expression of over twenty cellular genes (Staeheli, 1990; Samuel, 1991; Samuel, 1988) and decreases expression of some genes including c-myc (Dani *et al.*, 1985; Harel-Bellan *et al.*, 1988; Knight *et al.*,1985a), immunoglobulin M μ chain (Meurs and Hovanessian, 1988), and collagen (Goldring *et al.*, 1986). Of the IFN

Table 2.1 The IFN gene family

Type I			Type II
α: leukocyte (αi)	β: fibroblast	ω: trophoblast (αii)	γ: immune
>20 genes	1 gene	5 genes	1 gene
166 (165) aa	166 aa	172 aa	146 aa
major not glycosylated	N-glycosylated	N-glycosylated	N glycosylated
	chromosome 9		chromosome12
	α/β receptor		γ receptor

induced proteins, the 2'5' oligoadenylate synthetase, the P1/eIF-2 protein kinase and the Mx proteins have been shown to be involved with the development of the antiviral state at the cellular level.

Regulation of the IFN State

Normal cells do not contain or synthesize IFN. When cells are induced to produce IFN, an increase of transcription of the appropriate IFN gene(s) occurs rapidly by a process that does not require protein synthesis. The IFN gene regulatory element (IRE) (Fujita *et al.*, 1985; Goodbourn and Maniatis, 1988) of the beta IFN gene is comprised of three regulatory domains which interact with several proteins (Enoch *et al.*, 1986; Keller and Maniatis, 1988). Under normal conditions a repressor occupies a region known as the negative regulatory domain I (NRD). Upon activation the repressor is removed and two positive regulatory domain binding factors, PRD-I-BFc and PRD-II-BF are modified and bind to the positive regulatory domain activating transcription of the IFN beta gene.

Most inducers of inteferon are toxic to the cell and inhibit protein synthesis. Viruses, the most important inducers of IFNs, most likely induce IFN by inhibiting host protein synthesis. The IFN repressors are labile proteins and even a moderate reduction of the rate at which they are replaced may be sufficient to permit the two positive regulatory proteins to bind the IFN promoter (Joklik, 1990). In general, RNA viruses are good IFN inducers while DNA viruses, with the exception of the poxviruses, are poor IFN inducers. It is not clear what viral function(s) might be responsible for IFN induction although these studies have been performed using temperature-sensitive mutants (Lockhart *et al.*, 1968; Lomniczi and Burke, 1970). Virulent strains of lymphocytic choriomeningitis (LCM) virus (Jacobsen *et al.*, 1981) and polio virus (Pitkaranta *et al.*, 1988) are more efficient at inducing IFN than attenuated strains.

Viruses unable to replicate such as inactivated viruses and viruses in non-permissive cells can also induce IFN synthesis. Examples include the heat inactivated influenza used originally by Isaacs and Lindenmann (1957) and UV-inactivated viruses such as NDV (Youngner *et al.*, 1966), Colorado tick fever virus (Dubovni and Akers, 1972), and reovirus (Lai and Joklik, 1973). These effects are host cell and virus dependent. The basis of the induction is again probably due to inhibition of host protein synthesis either by viral components or due to the release of double stranded RNA.

Double stranded RNA (dsRNA) is cytotoxic, inhibits protein synthesis, and is a potent artificial inducer of IFN. Effective synthetic dsRNA molecules have the following properties; they possess a stable secondary structure, are relatively resistant to ribonuclease and they have a high molecular weight (Field *et al.*, 1970). Poly inosinic-cytidylic (poly IC) dsRNA is the most common artificial inducer and it affects the same region of the beta IFN promoter as viral infection (Goodbourn and Maniatis, 1985). Chemicals that stabilize poly IC from ribonuclease attack such the polycations DEAE-dextran, methylated albumin, protamine, polylysine or histones increase its effectiveness as an IFN inducer (Lampson *et al.*, 1981).

Virally infected or artificially induced cells synthesize and export IFN which then acts on neighboring and distant cells. Almost all cell types can produce one or more of the IFNs (Grossberg, 1972 , Ho and Armstrong, 1975) including fibroblasts and epithelial cells as well as cells of reticuloendothelial system. In animals inoculated with potent IFN inducers, IFN is produced by most organs from which tissues have been explanted (Grossberg, 1987). The effects of IFNs are dependent on binding of the exported IFN with an IFN receptor on the target cell. This is demonstrated by the fact that microinjected IFN fails to induce an antiviral state (Huez *et al.*, 1983), and that IFN coupled to sepharose beads is biologically active.

There are two IFN receptors (Branca and Baglioni, 1981; Orchansky *et al* .,1984), one for type I IFNs on chromosome 21 (Fournier *et al.*, 1985) and one for type two IFN on chromosome 6 (Rashidbaigi *et al.*, 1986). The type I IFN receptor (IFNABR) has a multisubunit structure composed of alpha and beta transmembrane subunits and the associated intracellular kinases, Jak 1 and Tyk 2 (Domanski *et al.*, 1995). Recent evidence indicates there are additional components required to confer high affinity binding and IFN-inducible growth inhibition (Ghislain *et al.*, 1995).

Binding of IFNs to their cognate receptors stimulates phosphorylation on tyrosine of several proteins including the receptors, members of the Jak family of tyrosine kinases, and transcription factor subunits called STATs (signal transducers and activators of transcription). Phosphorylated STATs associate to form homo- and heterodimers, with or without additional cofactors, and migrate to the nucleus where they activate the transcription of IFN-responsive genes (Darnell *et al.*, 1994). IFN alpha and beta induce formation of the transcription factor IFN-stimulated gene factor 3 (ISGF3) (Dale *et al.*, 1989; Levy *et al.*, 1989). The ISGF3 transcription factor is an oligomeric protein composed of three subunits; STAT2, STAT1alpha or STAT1beta, and a 48 kD DNA binding protein (Qureshi *et al.*, 1995). The activated ISGF3 transcription factor binds to

an IFN-stimulated response element (ISRE) and stimulates the transcription of IFN-regulated genes.

IFN Mediators

All activities of IFNs are thought to be mediated by IFN regulated cellular proteins. A variety of genes have been discovered whose transcription rates are rapidly activated by IFNs (Table 2.2). The contribution of some of these genes to the antiviral and cell proliferation control of IFNs are now beginning to be understood. For the P1 kinase, the 2'-5' oligoadenylate synthetase, and Mx proteins, specific intracellular antiviral activity has been demonstrated (Koromilas *et al.*, 1992; Meurs *et al.*, 1990; Staeheli *et al.*, 1986). In addition to increasing intracellular resistance to viruses, it is likely that IFN induction of MHC molecules increases cell recognition based immunity. IFN-induced increases in levels of class I and class II MHC molecules may increase the efficiency of antigen presentation, thereby increasing the efficiency of cellular immunity to viral infection.

The P1/Eif-2 Protein Kinase (PKR/p68 Kinase/DAI/dsI)

Treatment of most cells with IFN increases the level of the P1/eIF-2 protein kinase 20-fold. This kinase is normally inactive but when activated by dsRNA or virus it autophosphorylates. Following activation, P1 catalyzes the phosphorylation of protein synthesis initiation factor eIf-2 at serine 51 of the alpha subunit (Samuel, 1993). The P1/eIF-2 catalyzed phosphorylation of eIF-2alpha leads to an inhibition of protein synthesis, characterized by the selective inhibition of translation of certain mRNAs in transfected cells (Kaufman *et al.*, 1989; Samuel and Brody, 1990). There is considerable evidence that the P1/eIF-2 protein kinase plays a central role in the establishment of an antiviral state effective against a number of different types of animal viruses (Samuel, 1988).

Table 2.2 IFN Inducible Genes

Gene/Gene Product	IFN inducer	Reference
6-16	α/β	Kelly *et al.*, 1986
6-26	α/β	McMahon *et al.*, 1986
17 kD protein	α/β	Knight *et al.*, 1985a
ISG15	α/β	Reich *et al.*, 1987
ISG54	α/β	Larner *et al.*, 1984
Metallothionein II	α/β	Friedman *et al.*, 1984
Mx protein	α/β	Staeheli *et al.*, 1986a
P1-eIF-2alpha-kinase	α/β	Roberts *et al.*, 1976
p68-kinase	α/β	Meurs *et al.*, 1990
Xanthine oxidase	α/β	Ghezzi *et al.*, 1984
1-8	α/β/γ	Friedman *et al.*, 1984
2'-5'-A-Synthetase	α/β/γ	Merlin *et al.*, 1983
9-27	α/β/γ	Reid *et al.*, 1989
Complement factor B	α/β/γ	Strunk *et al.*, 1985
Egr-1	α/β/γ	Cao *et al.*, 1992
GBP	α/β/γ	Lew *et al.*, 1989
Indoleamine 2,3-dioxygenase	α/β/γ	Dai and Gupta, 1990
IRF1 (ISGF2)	α/β/γ	Miyamoto *et al.*, 1988
MHC class I	α/β/γ	Shirayosi *et al.*, 1988
Beta-2 microglobulin	γ	Wallach *et al.*, 1982
Fc gamma receptor	γ	Pearse *et al.*, 1991
Gamma.1	γ	Fan *et al.*, 1989
GM-CSF-Receptor	γ	Hallek *et al.*, 1992
ICSBP	γ	Driggers *et al.*, 1990
IP10	γ	Luster and Ravetch, 1987
IP30	γ	Luster *et al.*, 1988
MHC class II	γ	Finn *et al.*, 1990

The 2'-5' Oligoadenylate Synthetase

The 2'-5' oligoadenylate synthetase-nuclease system has been shown to confer antiviral resistance to picornaviruses in IFN treated cells (Chebath, 1987; Rysiecki *et al.*, 1989; Coccia *et al.*, 1990). The 2' 5'-oligoadenylate synthetase is induced by IFN and is dependent upon RNA for its activation. The activated enzyme catalyzes the synthesis of a family of oligonucleotides of the structure ppp (A2'p5')nA, with n greater or equal to 2 (designated 2,5A). These oligonucleotides in turn activate an endoribonuclease, designated RNase L or F. This 2,5A-dependent RNase is present in a latent form in both untreated and IFN-treated cells. Upon activation by 2,5A this enzyme degrades viral and cellular RNAs by cleaving single-stranded RNAs on the 3' side of -UpXp- sequences. A third enzyme, 2'-5' phosphodiesterase, is responsible for the degradation of the 2,5A to AMP and ATP, thus down regulating the pathway. Recently an inhibitor of the endoribonuclease L has been described and designated RLI (Ribonuclease L Inhibitor) (Bibal *et al.*, 1995). This 68 kD polypeptide reversibly binds to RNase L regulating its activity. In addition to its antiviral activity, the 2'5' synthetase-nuclease pathway may serve a general physiological role, regulating mRNA stability.

The IFN Induced Mx Proteins

Discovery of the Myxovirus Resistance Allele, Mx

The Mx locus and its function were discovered 17 years before it was known that Mx proteins were IFN-induced. Jean Lindenmann noticed that the inbred line of mice, A2G was resistant to doses of mouse-adapted influenza that were lethal to most other strains (Lindenmann, 1962). Resistance was inherited as a single dominant trait that was named Mx, for MyXovirus resistance. Expression of this resistance was found to be independent of the route of viral infection (Lindenmann *et al.*, 1963; Lindenmann, 1964; Lindenmann and Klein, 1966). The A2G Mx resistance to influenza was still present in both immunosuppressed (Haller *et al.*, 1976; Fiske and Klein, 1975) and athymic (nude) (Haller and Lindenmann, 1974) mice, demonstrating the Mx gene was not a component of acquired humoral, or cell mediated immunity. Further, the influenza resistance could be demonstrated in A2G hepatocytes (Arnheiter, 1980) establishing the resistance was mediated at the host cell level. Haller et al (1979) demonstrated that Mx resistance was

mediated by IFN. These authors showed that injection of anti-IFN antibody at the time of challenge with influenza virus abrogated the influenza resistance of A2G mice. In addition, cultured A2G cells were found to be permissive for influenza replication if not treated with IFN. Upon IFN treatment, A2G mice are protected against influenza virus while IFN treated Mx negative cells remain largely susceptible. This difference in virus susceptibility between Mx positive A2G cells and Mx negative cells was not seen when other challenge viruses were used (Haller *et al.*, 1980a; Arnheiter *et al.*, 1980; Arnheiter and Haller, 1983).

Identification and Cloning of the 72kD Murine Mx1 Protein

Before the Mx protein of mice was identified, it was known that IFN treatment of cells induced a variety of responses including increased levels of certain enzymes (Kerr and Brown, 1978) and the synthesis of new proteins (Broeze *et al.*, 1981). Horisberger *et al.*, (1983) compared macrophage cytoplasmic extracts from A2G mice and BALB/c mice (Mx negative) by two dimensional gel electrophoresis. A 72.5 kd protein with an isoelectric point of 6.3 was found only in Mx positive A2G cells and only after induction with a mixture of type I IFN. The presence of this 72.5 kd protein was found to correlate with the expression of the Mx allele influenza resistance. The Mx gene was introduced into BALB/c mice by crossing A2G (mx+/mx+) with BALB/c mice (mx-/mx-) followed by challenge with lethal doses of influenza. Surviving males were backcrossed to BALB/c females. The process was repeated to the 11th backcross and a clear correlation between Mx allele influenza resistance, and the presence of an inducible 72.5 kd protein was observed. This murine Mx protein was later named Mx1.

Polyclonal and monoclonal antibodies were generated to the Mx1 protein by immunizing Mx- BALB/c mice with extracts of IFN-treated cells from congenic Mx+ BALB.A2G Mx mice (Staeheli *et al.*, 1985). *In vitro* translation of poly A mRNA from IFN induced cells followed by immunoprecipitation, produced the 75 kd (formerly identified as 72.5 kd) Mx1. This assay system was then exploited to clone the murine Mx1 (Staeheli *et al.*, 1986a). IFN-induced mRNA was size fractionated followed by *in vitro* translation and immunoprecipitated with the anti-Mx antisera. mRNA fractions of approximately 3 Kb were then used to direct synthesis of a cDNA library. This library was screened by differential colony hybridization using cDNA probes derived from IFN-induced BALB/c mice and the congenic BALB/c.A2G mice. A murine Mx1 cDNA clone was isolated that encoded a 631 aa, 72,037 Da protein.

The murine Mx1 protein is very hydrophilic and contains an unusually high percentage of charged amino acids (Staeheli,1986a). Dense arrays of acidic residues, clusters of alternating basic and acidic residues, and arrays of predominantly basic residues are found. Near the carboxylic acid terminus there is a stretch of basic amino acids, with homology to the SV40 large T antigen, responsible for the nuclear localization of Mx1 (Noteborn *et al.*, 1987; Melen *et al.*, 1992; Melen and Julkunen, 1994). The murine Mx1 has a distinct granular or punctate pattern of expression in the nucleus of induced A2G cells and cells transfected with Mx1 (Dreiding *et al.*, 1985; Staeheli, 1986a).

The Murine Mx1 Protein is Antiviral

Staeheli *et al.* (1986a) used the murine Mx1 cDNA clone to demonstrate that the Mx1 protein was responsible for influenza resistance. The Mx1 631 aa open reading frame (ORF) was cloned downstream of a the SV40 early promoter and transfected into 3T3 (Mx negative) cells. After cloning by limiting dilution, the expression of Mx protein in individual cells was not uniform so these authors looked at the single cell level to assay Mx inhibition of viral replication. Using a double-label immunofluorescence study with anti-Mx sera and antisera to viral proteins, it was determined that expression of Mx protein was inversely correlated with expression of influenza proteins but not with control vesicular stomatitis virus (VSV) G protein. This study using an Mx protein in an Mx negative background was the first direct evidence that the Mx protein was responsible for specific inhibition of influenza replication. Further, by using the constitutive SV40 promoter these authors demonstrated that the Mx antiviral action was independent of concurrent activation of other IFN-regulated genes; the Mx protein was sufficient for influenza resistance.

Mx Proteins are Ubiquitous

After the discovery that the murine Mx1 protein was responsible for influenza resistance conferred by the Mx allele, Mx homologs were looked for in other species. Mx genes have since been cloned from humans (Horisberger *et al.*, 1988a; Aebi *et al.*, 1989), rats (Meier *et al.*, 1988; Meier *et al.*, 1990), sheep (Charleston and Stewart,

1993), pigs (Muller *et al.*, 1992), ducks (Bazzhiger *et al.*, 1993), chickens (Bernasconi *et al.*, 1995) and a partial clone was obtained from perch (Staeheli, 1989). Two Mx-related proteins were also found in bovine cells (Horisberger, 1988). Horisberger and Gunst (1991) conducted a survey of Mx proteins based on inducibility by IFN alpha, immunological relatedness, relative molecular weight, pI, and intracellular distribution. All mammals tested contained one or more Mx proteins. Expression of Mx protein was confined to the cytoplasm with the exception of the rodents. Hamsters, guinea pigs, mice, and rats expressed Mx protein in both the nucleus and cytoplasm. Cloned Mx proteins have been tested for antiviral activity using both transient and stable transfection assays. The methods of testing have varied and will be discussed below. Table 2.3 lists the Mx proteins tested for antiviral activity, their known antiviral spectrum, and their localization in the cell.

Mx Proteins are GTPases

All known Mx proteins contain in their highly conserved amino-terminal halves, a tripartite GTP-binding motif (Bourne *et al.*, 1991; Horisberger et al 1990). Mx proteins have homology in this GTP-binding domain with rat brain dynamins (Obar *et al.*, 1990), Drosophila dynamins (Chen *et al.*, 1991) and VPS1, a protein sorting protein of yeast (Rothman *et al.*, 1990). Studies using site-directed mutagenesis of this GTP-binding region have demonstrated that the ability of human MxA and murine Mx1 to bind and hydrolyze GTP correlates with antiviral activity (Nakayama *et al.*, 1991; Pitossi *et al.*, 1993; Garber *et al.*, 1993; Melen and Julkunen, 1994; Toyoda *et al.*, 1995). These studies all suggest that the GTP-binding domains are critical for antiviral activity but that there are other regions of the protein that affect antiviral activity. Recent studies on the biochemical characteristics of *E. coli* expressed human MxA suggest that the GTP-binding consensus element located in the amino terminal half of the protein is held in an active conformation by strong physical interactions with amino acids from the carboxyl terminal region (Schwemmle *et al.*, 1995). Richter *et al.* (1995) performed competitive binding studies of GDP and GTP that suggest that a high percentage of human MxA may be complexed with GTP *in vivo*.

Table 2.3 Subcellular Localization and Antiviral Spectrum of Mx Proteins

Mx Protein	Subcellular Localization	Antiviral Spectrum Determined To Date
Murine Mx1	Nuclear	Influenza/Dhori/Thogoto/VSV
Murine Mx2	Cytoplasmic	VSV
Rat Mx1	Nuclear	Influenza/VSV
Rat Mx2	Cytoplasmic	VSV
Rat Mx3	Cytoplasmic	None
Human MxA	Cytoplasmic	Influenza/VSV/Thogoto
Human MxB	Cytoplasmic	None
Duck Mx	Predominantly Cytoplasmic	None
Chicken Mx	Predominantly Cytoplasmic	None

Mx Proteins Contain Leucine Zippers

Melen et al (1992) expressed mouse Mx1 protein in a baculovirus expression system and purified it to homogeneity. The purified murine Mx1 protein was found to aggregate in dimers and trimers. This aggregation of Mx had been suggested by previous immunofluorescence studies showing the cytoplasmic human Mx proteins and the nuclear mouse and rat proteins Mx proteins form distinct punctate fluorescence patterns (Pavlovic *et al.*, 1992; Zurcher *et al.*, 1992; Meier *et al.*, 1990; Meier *et al.*, 1988; Staeheli *et al.*, 1986a). Crosslinking studies using the baculovirus expressed murine Mx1, rat Mx, and human Mx proteins demonstrated that the predominant form of Mx proteins is the trimer and human MxA was found almost exclusively in trimeric form. Protein sequence analysis identified a potential leucine zipper motif in the carboxyl-terminal ends of all Mx proteins. Leucine zippers are responsible for the dimerization of Jun-Fos heterodimers, although sequences surrounding the leucine zipper dictate the specificity of the Jun-Fos heterodimerization (O'Shea *et al.*, 1992). Amino acids 602 to 631 of murine Mx1 were introduced at the end of a foreign protein, catechol-O-methyltransferase (COMT)-Mx1. The Mx1 leucine zipper region was able to trimerize the normally monomeric COMT enzyme. Immunoelectron microscopy revealed that Mx1 in IFN treated A2G cells aggregated in the nucleus in distinct large structures, clearly discernable from the nuclear matrix and separated from the nuclear membrane, chromatin or nucleoli (Melen *et al.*, 1992).

Antiviral Activity of Cloned Mx Proteins

Mouse Mx1

Several studies have confirmed that the murine Mx1 protein is responsible for specific resistance to influenza infection. Arnheiter and Haller (1988) microinjected the anti-Mx monoclonal antibody 2C12 into IFN-treated Mx+ cells and found that influenza virus resistance was lowered to the level of IFN Mx- cells. The ability of the monoclonal antibody to specifically interfere with the Mx protein, and presumably not interfere with other IFN-mediated antiviral effects, lent support to the role of Mx1 protein as the major effector of inhibition of influenza replication. Murine cell lines that stably express Mx1 protein are specifically resistant to influenza virus replication as evidenced by a reduction

in plaques (Pavlovic *et al.*, 1990). This cell line remained sensitive to: the rhabdovirus VSV; the togavirus semliki forest virus (SFV); two picornaviruses, mengovirus, and encephalomyocarditis virus (EMC); and a herpes virus, herpes simplex virus 1 (HSV-1). Avian cell lines expressing the murine Mx1 had a reduced viral yield of influenza virus (Garber *et al.*, 1991). There was no inhibiton of replication of the two controls, VSV or Sindbis virus.

Transgenic expression of Mx1 also inhibits Influenza virus replication *in vivo*. Arnheiter *et al.* (1990) injected pronuclear Mx1- embryos with an Mx1 cDNA driven by a 1.8 Kb fragment of the murine Mx promoter. Three transgenic lines, high, low, and non-responders were generated. In the high responder line, Mx protein was inducible by both dsRNA and IFN to levels approximately one half of the positive control, A2G mice. Further, the transgenic Mx protein showed similar tissue distribution of induced Mx protein to Mx+ A2G mice. Upon infection with influenza virus mice of the high responder line produced Mx1 protein locally at the sites of initial viral replication, exhibited little viral spread, and survived infection. Mice of the low responder line showed more extensive viral spread and survived infection only when given high doses of virus. To survive low dose infection, these mice required co-injection of IFN with the virus, demonstrating a need for increased induction of the Mx protein. The non-responder line did not survive infection.

Recently the murine Mx1 was shown to confer resistance to two tick transmitted orthomyxovirus, Dhori virus and Thogoto virus (Haller *et al.*, 1995; Thimme *et al.*, 1995). Unlike the mice adapted influenza used by Lindenmann (1962), rodents are exposed to these arboviruses in their natural environments. In these studies both Mx positive stably transfected cell lines and transgenic animals were used. Virus susceptible BALB/c mice succumbed to infection while their congenic BALB.A2G-Mx1 counterparts remained healthy.

Mouse Mx2

A second murine Mx protein was identified (Staeheli and Sutcliffe, 1988) which had mutations in the deduced ORF in BALB/c and CBA mice. The murine Mx2 cDNA was corrected by site directed mutagenesis and expressed in 3T3 cells. Double-label immunofluorescence studies showed that the murine Mx2 was localized in the cytoplasm and inhibited VSV replication but not influenza replication.

Human MxA and MxB

Both IFN-induced human MxA and MxB are cytoplasmic but only MxA has antiviral activity. Pavlovic *et al.*, 1990 stably transfected 3T3 cells with either MxA or MxB and tested for viral resistance by reduction of virus yield or plaque assay. A reduction of virus yield for Influenza and VSV was seen in MxA transfected cells but not MxB transfected cells. Mengovirus, HSV-1, EMCV and SFV viral titres were not reduced by either MxA or MxB. MxA transfected 3T3 cells also inhibited plaque formation by Influenza and VSV. MxA transfected in the human monocytic cell line, U937, reduced the release of infectious VSV and the measles paramyxovirus 100-fold (Schnorr *et al.*, 1993). Measles virus replication was also inhibited in a human glioblastoma cell line, U-87-MxA, although the mechanism of resistance seemed to vary, see below (Schneider-Schaulies *et al.*, 1994).

MxA and MxB were stably transfected into the Vero cell line and plaque assays were performed with influenza, Thogoto and Dhori orthomyxoviruses (Frese *et al.*, 1995). MxA inhibited influenza and Thogoto virus, but not Dhori virus. MxB had no antiviral activity. Transgenic mice expressing human MxA were constructed that expressed human MxA under control of the ubiquitously expressed constitutive promoter mouse hydroxymethylglutaryl coenzyme A reductase (Pavlovic *et al.*, 1995). Embryonic fibroblasts derived from the transgenic mice were nonpermissive for Thogoto virus and showed reduced susceptibility for influenza and VSV as shown by viral reduction assay. The MxA transgenic mice survived challenges with high doses of Thogoto virus by intracerebral or intraperitoneal infection. These MxA mice remained susceptible to Dhori virus infection (Thimme *et al.*, 1995). Inhibition of VSV and Influenza virus by MxA required higher levels of expression of MxA.

Rat Mx1, Mx2, and Mx3

Rats contain three IFN-inducible Mx proteins (Meier *et al.*, 1988). Rat Mx1 is localized in the nucleus and shares 79% identity to the nuclear murine Mx1. Rat Mx2 and Mx3 are cytoplasmic and differ by only by eight amino acids (Meier *et al.*, 1990). 3T3 cell were micro-injected with cDNAs of rat Mx1, Mx2 or Mx3, and challenged with

influenza virus or VSV. Double label indirect immunofluorescent staining showed that nuclear Rat Mx1 strongly inhibited Influenza virus, and to a lesser degree inhibited VSV replication. The cytoplasmic rat Mx2 strongly inhibited VSV replication. The rat Mx3 did not inhibit replication of either virus.

Duck and Chicken Mx Proteins

Duck and chicken Mx cDNAs have been cloned and tested for antiviral activity. Two duck Mx cDNA clones, considered to represent alleles, were tested for anti-influenza activity in 3T3 cells and chick cells (Bazzigher *et al.*, 1993). 3T3 Cells permanently transfected with the duck Mx clones did not inhibit plaque formation by influenza virus. The antiviral activity of chicken Mx protein was tested at the single cell level using double-label immunofluorescent techniques (Bernasconi *et al.*, 1995). Transfected cells expressing chicken Mx protein showed no enhanced resistance to influenza virus, VSV, Thogoto virus, or sendai virus.

Mechanism of Mx Action

Mouse Mx1 Inhibits Primary Transcription

Virus adsorption, penetration, uncoating and transport of influenza virus nucleocapsids into the nucleus of infected cells are not affected by Mx protein (Horisberger *et al.*, 1980; Meyer and Horisberger, 1984; Broni *et al.*, 1990). Krug *et al.* (1985) demonstrated that primary transcription of influenza in the nucleus was reduced in IFN treated Mx+ cell. This is supported by the finding that 3T3 cells that constitutively express Mx1 reduce the level of the three influenza polymerase transcripts, PB1, PB2, and PA at least 50-fold (Pavlovic *et al.*, 1992). The authors suggested that Mx1 may interfere with the elongation step of transcription, because the longer viral transcripts were decreased the most. Overexpression of the influenza polymerase subunit PB2 abrogates the decrease in primary transcription caused by Mx1 (Huang *et al.*, 1992; Stranden *et al.*, 1993). This result, taken with the finding that there is an inverse correlation of influenza mRNA synthesis and Mx1 expression (Pavlovic *et al.*, 1990) suggest that inhibition of influenza virus is Mx1 concentration dependent. The simplest

interpretation is that Mx1 interacts with PB2, interfering with its action. However, extensive coimmunoprecipitation experiments with cell extracts from infected A2G cells did not reveal any physical interaction of Mx1 with subunits of the viral polymerase.

Human MxA Inhibits Protein Synthesis

Using 3T3 cells that express MxA constitutively, Pavlovic et al (1992) found that all influenza viral mRNAs accumulated in the nucleus to normal levels. These primary viral transcripts were polyadenylated, able to direct viral protein synthesis *in vitro*, and appeared to be efficiently transported to the cytoplasm. However viral protein synthesis and genome amplification were strongly inhibited. These authors suggested that MxA might interfere with intracytoplasmic transport of viral mRNAs, viral protein synthesis, or translocation of newly synthesized viral proteins to the cell nucleus.

Mutational Analyses of Mx Proteins

Mx protein mutants were constructed in an attempt to understand which regions of Mx proteins were responsible for their antiviral activities. Zurcher *et al.*, 1992 identified a MxA mutant, MxA (R645) which inhibited influenza virus but had lost the ability to inhibit VSV. This single amino acid change near the carboxyl terminal was sufficient to knock out part of the proteins antiviral repertoire. Like wild type MxA, this mutant was localized in the cytoplasm and still blocked influenza virus at a step after primary transcription. A nuclear transport signal was added to this mutant MxA to see if it would retain antiviral activity when moved to the nucleus. Like mouse Mx1, the nuclear MxA now inhibited influenza virus replication by interfering with primary transcription. Mutational analyses of murine Mx1 demonstrate that the amino terminal region of the protein is critical to its antiviral activity. Other regions including the leucine zipper and nuclear localization are also critical (Melen and Julkunen, 1994; Garber *et al.*, 1993). Several regions are important for its characteristic punctate distribution. Mutations in the nuclear localization signal of Mx1 that made the protein cytoplasmic, abrogated its antiviral action.

In Vitro Studies of Mx Activity

The mechanism of MxA antiviral activity was further characterized using a VSV *in vitro* transcription system (Peluso and Moyer, 1983). Purified MxA expressed in *E. coli* was tested for its ability to inhibit leader RNA and mRNA synthesis of VSV (Schwemmle *et al.*, 1995). The purified MxA inhibited both leader RNA and mRNA synthesis. The degree of VSV inhibition correlated with the GTPase activities of the purified MxA preparations. MxA was inactive when added to preformed VSV mRNAs, suggesting it inhibits the accumulation of VSV transcripts by interfering with the VSV polymerase, not by destabilizing VSV mRNAs. To distinguish between MxA effects on transcription initiation and chain elongation, the authors determined whether the 47 nt VSV leader RNA was synthesized at a reduced rate in the presence of MxA. Leader RNA was dramatically inhibited, suggesting MxA affects transcription initiation.

The transcriptional inhibition of MxA is not due to depletion of GTP and the activity of MxA does not require cleavage of GTP. The VSV transcription reaction with MxA was carried out in the presence of GMP-PNP or GTPgammaS. These GTP analogues have a non-cleavable bond between the beta and gamma phosphates and cannot be cleaved by MxA. However, the analogues can serve as substrates for the VSV polymerase. The purified MxA inhibited VSV transcription independently of whether GTP, GMP-PNP or GTPgammaS were used as the single sources of guanine nucleotides.

In vitro inhibition of influenza virus transcription has been reported (Landis *et al.*, 1995 in Schwemmle *et al.*, 1995), but remains unpublished.

Regulation of Mx Expression

The Mx Promoter

Mx promoters contains an interferon stimulated response element (ISRE) of the consensus 5' A/GGTTTCN(1-2)TTTCC/T3' (Hug *et al.*, 1988; Schumacher *et al.*, 1994) or its reverse complement, shared with other IFN stimulated genes (Reid *et al.*, 1989). Mouse and chicken Mx promoter fragments containing these ISRE elements mediate IFN inducibility to reporter genes (Hug *et al.*, 1988; Schumacher *et al.*, 1994). Transcription of Mx mRNA following induction by IFN is detectable in a few hours

(Ronni *et al.*, 1993). A substantial pool of Mx mRNA, an estimated 0.1% of the cytoplasmic poly A mRNA, is produced. *In vitro* nuclear run-off experiments suggest that IFN mediated regulation of Mx gene expression occurs mainly at the level of gene transcription (Staeheli *et al.*, 1986b). The steady state mRNA levels of MxA in human mononuclear cells are highest at 4 to 6h postinduction and return to basal levels at 24 to 48h postinduction demonstrating a rapid down-regulation of transcription following induction (Ronni *et al.*, 1993). MxA can be restimulated 24h after the initial induction to similar levels as the primary induction.

Studies using cloned Mx promoter fragments demonstrated that Mx transcription could be activated directly by viral infection, without the need for IFN (Hug *et al.*, 1988; Lleonart *et al.*, 1990). However, Bazzhiger et al (1992) found that viral induction of Mx in cultured embryo cells and macrophages was poor. IFN receptor minus cells and cells treated with cycloheximide to prevent IFN production did not produce high quantities of Mx mRNA. These authors suggested that direct viral inducibility of the Mx promoter may be a property of some established cell lines or the result of a promoter not in proper chromosomal context.

Cytokine Control of Mx

The expression of Mx is tightly regulated by IFN alpha/beta (Aebi *et al.*, 1989; Simon *et al.*, 1991; von Wussow *et al.*, 1990). IFN gamma, tumor necrosis factor (TNF), or interleukins do not seem to induce Mx directly (Simon *et al.*, 1991). IFN gamma does not induce Mx (Staeheli *et al.*, 1984).

In Vivo Expression of Mx

Mx protein or mRNA has been detected in all organs examined of infected or poly IC induced animals; lung, brain, liver, spleen, heart, kidney, thymus, pancreas, testis, uterus and muscle (Wuethrich *et al.*, 1985; Staeheli and Haller, 1987; Chang *et al.*, 1990). Staeheli and Haller (1987) detected nuclear Mx protein in virtually all cell types using immunofluorescence with monoclonal and polyclonal antibodies. However, Chang *et al.*, (1990) did not detect Mx mRNA in some specialized cell types using *in situ* hybridization. Both investigators found that mononuclear cells expressed high amounts of Mx.

Some cells of control mice, considered to be sessile macrophages, not treated with IFN were found to constitutively express Mx protein. In addition, macrophages freshly explanted from the peritoneal cavity of Mx+ mice contain high levels of Mx protein (Dreiding *et al.*, 1985). This constitutive expression of Mx in macrophages is thought to result from their sensitivity to low levels of circulating IFN (Staeheli and Haller, 1987).

Construction of Transgenic Mx Animals

The construction of animals, transgenic for murine Mx1 or human MxA has confirmed the role of the Mx protein in antiviral defence and shed light on its mechanism of action *in vivo*. Constitutive expression of murine Mx1 or human MxA seems to be deleterious to transgenic mice (Arnheiter *et al.*, 1990; Kolb *et al.*, 1992; Pavlovic *et al.*, 1995). However, transgenics expressing Mx under control of constitutive promoters have demonstrated enhanced resistance to viruses (Kolb *et al.*, 1992; Pavlovic *et al.*, 1995). Transgenic mice expressing murine Mx1 under control of a 1.8 Kb fragment of the Mx1 promoter express Mx rapidly and transiently in a number of organs (Arnheiter *et al.*, 1990). Infection of these Mx1 transgenic mice with a pneumotropic influenza virus followed by double label immunofluorescence suggests how Mx works *in vivo* (Arnheiter *et al.*, 1990). Following first contact with influenza virus, a few cells become infected and release IFN. This IFN induces expression of Mx1 in neighboring cells, such that the cells become demarcated by a barrier of virus-protected cells. Consequently, the virus cannot spread efficiently, giving the adaptive immune system time to mount a response and eliminate the virus.

Use of Mx Protein as a Marker for Type I IFN

Several characteristics make the Mx protein an ideal marker for IFN. Mx proteins are rapidly and transiently induced specifically by type I IFNs. More importantly, unlike IFN, Mx is produced in large amounts, and has a long halflife. Mx in induced cells can comprise up to 1% of the total cytoplasmic protein (Horisberger, 1992) with a biological half-life of 2.5 days (Ronni *et al.*, 1993). Moreover, cellular induction of Mx protein is not subject to feed-back inhibition, even at high doses of IFN therapy (von Wussow *et al.*, 1990). Immunoassays have been developed to detect Mx in blood (Oh *et al.*, 1994;

Towbin *et al.*, 1992) and Mx has been used as a marker for IFN therapy (Jakschies *et al.*, 1989). Mx has been used in several studies as a marker for type I IFN (Kraus *et al.*, 1992; Yamada *et al.*, 1994; Abrams *et al.*, 1995). Roers et al (1994) used the presence of Mx, and therefore type I IFN, to assess vaccination efficacy of attenuated yellow fever virus in volunteers.

The IFN System of Fish

In Vitro IFN Studies

Several studies have shown that fish cells are able to produce an IFN, or IFN-like activity. Gravell and Malsberger (1965) first demonstrated the *in vitro* production of fish IFN in fathead minnow (*Pimephalus promelas*) cells (FHM) when exposed to infectious pancreatic necrosis virus (IPNV). *In vitro* production of a fish IFN with properties similar to mammalian and avian IFN has since been reported by several investigators (Beasley *et al.*, 1966; Oie and Loh, 1971; DeSena and Rio, 1975; MacDonald and Kennedy, 1979; Sano and Nagakura, 1982; Tengelsen *et al.*, 1989). The IFN substance is heat stable, non-dialyzable, non sedimentable, pH stable, sensitive to trypsin and resistant to nucleases. IFN production may be involved in persistent infections of rainbpw trout gonad cells (RTG-2) (Hedrick and Fryer, 1981) and in reduced viral titres of highly confluent monolayers with IHNV (Okamoto *et al.*, 1983).

In Vivo IFN Studies

Several studies have suggested that IFN may be an important innate immune response to viral pathogens of fish. Amend (1970) was able to prevent IHN virus pathology by elevating water temperature. The rapid onset and disappearance of the protection suggested an IFN was responsible for the protection. Another fish rhabdovirus, viral hemorrhagic septicemia (VHS), causes disease when temperatures are between 6°C and 12°C but not at 15°C. However, 15°C is the optimal temperature for VHS replication *in vitro*. deKinkelin and Scherrer (1970) considered that this temperature related antiviral effect was mediated by IFN. deKinkelin and Dorson (1973)

confirmed that trout injected with VHS and kept at 15°C produced an IFN-like substance that was able to confer protection to RTG-2 cells against IPN, VHS, and IHN virus.

Eaton, 1990 induced antiviral protection in chum (*Oncorhynchus keta*) and sockeye (*Oncorhynchus nerka*) salmon by pre-injection with poly IC dsRNA. Fish pretreated with poly IC and challenged with IHNV developed an antiviral response, resulting in decreased IHNV titre, lower cumulative mortalities, and a delay in virus replication when compared to untreated fish. Further, purified sera from poly IC injected salmon and trout was able to protect RTG-2 cells from IHNV cytopathic effect. This IFN-like substance exhibited type I IFN properties.

Fish IFN Genes

Wilson *et al.*, (1983) examined perch (*Perca fluviatis*), minnow (*Phoxinus phoxinus*), dace (*Leuciscus leuciscus*) and stone loach (*Noemacheilus barbatulus*) fishes for the presence of IFN homologous genes. Using a beta IFN gene probe under low stringency, faint but reproducible bands were seen. A flatfish (*Paralichthys olivaceus*) IFN cDNA clone has been published (Tamai *et al.*, 1993) encoding a 138 aa polypeptide. This protein has between 12% and 24% sequence identity to mammalian IFNs. The cDNA was expressed in transfected BHK-21 cells and supernatants were used treat EPC (*Epithelioma papilloma cyprio*) cells. EPC cells treated with supernatants of IFN cDNA transfected BHK cells survived challenge with Hirame rhabdovirus as evidenced by a neutral red dye uptake assay. Further studies characterizing this cDNA clone are needed to confirm it is responsible for the antiviral effects of fish IFN seen *in vitro* and *in vivo*.

Mx Genes of Fish

Although piscine IFN activity has been well characterized, there is only one report of IFN-induced proteins in fish cells. Staeheli et al (1989) used a murine Mx1 cDNA probe to identify a Mx homologous gene in perch. A 2.35 Kb Mx genomic fragment was cloned and sequenced. This cloned fish DNA contained blocks of sequences related Mx1 gene exons 3 to 8. The exon lengths and the exon-intron junctions were exactly the same between the perch Mx gene and the murine Mx1 gene. These authors injected rainbow trout (*Oncorhynchus mykiss*) with poly IC dsRNA and

Newcastle disease virus (NDV), a potent IFN inducer. Two Mx mRNA transcripts of 2.5 and 2.0 Kb were detected by Northern blot analysis of the livers 18h after injection. The Mx1 monoclonal antibody 2C12 failed to detect fish Mx protein by immunoprecipitation or immunofluorescence of induced trout livers or spleens.

CHAPTER 3
CHARACTERIZATION OF A RAINBOW TROUT MX GENE

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Abstract

A full length cDNA clone of a rainbow trout (*Oncorhynchus mykiss*) Mx gene was obtained using RACE (rapid amplification of cDNA ends) polymerase chain reaction (PCR) amplification of RNA extracted from poly I:C induced rainbow trout gonad cells (RTG-2). Mx had been previously identified in rainbow trout by Staeheli et al.(1) by hybridization with a partial perch genomic Mx probe to induced rainbow trout mRNA. The 2.5 Kb rainbow trout cDNA clone contains an open reading frame of 1863 nt encoding a 621 amino acid protein. The deduced rainbow trout Mx protein is 70.6 kD and contains the characteristic tripartite GTP binding motif common to all Mx proteins. Southern blot analysis with the rainbow trout Mx probe demonstrated the presence of Mx homologous genes in four other salmonid fish species including chinook, coho, and kokanee salmon and brook trout. Poly I:C treatment of both RTG-2 and chinook salmon cells (CHSE-214) induced two transcripts whose appearance was observed first at 24 h and as long as 72 h after treatment. Infection of rainbow trout with the salmonid rhabdovirus, IHNV (infectious hematopoietic necrosis virus) also induced the synthesis of Mx mRNA. A comparison of the rainbow trout Mx protein with other reported Mx proteins indicates that the piscine Mx is highly homologous to the mammalian Mx proteins.

Introduction

The vertebrate IFN system provides an initial line of defense against viral infection by inducing the synthesis of proteins that inhibit virus replication. These intracellular mediators of viral resistance include such proteins as protein kinase P1, the 2',5'-oligoadenylate synthetase, and the Mx proteins (2-4). In the case of the Mx proteins, the role of these proteins in resistance to negative-stranded RNA viruses has been well established (5-8). Their antiviral activity has been shown to be dependent upon a GTPase activity that correlates with a consensus tripartite GTP binding domain in the amino terminal half of the protein (9). Although the actual mechanism for viral inhibition by Mx is not understood, studies with the nuclear murine Mx1 protein have shown that overexpression of the influenza virus polymerase protein, PB2, will counter the antiviral activity of Mx1. This finding suggests that murine Mx1 inhibits viral replication by interfering with the function of the viral polymerase (10). However, these authors did

not find a high affinity association between the murine Mx1 and the influenza polymerase subunit, PB2.

Mx genes are ubiquitous in nature. They are found in a wide variety of organisms including such vertebrates as humans (11, 12), mice (5), rats (7, 13), sheep (14), pigs (15), ducks (16), chickens (17), and perch (1). A gene resembling Mx has even been found in yeast (18). In humans, mice and rats, there are two to three Mx genes expressed in either the nucleus or cytoplasm of the cell (13, 19, 20). The presence of Mx genes in vertebrates as diverse as humans, mice, ducks and fish suggests these proteins must play an important role in the survival of these organisms. Although some wild strains of mice survive quite well with no functional Mx1 (21), it is possible that the Mx is important in the survival of the species over evolutionary time spans. The Mx proteins may not always be necessary, but they are necessary for survival often enough for them to be kept functional in a proportion of the population. Further, the high degree of conservation of Mx sequence and the similarities in the control of Mx expression, suggest they have similar functions and may interact with conserved cellular and/or viral mechanisms.

IFN-like activity has been reported for the tissue cultured cells of several fish species including grunt fin (22), fathead minnow (23), swordtail (Xiphophorus) (24), goldfish (24), carp (26), and rainbow trout (27, 28). In rainbow trout, this IFN-like substance can protect fish from several types of viruses including two rhabdoviruses, infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV); a birnavirus, infectious pancreatic necrosis virus; and an iridovirus, viral erythrocytic necrosis virus (VEN) (29,30). A flatfish (*Paralichthys olivaceus*) IFN cDNA has been cloned that confers resistance to a fish rhabdovirus on cultured fish cells (31). How fish IFNs induce antiviral resistance in cells has never been determined and the IFN-inducible mediators of antiviral activity in fish have not been well characterized. Thus, we were interested in defining the role of Mx proteins in the salmonid fish. A previous report by Staeheli et al. (1) provided evidence of a rainbow trout Mx gene in the RNA of a liver treated with poly I:C, a potent IFN inducer. The trout Mx-related sequences were detected with an Mx gene probe that had been originally isolated from perch. We report here the cDNA cloning of a rainbow trout Mx gene and demonstrate its expression *in vivo* in response to infection with the fish rhabdovirus, IHNV.

Materials and Methods

Cell Lines and Viruses

The chinook salmon embryo (CHSE-214) and rainbow trout gonad (RTG-2) cell lines used for propagating IHNV and producing mRNA were obtained from J.L. Fryer, Oregon State University, Corvallis. The cells were grown as monolayers in minimal essential medium (Gibco Laboratories) supplemented with 10% FBS and 2 mM L-glutamine. For virus propagation, CHSE-214 cells were supplemented with 5% fetal bovine serum, 2mM L-glutamine, 14mM HEPES (N-2-Hydroxyethylpiperazine - N'-ethane sulfonic acid), pH 7.8, 100 IU/ml Penicillin G, 100 μg/ml Streptomycin and 0.25 μg/ml amphotericin B. The IHNV used in this study was isolated in 1975 from an adult steelhead trout at the Round Butte Hatchery in Oregon. The chinook cells were infected with IHNV at multiplicity of infection of 0.001 and incubated at 16°C for 7 days. At that time the supernatant was harvested and centrifuged at 2,500 X g for 10 min at 4°C, then filtered through a 0.22 mm low protein binding filter (Gelman). The cell-free supernatant contained 7.5 X 10^6 tissue culture infective 50% doses per ml (TCID50/ml).

PCR Cloning of a Rainbow Trout Mx Gene Fragment

Primers for PCR were designed from the published partial sequence of a perch Mx genomic DNA sequence (1). The template was genomic DNA extracted from the liver of a rainbow trout, Shasta strain according to the method of Gross-Bellard (32). The forward primer 5'-TGAGGAGAAGGTGCGTCC-3' and the reverse primer 5'-GCGCCTCCAACACGGAGCTC-3' were used to PCR amplify a 135 bp target corresponding to nt 75 to 209 of the rainbow trout gene. Five cycles of 1 min, 95°C; 2 min, 45°C; 1 min, 72°C were followed by 30 cycles of 1 min, 95°C; 2 min 54°C and 1 min 72°C. The cycles were performed with final concentrations of 0.5 mM each primer, 200 mM each dNTP, and 2.5 U Taq polymerase in standard PCR buffer (33). The PCR product was cloned into the PCR II vector (Invitrogen) and sequenced.

Southern Blot and Northern Blots

Probes to the rainbow trout Mx genes were generated by PCR incorporation of digoxigenin into the rainbow trout Mx gene fragment as previously described by Lanzillo (34). DNA for Southern blots was extracted from rainbow trout livers by proteinase K digestion and phenol chloroform extraction as described (32). After extraction, 15 µg of DNA was cut with either EcoRI or BamHI (Promega) and Southern blots were carried out as described by Maniatis *et al.* (35) and Southern (36). Northern blots to detect rainbow trout Mx mRNA *in vitro* were performed using 150 cm2 cell monolayers induced with 50 µg/ml poly I.C (Boehringer Mannheim) and 200 µg/ml DEAE-dextran 200 in minimal essential media. *In vivo* studies were performed by injecting 40 gm rainbow trout with 2 mls of 1 mg/ml poly I.C or control phosphate buffered saline. RNA for Northern blots was extracted from cell monolayers or the frozen livers using RNAzol (Cinna Biotech) following the manufacturer's protocol. Total RNA was electrophoresed in formaldehyde gels and Northern blots performed as described by Maniatis et al. (35). After transfer to Nytran (Schleicher and Schuell) all blots were hybridized to the PCR-labeled digoxigenin probe (Boehringer Mannheim). For all blots, the hybridization and chemiluminescent detection were performed using the methods of Holtke et al. (37) with modification of the hybridization and washing temperatures. The hybridization solution was 50% formamide with 5X SSC (1X SSC is 0.15 M NaCl, 0.015 M Na citrate), 2% wt/vol blocking solution (Boehringer Mannheim) 0.1% (wt/vol) N-lauryl sarkosine and 0.02% (wt/vol) SDS. Membranes were washed twice for 5 min at room temperature with 2X SSC, 0.1% SDS, followed by two washes of 15 min each at 55°C with 0.1X SSC, 0.15% SDS.

RACE Cloning of a Full Length Rainbow Trout Mx cDNA

Both 5' and 3' RACE amplifications were carried out essentially as described (38) with the modifications of Jain and Gomer (39). For 5' RACE, reverse transcriptions were carried out on 5 µg total RNA obtained from RTG-2 cells after treatment with poly I.C for 24 hours. The RNA was resuspended in 11 µl of diethylpyrocarbonate-treated water with 1 µl (20 U) of RNAsin (Promega) and the mixture was heated to 65°C for 3 min followed immediately by incubation on ice. The RNA was then added to the prepared reverse transcription mix consisting of 4 µl 5X

reverse transcription buffer (1X reverse transcription buffer is 50 mM Tris HCL, pH 8.3, 40 mM KCL, 10 mM DTT, 7 mM MgCl2, 0.1 mg/ml BSA), 2 µl of 2mM of each of the four deoxynucleoside triphosphates (dNTP's), 0.8 µl of 20 pmol of the first antisense gene specific primer 5' CTGGTCGCCTATCACGGC 3', and 1 µl (200U) M-MLV reverse transcriptase (Promega) for a total volume of 20 µl. The reverse transcription was carried out at 42°C for 1 hour, then 52°C for 30 min., and the reaction was stopped by incubation at 95°C for 5 min. The RNA template was then removed by digestion with RNAseH (0. 4U) (Promega) for 20 min at 37°C followed by an incubation at 95°C for 5 min. Unincorporated primers were removed by filtration centrifugation in a Centricon 100 spin filter (Amicon) and the cDNA pool was concentrated to 10 µl in a speed vacuum. A polyA tail was synthesized onto the ends of the cDNA with 12 U terminal transferase (Promega) in 5 µl of 5X tailing buffer (1X tailing buffer is 100mM cacodylate buffer pH 6.8, 1mM CoCl2, and 0.1mM DTT), 5 µl of 1 mM dATP after incubation at 37oC for 10 min. The reaction was then heated at 95°C for 5 min, and diluted with 400 µl of TE (10 mM Tris-HCL, pH 7.8, 1 mM EDTA pH 8.0) to make a cDNA pool.

An aliquot (5 µl) of the cDNA pool was amplified by PCR with 5 pmol of the poly dT RACE primer 5' GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT 3' and 25 pmol of the first gene specific primer in standard PCR buffer. The reaction was heated to 95°C for 5 min., cooled to 72°C, and 1 µl Taq (4U) added, annealed at 45°C for 5 min, and extended at 72°C for 20 min. This was followed by 3 cycles of 1.5 min., 48°C for 1.5 min., and 72°C for 2 min. This initial amplification reaction was filter-centrifuged to remove unincorporated primers, and then PCR amplified with 25 pmol of the RACE adapter primer 5' GACTCGAGTCGACATCGA 3' and 25 pmol of the second nested gene specific primer 5' CAGCGCAAGGTCCTTCTC 3' in standard PCR buffer. The reaction was heated to 95°C for 3 min followed by 35 cycles of 95°C, 1 min, 48°C, 1.5 min., 72°C, 2.0 min and a final 72°C 10 min extension. Using this procedure a 221 bp PCR product was cloned into the PCRII cloning vector (Invitrogen) and designated RBTMx5-C3.

For the 3' RACE procedure the reverse transcription was carried out as described above except 400 ng of poly A+ RNA was used for template with 20 pmol of the poly dT RACE primer. After reverse transcription the reaction was diluted to a final volume of 1 ml in TE. PCR amplification was carried out with 25 pmol of the RACE adapter primer and 25 pmol of a sense gene specific primer, 5' TACGAAGAGAAGGTGCGG-3',

constructed from the sequence of the 5' RACE clone RBTMx5-C3. The reaction was heated to 95°C, cooled to 72°C and then Taq polymerase was added. The mixture was then incubated at 48°C for 5 min and 72°C for 3 min, followed by 35 cycles of 95°C, 1 min., 48°C, 1.5 min., 72°C, 2.0 min. and a final 72°C 10 min extension. A clone, RBTMx3-1, of 2406 nt was obtained and subsequent sequence analysis of this clone indicated that it overlapped the 5' clone by 54 bp. Confirmation that these two clones originated from the same mRNA sequence was obtained from a second 3' RACE-PCR using two gene specific primers derived from the untranslated regions of the 5' and 3' RACE clones. Two clones, RBTMx1-1 and RBTMx1-2, from two separate PCR reactions were generated containing the complete open reading frame. A consensus sequence of the four clones, including the entire 5' and 3' noncoding regions as determined by RACE PCR, is reported here.

Nucleotide Sequence Analysis

Sequencing was performed at the Oregon State University's Center for Gene Research and Biotechnology Central Services Facility on a 373A DNA sequencer (Applied Biosystems, Inc., Foster City, CA). The Taq Dyedeoxy terminator cycle sequencing kit with Amplitaq (Perkin Elmer) was used. The entire sequence of the clones was determined on both strands.

The coding regions of the Mx gene sequences for; human MxA, human MxB, murine Mx1, murine Mx2, rat Mx1, rat Mx2, rat Mx3, perch partial Mx clone, pig Mx1, Duck Mx clone 15, and Chicken Mx were obtained from genetic data banks, GenBank and EMBL. For the percentage similarity calculations of Table 1 each Mx protein sequence was aligned separately with the rainbow trout Mx protein sequence using the Bestfit program (40) in Genetics Computer Group (GCG) sequence analysis set of programs. The yeast VSPo1 protein (18) was also used in this comparison. A phylogenetic tree of Mx genes was constructed using the Genetics Data Environment (GDE) set of programs for manipulating and analyzing genetic data developed by Steven Smith (University of Illinois and Harvard University). The Mx protein sequences were aligned using ClustalV (41) with minor adjustment. Only selected regions for which the protein alignment was reasonably certain were compared. The regions used in the comparison were: position 107-179, 186-485, 502-615, and 650-736 (Figure 3.5). The

Figure 3.1 Salmonid fish genome contains Mx related genes. Liver tissue was harvested and frozen from euthanized adult salmon and trout. The extracted genomic DNA was digested with EcoRI (lanes 2, 4, 6, 8, 10) or BamHI (Lanes 3, 5, 7, 9, 11). The restricted DNA was electrophoresed in agarose and transferred to membranes for Southern blotting. Specific hybridization of Mx-specific sequences was detected with a 135 nt digoxigenin-labeled probe to rainbow trout Mx. Lane 1, 100 pg plasmid control vector with the rainbow trout 135 nt Mx-gene insert; lanes 2 and 3, rainbow trout (Rbt) (Oncorhynchus mykiss); lanes 4 and 5, brook trout (BrT) (Salvelinus fontinalis); lanes 6 and 7, coho salmon (Coh) (O. kisutch); lanes 8 and 9, chinook salmon (Chn) (O. tshawytscha); lanes 10 and 11, kokanee salmon (Kok) (O. nerka).

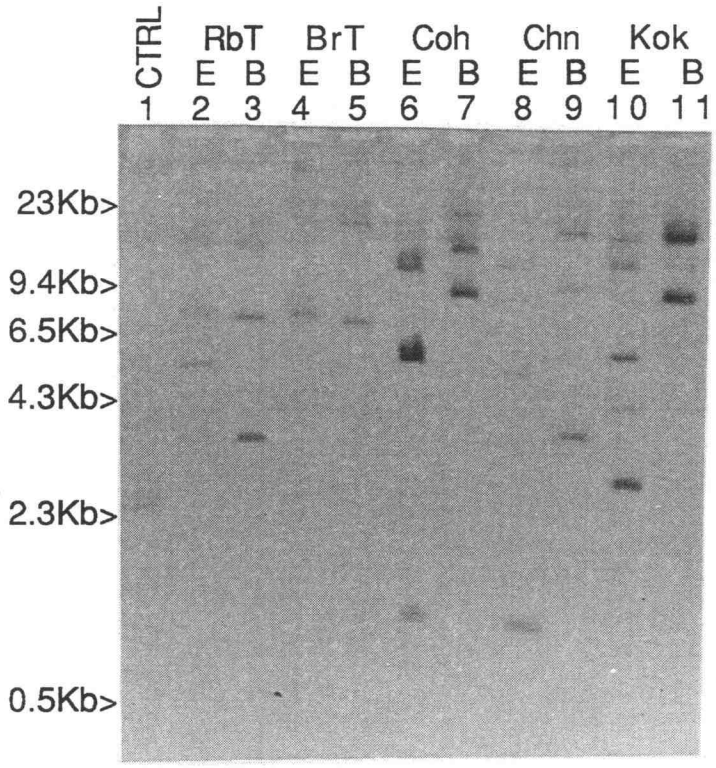
CTRL
RbT
BrT
Coh
Chn
Kok
E B E B E B E B E B
1 2 3 4 5 6 7 8 9 10 11
23Kb>
9.4Kb>
6.5Kb>
4.3Kb>
2.3Kb>
0.5Kb>

Figure 3.1

DeSoete least squares algorithm for fitting additive trees to proximity data with the Treetool program of GDE (42) was used to construct the distance trees. Parsimony analysis was performed using the protein parsimony program of PHYLIP 3.5 (43) by Joe Felsenstein in GDE.

Results

Salmonid Fish Contain Mx Gene Homologs

Primers for PCR amplification of fish genomic DNA were generated for Mx genes by selecting a conserved sequence common to that reported for the perch and the mammalian Mx genes. These primers amplified a 135 bp fragment from rainbow trout genomic DNA whose sequence was found to be highly homologous to Mx. The sequence corresponded to nt 171 to 305 of the trout Mx gene shown in Figure 3.4. A comparison of the 30 amino acid sequence derived from this 135 bp fragment with that of the perch Mx sequence indicated 100% conservation. This region of mammalian Mx proteins is also highly conserved; there is only one amino acid difference in this region of the trout Mx protein in comparison to most of the mammalian Mx proteins with the exception of the murine Mx1 protein which has two amino acid differences. The 135 bp trout Mx fragment was used to probe the genomic DNA of several different species of salmonid fish in a Southern analysis (Figure 3.1). All of the species tested contained Mx homologous sequences that were present in two to five bands in DNA digested with either EcoRI or BamHI. Hybridization of the 135 bp probe to multiple restriction fragments suggested that salmonids have more than one Mx gene. These results are in marked contrast to that obtained for the genome of the perch (*Perca fluviatis*) where only a single band was detected in EcoRI digested DNA that hybridized to a probe of 831 nt fragment from the 5' end of the murine Mx1 gene (1).

Salmonid Mx mRNA is Inducible In Vitro by Poly IC

Two salmonid cell lines were tested for Mx gene expression after poly IC induction. RTG-2 and CHSE-214 cells were induced with poly IC and the induced total RNA from each cell preparation was examined by Northern blot analysis with the 135 bp

Figure 3.2 Appearance of Mx mRNA with time after induction by poly IC in fish cells. CHSE-214 (A) and RTG-2 (B) cells were induced with 50 mg/ml poly IC in 200 mg/ml DEAE dextran and total cellular RNA extracted for RNA blot analysis. At five different time points (0, 12, 24, 48, and 72 h posttreatment), cells were harvested and RNA extracted. The RNAs (20 mg/sample) were separated on a formaldehyde-agarose gel and transferred to a nylon membrane. The Mx-specific RNA was detected by hybridization to the 135 nt rainbow trout Mx probe. Lane 1, Control at time 0; lanes 2, 3, 4, and 5 respectively are 12 h, 24 h, 48 h, and 72 h postinduction.

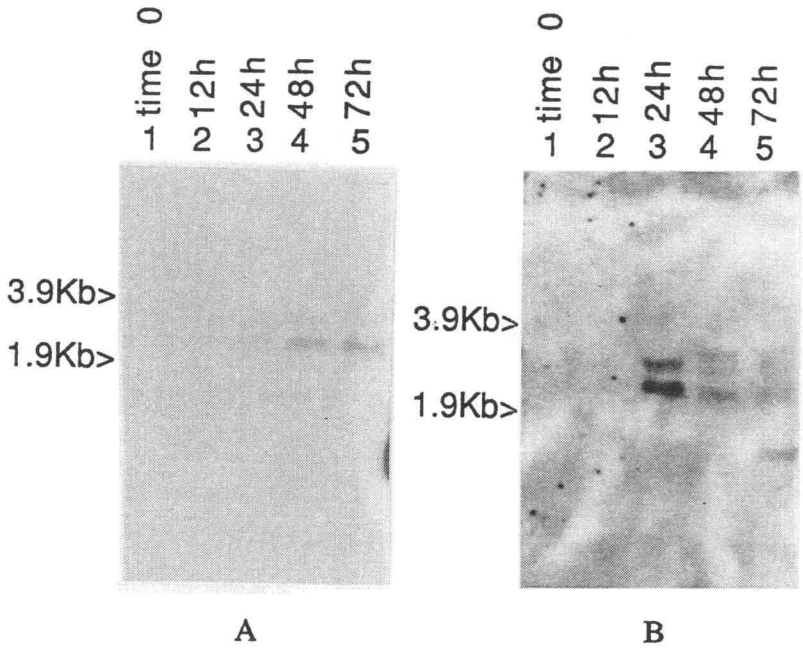
time 0
1
2 12h
3 24h
4 48h
5 72h
3.9Kb>
1.9Kb>
A
time 0
1
2 12h
3 24h
4 48h
5 72h
3.9Kb>
1.9Kb>
B

Figure 3.2

rainbow trout Mx fragment (Figure 3.2). In RTG-2 cells, two transcripts of approximately 2.9 and 2.6 Kb were detected at 24 h. At 48 h post induction three transcripts of 3.1, 2.9, and 2.6 Kb were observed. At 72 h, the detectable Mx transcripts were substantially decreased. Each lane contained 10 mg of total cellular RNA. The time and duration of appearance of the Mx transcripts in CHSE-214 cells was very different. At 24 h after poly I:C treatment, CHSE-214 cells produced two transcripts of approximately 3.4 and 2.5 Kb and the apparent amount of Mx mRNA per cell increased to 72 h postinduction. No hybridization signal was present in any untreated control cells. It was not determined whether the altered RTG-2 mRNA size transcripts observed at 48 h resulted from differentially polyadenylated transcripts, as reported for the human MxB gene (11), or from alternative splicing.

Mx mRNA Synthesis is Induced In Vivo

It had been shown previously that rainbow trout express two Mx mRNA transcripts upon induction with poly I.C or with Newcastle Disease Virus (1). In order to determine whether the fish rhabdovirus, IHNV, was capable of inducing Mx expression as well, rainbow trout were injected with either phosphate buffered saline (PBS), poly I:C, or live IHNV. The fish were sacrificed at 2, 4, 6 and 8 days after the injections and the livers were removed for RNA extraction. Northern blot analysis of the liver RNA showed two mRNAs of approximately 2.9 and 2.6 Kb at 2 days after injection with either poly I:C or IHNV (Figure 3.3). By day 4 after induction, these Mx transcripts were almost completely gone and by day 8, no transcripts were detectable. Liver RNA from the PBS-injected control fish taken at the same times did not contain any observable Mx RNA (data not shown). The liver RNA from untreated fish also did not contain any detectable Mx RNA species.

Sequence Analysis of the Rainbow Trout Mx Gene

A full length rainbow trout Mx gene was amplified at both the 5' and 3' ends by RACE-PCR . The 2.5 Kb rainbow trout Mx sequence and its deduced amino acid sequence is shown in Figure 3.4. The RBTMX1 consisted of 2,514 nt followed by a poly (A) tail. A single long open reading frame from the ATG start at nt 145 to a TAG

Figure 3.3 Induction of Mx mRNA synthesis in rainbow trout liver. Rainbow trout were injected intraperitoneally with either poly I:C, live IHNV, or phosphate buffered saline. At five different time points (0, 2, 4, 6, and 8 days postinjection), a trout from each treatment group was euthanized and the liver tissue was excised and frozen in liquid nitrogen. Total RNA was extracted from these livers. The RNA (20 mg/sample) was analyzed by Northern blotting. Lane 1, positive control RNA from CHSE-214 cells treated with poly I:C for 48 h; lane 2, untreated liver cellular RNA; lanes 3, 4, 5, and 6 respectively are RNAs from fish treated with poly I:C for 2, 4, 6, and 8 days postinjection; and lanes 7, 8, 9, and 10 respectively are RNAs from fish infected with IHNV at 2, 4, 6, and 8 days postinfection.

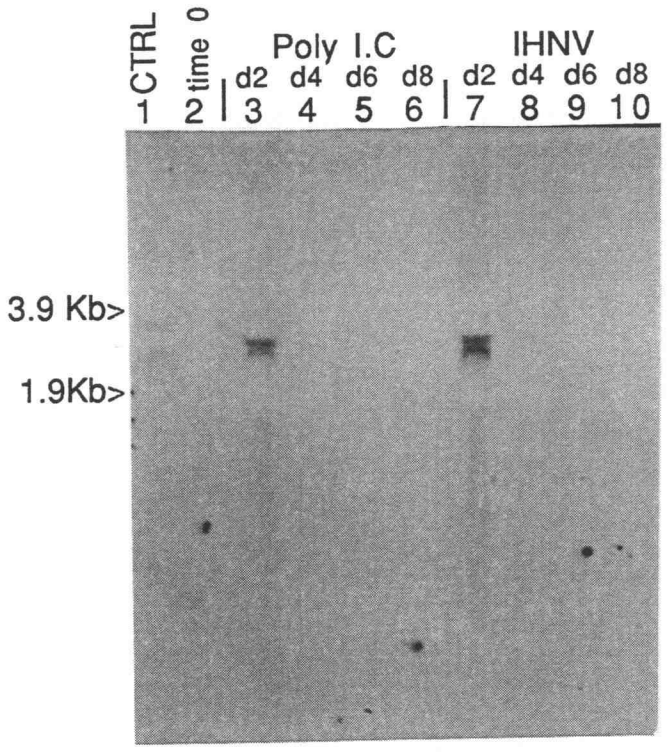
CTRL
time 0
Poly I.C
IHNV
d2 d4 d6 d8
d2 d4 d6 d8
1 2 3 4 5 6 7 8 9 10
3.9 Kb>
1.9Kb>

Figure 3.3

Figure 3.4 Rainbow trout Mx clone RBTMX1 cDNA sequence. The sequence of the rainbow trout Mx1 gene was determined for three separate clones obtained from three separate PCR amplifications. The tripartite GTP binding consensus domain is enclosed by boxes and the putative leucine zipper is depicted in the region by circles enclosing leucine residues. The circled methionine residue lines up with the putative leucine zipper repeats of Mx sequences.

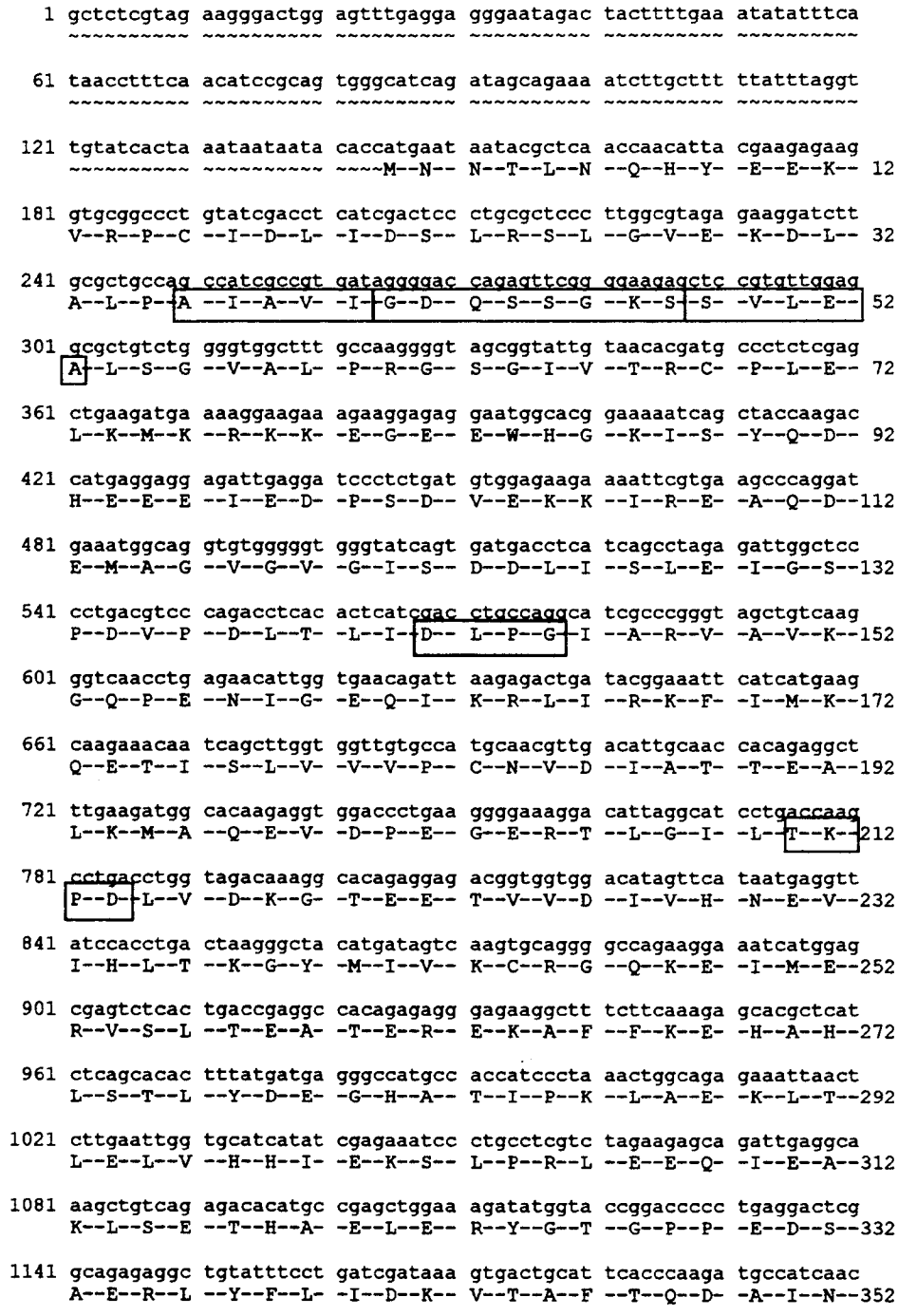

Figure 3.4

```
1201 ctgagcactg gggaggagat gaaaagcgga gttcgtctca acgtcttctc cacactcaga
     L--S--T--G --E--E--M- -K--S--G-- V--R--L--N --V--F--S- -T--L--R--372

1261 aaagagtttg ggaaatggaa gttacacctg gaacgctctg gagaaatctt taaccagagg
     K--E--F--G --K--W--K- -L--H--L-- E--R--S--G --E--I--F- -N--Q--R--392

1321 attgagggag aagtggatga ttatgagaag acgtaccgtg gaagggagct cccagggttc
     I--E--G--E --V--D--D- -Y--E--K-- T--Y--R--G --R--E--L- -P--G--F--412

1381 atcaactaca agacatttga ggtgatggtg aaagaccaga tcaaacaact ggagggacca
     I--N--Y--K --T--F--E- -V--M--V-- K--D--Q--I --K--Q--L- -E--G--P--432

1441 gcagtcaaga aactgaagga gatttcagat gccgttagga aggtgttctt actgctggct
     A--V--K--K --L--K--E- -I--S--D-- A--V--R--K --V--F--L- -L--L--A--452

1501 cagagcagct tcactggatt tcctaacctc ctgaaatcag cgaagacaaa gattgaggcc
     Q--S--S--F --T--G--F- -P--N--L-- L--K--S--A --K--T--K- -I--E--A--472

1561 attaagcagg tgaatgagtc tacagctgag tccatgttga ggactcagtt caagatggag
     I--K--Q--V --N--E--S- -T--A--E-- S--M--L--R --T--Q--F- -K--M--E--492

1621 ctgatagtgt acacacagga cagcacctac agccacagtc tgtgtgagag gaagagggag
     L--I--V--Y --T--Q--D- -S--T--Y-- S--H--S--L --C--E--R- -K--R--E--512

1681 gaggacgaag accaaccctt aactgagata aggagtacga tctttagcac agacaaccat
     E--D--E--D --Q--P--L- -T--E--I-- R--S--T--I --F--S--T- -D--N--H--532

1741 gccaccctac aggagatgat gctgcacctc aagtcctact actggatatc cagtcagcgt
     A--T--L--Q --E--M--M- -L--H--L-- K--S--Y--Y --W--I--S- -S--Q--R--552

1801 ctggctgatc agattcccat ggtgatccgc tacctggtgc tgcaggagtt tgcttcccag
     L--A--D--Q --I--P--M- -V--I--R-- Y--L--V--L --Q--E--F- -A--S--Q--572

1861 ctgcagaggg agatgcttca gactctgcag gagaaggaca acatcgagca gctgctgaag
     L--Q--R--E --M--L--Q- -T--L--Q-- E--K--D--N --I--E--Q- -L--L--K--592

1921 gaggacatcg acatcggcag caaaagggct gcactgcaga gcaagctcaa acgcctgatg
     E--D--I--D --I--G--S- -K--R--A-- A--L--Q--S --K--L--K- -R--L--M--612

1981 aaggcacgca gctacctagt tgagttctag tatggacagc tgcttgttaa catttaggat
     K--A--R--S --Y--L--V- -E--F--*                                  621

2041 ggtcttgact aatgccagat ggattggtca atggaagtag acctacaggt gtgtttcagt

2101 ggggtttggc gtagattctg tgatctcagt tcattgcata gtaaatgtaa tagaagttac

2161 tatgcaatca agtcctttgt tatccttttt tgttgaatag taaactaaaa tgtctgatga

2221 acgcaagcag ctagtctacc aggtggagtg ggaacctgcc tgataacatt tgggaacaga

2281 gaaggaagtg caatcatttt attgaaaggc aatgtataac ttgtggctta tattcctgct

2341 gacatacatt ttattgttat tgtgtctcct ttttatcatg tctggatggt gggcctgtgg

2401 accactgggc atcaactaat ggcctcacaa ttagggttta tctatgttaa accatgttgt

2461 atatttctta taatttcttt aaaaacattt aagccaaata aaaaatgtgt gaagaaa
```

Figure 3.4 (Continued)

stop at nt 2008 encoded a 621 aa polypeptide with a predicted molecular weight of 70,560 D. The deduced amino acid composition of the RBTMx1 is very hydrophilic, and rich in both positively and negatively charged amino acids: 16.1% basic residues (5.3% Arg, 2.4% His, 8.4% Lys), and 17.1% acidic residues (5.5% Asp, 11.6% Glu) and 24.3% other polar residues (2.3% Asn, 0.8% Cys, 4.7% Gln, 6.6% Ser, 5.6% Thr, 0.5% Trp, and 2.4% Tyr). In this regard, the trout Mx1 protein is similar to other Mx proteins (5, 11).

The putative translational start sequence of the rainbow trout Mx (TAA)TACACCATGA does not conform to the consensus start sequence for vertebrate genes, (GCC)GCCGCCA/GCCATGG (44) but does fit the reduced consensus start sequence, A/GNCATG, of Cavener and Ray (45). Other published Mx mammalian start sequences have poor homology to these consensus sequences (11, 15). There are no additional ATG start codons upstream of the proposed ATG start codon but there are two in-frame and one out-of-frame stop codons (TAA) before the start codon. The 3' untranslated region contains 411 nt and has a -AAUAAA- polyadenylation signal 18 nt upstream of the poly A tail.

Comparison of the Rainbow Trout Mx Protein to Other Mx Proteins

The alignment of the deduced rainbow trout Mx protein sequence with 12 other Mx proteins is shown in Figure 3.5. Mx proteins have the highest similarity in the amino terminal halves and all Mx proteins, including the rainbow trout Mx, contain a GTP binding tripartite consensus sequence marked by boxes. The percentage of identical amino acids between the rainbow trout Mx protein and other Mx proteins is shown in Table 1. These figures were calculated by pairwise alignments of the rainbow trout with each Mx protein. Only the regions that could be aligned with confidence were used in the comparison and these regions are given in Table 3.1. The greatest conservation of sequence between the rainbow trout deduced amino acid sequence and that of the other Mx proteins was found in the amino terminal half of the protein containing the consensus GTP binding sites. There was less similarity of sequence in the carboxyl terminal half of the protein; yet, there was still conservation at several amino acid positions and few gaps were required in the alignment. An unrooted distance tree relating the rainbow trout Mx gene with other reported Mx genes was constructed with Treetool, an algorithm for measuring similarities between genetic sequences (42). The neighbor-joining tree with

added bootstrap values from a parsimony analysis is shown in Figure 3.6 and resulted from an analysis restricted to those regions of the Mx genes that could be aligned with confidence as shown in Figure 3.5. The analysis indicated that there were four phylogenetically distinct classes of Mx genes consisting of the avian Mx genes, the fish Mx genes, the human MxB gene, and a class containing a cluster of all the other mammalian Mx genes. The general topology of the neighbor-joining distance tree depicted in Figure 3.6 remained unchanged when the sequence comparisons were made with the entire nucleic acid sequence, with the protein sequence, or with sequences restricted by selection for optimal alignment. The same distribution of Mx genes was also observed when the sequences were used to generate distance matrix trees or maximum parsimony trees (data not shown). In all analyses the branch orders corresponded to the accepted phylogenetic relationships of the organisms.

Discussion

A cDNA clone to a rainbow trout Mx gene has been constructed from rainbow trout liver RNA extracted after exposure of the fish to poly I:C. Sequence analysis of the cloned Mx cDNA indicated that the trout Mx gene is highly conserved in comparison with other reported Mx genes. In mammals, the expression of the Mx genes is induced by IFN and is correlated with resistance to the myxovirus, influenza, and to the rhabdovirus, VSV. In particular, the nuclear rat Mx1, the cytoplasmic rat Mx2 (7), the functional murine Mx2 (43), and the cytoplasmic human MxA (6) have been shown to confer resistance to rhabdovirus infection. Transcription of the trout Mx gene was also induced by exposure to poly I:C or infection with the IHNV, both potent IFN inducers. The similarity of these findings with those of the mammalian Mx genes suggest that the trout Mx gene may have a similar role in virus resistance. These observations are important because rhabdoviruses are severe pathogens in fish and an understanding of how fish Mx proteins mediate virus resistance are very important in developing prophylactic control measures for the rhabdoviruses, IHNV and VHSV, in salmon and trout.

At least two or three Mx-specific bands were detected in a Southern blot of DNA from several different species of salmon with a probe to a highly conserved region of the Mx protein (Figure 3.1). These findings suggest that salmonid fish are like mammalian species and contain multiple copies of Mx genes. Whether the multiple copies of Mx in

Figure 3.5 Amino acid alignment of the rainbow trout Mx with other Mx sequences. The deduced amino acid sequence of RBTMx1 was aligned using the Clustal V protein alignment program (41). Dashes indicate gaps in the alignment and dots indicate identity.

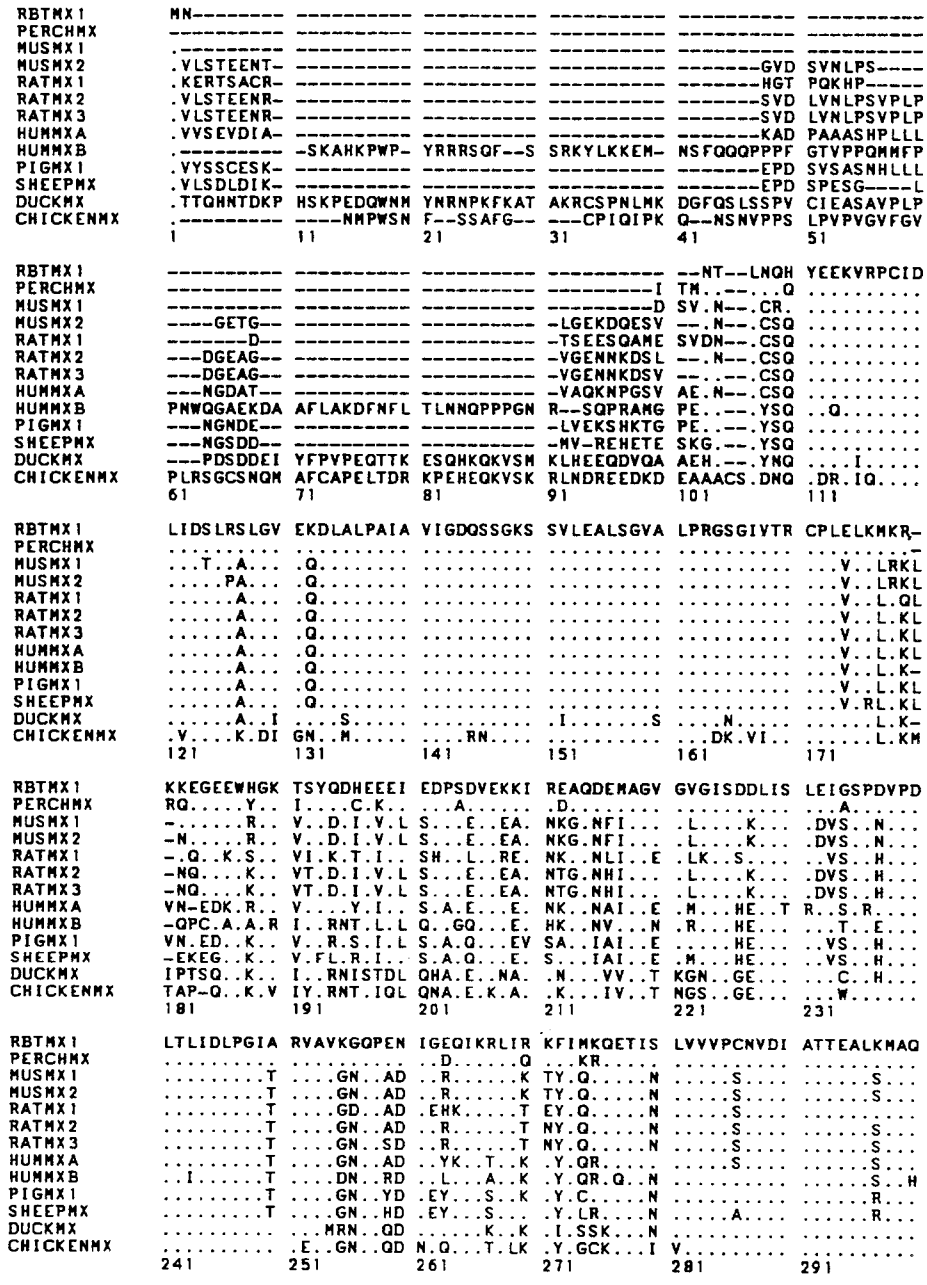

Figure 3.5

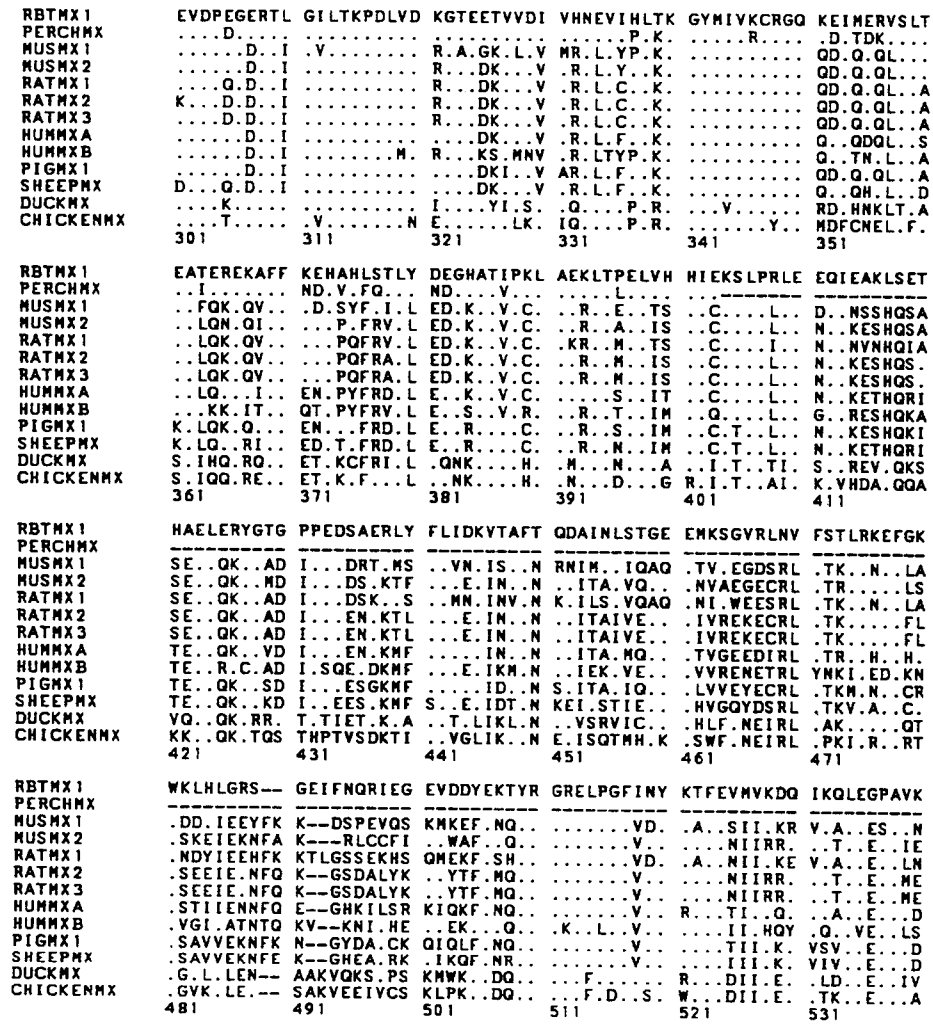

Figure 3.5 (Continued)

```
RBTMX1      YTQDSTYSHS LCERKRE--E DEDQPLT--- ---EIRSTIF STDNHATLQE MMLHLKSYYW
PERCHMX     ---------- ---------- ---------- ---------- ---------- ----------
MUSMX1      .C..QV.KET .KTIREK--. A.KEKTKALI NPATFQNNSQ FPQKGL.TT. .TQ...A..Q
MUSMX2      .C..QI.RGA .QKVRE.--. A.EEKK.--- KHGTSS.SQS QDLQTSSMA. IFQ..NA.RQ
RATMX1      .C..QA.KKA .Q.IREK--. A.KEKS.F-- --GAFQHNS- -PRKEL.TT. .TQ..NA..Q
RATMX2      .C..QI.RKA .QKVRE.--. A.EEER---- KHGKS..SQS KNLQTSSMD. IFQ..NA.RQ
RATMX3      .C..QI.RKA .QKVRE.--. A.EEER---- KHGKS..AQS PNLQTSSMD. IFQ..NA.RQ
HUMMXA      .C..QV.RGA .QKVREK--. L.EEKKK--- KSWDFGAFQS .SATDSSME. IFQ..MA.HQ
HUMMXB      FC..QI..VV .KKVRE.IFN PLGT.SQNMK LNSHFP.NES .V---SSFT. IGI..NA.FL
PIGMX1      .C..QV.RGA .QKVREK--. A.EEKNR--- KSNQYFLSSP APSSDPSIA. IFQ..IA.HQ
SHEEPMX     .C..QV.RRA .QQVREK--. A.EEKKK--- KSNHYYQSE- --.SEPSTA. IFQ..MA.HQ
DUCKMX      .C..NI.LDD .RST.A.--I LGKDGGK--- ---..SFASV .AK.AIFI.. .IS.T.A.FT
CHICKENMX   .C..NI.ADD .KAARA.GIS KDT------- KIKDLAFGCA .RQCPSFAL. .VS.V.A.FT
            601        611        621        631        641        651

RBTMX1      ISSQRLADQI PMVIRYLVLQ EFASQLQREM LQTLQEKDNI EQLLKEDIDI GSKRAALQSK
PERCHMX     ---------- ---------- ---------- ---------- ---------- ----------
MUSMX1      ECRRNIGR.. .LI.Q.FI.K T.GEEIEKM. ..L..DTSKC SWF.E.QS.T RE.KKF.KRR
MUSMX2      EAHN.ISSHV .LI.Q.FI.K M..ER..KG. ..L..D..SC SW....QS.T SE..KF.KER
RATMX1      ECGRNIGR.. .LI.Q.SI.. T.GQEMEKA. ..L..DTSKC NWF.T.QS.S RE.KKF.KRR
RATMX2      EAHN.ISSH. .LI.Q.FI.K M..EK..KG. ..L..D..SC SW....HS.T SE..RF.KER
RATMX3      EAHNCISSH. .LI.Q.FI.K M..EK..KG. ..L..D..SC SW....KS.T SE..RF.KER
HUMMXA      EA.K.ISSH. .LI.QFFM.. TYGQ...KA. ..L..D..TY SW....RS.T SD..KF.KER
HUMMXB      ET.K...N.. .FI.Q.FM.R .NGDS..KA. M.I....NRY SW..Q.QSET AT..RI.KER
PIGMX1      EVGK.ISSH. .LI.QFFI.R T.GQ...KS. ..L..N..QY DW..R.RS.T SD..KF.KER
SHEEPMX     EV.T.ISSH. .LI.QFF..R TYGE..KKS. ..L..D..QY DW....RT.T RD..KF.KER
DUCKMX      GA.K..SN.. .LI.LSA..H D.GDN..SS. .HL...REKL NS..Q.NSEA AKM.NY.SGR
CHICKENMX   GA.K..SN.. .LI.LST..H D.GNY..TS. .HL..G.EE. NY..Q..HEA ANQQKL.T.R
            661        671        681        691        701        711

RBTMX1      LKRLMKARSY LVEF------ *
PERCHMX     ---------- ---------- .
MUSMX1      .L..DE..QK .AK.SD---- .
MUSMX2      .A..AQ..RR .AK.PG---- .
RATMX1      .L..DE.QRK .AK.SN---- .
RATMX2      .A..AQ.QRR .AK.PG---- .
RATMX3      .A..AQ.QRR .AK.PG---- .
HUMMXA      .A..TQ..RR .AQ.PG---- .
HUMMXB      IY..TQ..HA .CQ.SSKEIH .
PIGMX1      .M..TQ..RR .AK.PG---- .
SHEEPMX     .E..SR..QR .AK.PG---- .
DUCKMX      VN..S..YQC .KD.SC---L .
CHICKENMX   ISH.N..YQ. ..D.KS---L .
            721        731
```

Figure 3.5 (Continued)

Figure 3.6 Mx Protein Parsimony tree. The amino acid alignment of Figure 3.5 was used to construct an unrooted parsimony tree of Mx proteins. Bootstrap values taken from 100 replicates are given at the nodes.

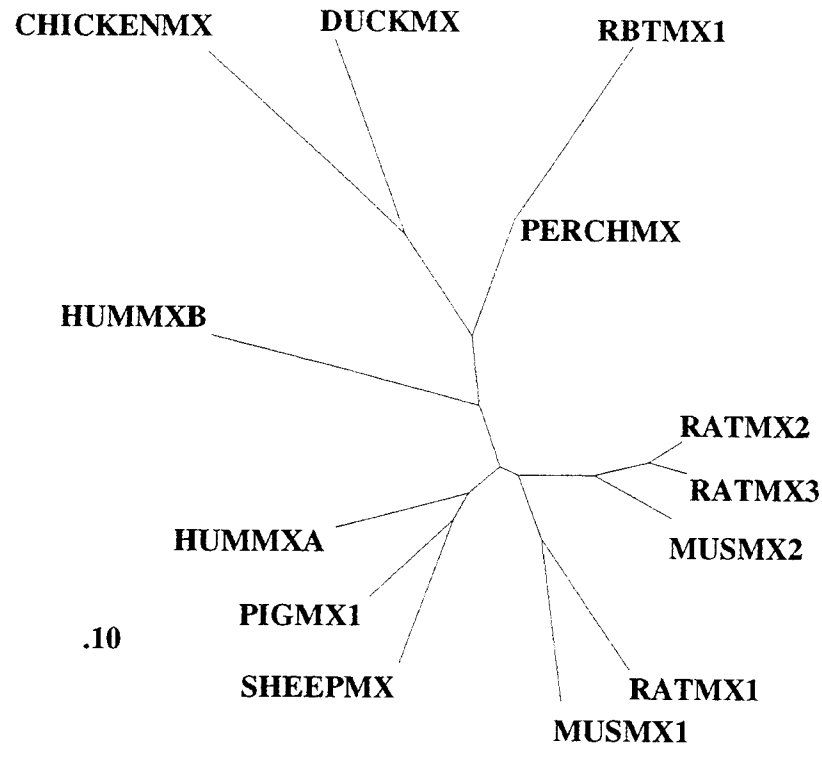
CHICKENMX
DUCKMX
RBTMX1
PERCHMX
HUMMXB
RATMX2
RATMX3
HUMMXA
MUSMX2
PIGMX1
.10
SHEEPMX
RATMX1
MUSMX1

Figure 3.6

Table 3.1 % sequence identity between rainbow trout Mx protein and the Mx proteins of perch, human, mice, rat, sheep, pig, duck, chicken, and the yeast Vsp1 protein.

Rainbow trout compared with:	Region Prot. Compared:	Region of RBT Mx	GenBank/EMBL Accession No.		Percent Identity
Perch Mx	aa 4-301	aa3-300	PEFMUR	M27252	89.3%
Human MxA	aa38-660	aa2-621	HUMMXA	M30817	55.2%
Human MxB	aa86-709	aa2-621	HUMMXB	M30818	52.5%
MusMx1	aa 5-629	aa3-621	MUSMX	M12279	52.5%
MusMx2	aa32-652	aa3-621	MUSMX2	J03368	56.7%
Rat Mx1	aa30-650	aa3-621	RNMX1	X52711	53.2%
Rat Mx2	aa37-657	aa3-621	RNMX2	X52712	54.6%
Rat Mx3	aa37-657	aa3-621	RNMX3	X52713	54.6%
Sheep Mx	aa33-652	aa2-621	OAMXHOMM	X66093	53.4%
Pig Mx1	aa38-661	aa2-621	PIGMX1A	M65087	54.2%
Duck Mx	aa99-718	aa2-621	APMXPC15	Z21550	53.1%
Chicken Mx	aa84-702	aa4-621	GDMXGNA	Z23168	48.7%
Yeast VSPS1	aa 3-702	aa10-620	YSCVPS1A	M33315	34.0%

the salmonid genome resulted from the chromosomal duplication event that produced the autotetraploid genome in these fish, or whether the multiple copies resulted from specific duplication of the Mx gene is not known. In the perch, however, only a single band was detected in the genomic DNA after digestion with either EcoRI, HindIII, or PstI (1). Interestingly the genome of the percidae is not considered tetraploid (47).

Northern blots of the Mx-specific mRNA induced upon poly I:C treatment or IHNV infection in rainbow trout revealed two distinct classes, a finding that supports the observation of Staeheli et al.(1). However, in RTG-2 cells, three different Mx-specific transcripts were detected upon induction with poly I:C. These transcripts may be similar to the alternatively spliced, unspliced or differentially polyadenylated RNA species of murine and human Mx RNA species. Primer extension analysis of murine Mx mRNA indicated a potential alternative splice site in the 5' region of the gene (43). Also, there were three transcript sizes for the human MxB mRNA; one transcript is thought to result from an alternative polyadenylation site (11).

RTG-2 cells have been shown to be resistant to viral infection after poly I:C induction. However, CHSE-214 cells were found to be have no detectable IFN activity after poly I:C treatment and remained sensitive to virus infection (49). These cells would respond to exogenously added IFN (30). These findings suggested that there was a defect in the regulation of IFN production in CHSE-214 cells. Mx transcripts were produced in CHSE-214 cells but the induction kinetics were very different from that observed for the RTG-2 cells. In RTG-2 cells maximum induction was reached at 24 h and no detectable RNA remained at 72 h. In CHSE-214 cells, the Mx gene transcripts were first observed at 24 h after induction and the quantity of Mx-specific RNA continued to increase until 72 h. One possible explanation for the differences between the cell lines is that in CHSE-214 cells, induction of Mx transcription is mediated directly by poly I:C or by an alternative, non-IFN mediator.

Comparison of the deduced amino acid sequence of the rainbow trout Mx protein with that of other Mx proteins shows a high degree of homology, especially in the amino terminal half of the protein. Phylogenetic analysis of the Mx gene family shows that the Mx genes branch in an order that correlates with the accepted evolutionary branching of the host organisms. Whatever the function(s) of the Mx in the host, an ancient Mx gene(s) has persisted, and in some cases, were duplicated with both Mx genes persisting in diverse organisms. This persistence suggests that the gene(s) are important to the survival of these species. Further, the similarity of sequence and control of expression of these genes suggests that they share common functions in their host. We have

recently obtained partial clones of two more distinct rainbow trout Mx genes (RBTMx2 and RBTMx3). Expression of the corresponding full length cDNAs will be necessary to elucidate the antiviral potential of these three fish Mx proteins.

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CHAPTER 4
CLONING OF THE RAINBOW TROUT MX2 AND MX3 PROTEINS AND CHARACTERIZATION OF TROUT MX PROTEINS BY EXPRESSION IN SALMON CELLS.

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This paper reports a portion of the work described in a thesis to be submitted to Oregon State University Department of Microbiology in partial fulfillment of the requirements for a PhD degree.

Abstract

Two rainbow trout (*Oncorhynchus mykiss*) Mx cDNAs were cloned and designated RBTMx2 and RBTMx3 using RACE (rapid amplification of cDNA ends) PCR. The deduced RBTMx2 and RBTMx3 proteins are 636 and 623 amino acids in length with molecular weights of 72 and 70.8 kD respectively. These proteins, along with the previously described RBTMx1 protein (chapter 3) share between 88.7 and 96.6% identity at the amino acid level. All three proteins contain the tripartite GTP binding domain and leucine zipper motif common to Mx proteins. Monospecific polyclonal antisera was generated to an *E. coli* expressed fragment of RBTMx3 that reacted to all three rainbow trout Mx proteins. Endogenous Mx production of RTG-2 cells induced with poly IC dsRNA was detcted by western blot. The nuclear localization of the rainbow trout proteins was determined by transient expression of the RBTMx cDNAs in a salmon cell line (CHSE-214). A single-cell transient transfection assay was used to test the Mx cDNA clones ability to inhibit replication of the fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV). Transient expression of the trout Mx proteins did not interfere with replication of the IHN rhabdovirus as evidenced by accumulation of the nucleoprotein, N.

Introduction

Mx proteins are a component of the IFN system of vertebrates and some members of this family are responsible for resistance to viral infection. Mx genes have been cloned from diverse vertebrate species such as humans (Aebi *et al.*, 1989; Horisberger *et al.*, 1990), rodents (Staeheli *et al.*, 1986; Meier *et al.*, 1988), piscine (Staeheli *et al.*, 1989; chapter 3), and avian hosts (Bazzhiger *et al.*, 1993; Bernasconi *et al.*, 1995). The Mx proteins are expressed either in the nucleus or cytoplasm and specific antiviral activity has been demonstrated for some members of the Mx protein family (for review see Arnheiter and Meier, 1990). Specific antiviral activity against the rhabdovirus VSV (Meier *et al.*, 1990; Zurcher *et al.*, 1992), influenza (Staeheli *et al.*, 1986; Pavlovic *et al.*, 1990), Dhori (Thimme *et al.*, 1995) and Thogoto orthomyxoviruses (Haller *et al.*, 1995), and the measles paramyxovirus (Schneider-Schaulies *et al.*, 1994) have been demonstrated. The role of certain Mx proteins in antiviral defense has been established, however the mechanism of their action is not yet well defined.

Mx proteins are 70 to 80 kD proteins that contain a tripartite GTP binding domain (Bourne *et al.*, 1991) essential to their antiviral activity (Nakayama *et al.*, 1991; Pitossi et al. 1993). Mx proteins also contain leucine zipper motifs near their carboxyl terminals (Melen *et al.*, 1992). This leucine zipper of murine Mx1 is thought to mediate the aggregation of murine Mx1 into dimers and trimers. The cytoplasmic human Mx proteins, and the nuclear rodent Mx proteins have a characteristic, highly punctate immunofluorescent staining pattern, suggesting aggregation of these proteins *in vivo* (Pavlovic *et al.*, 1992; Zurcher *et al.*, 1992; Meier *et al.*, 1990; Staeheli *et al.*, 1986).

There are Mx proteins such as the human MxB and the avian Mx proteins which have no defined antiviral activity to date. Further, the homology of Mx proteins to other GTPases not directly involved with viral defense such as the yeast vacuolar sorting protein (Rothman *et al.*, 1990) and to dynamins (Obar et al 1990; Chen *et al.*, 1991) has suggested that Mx proteins may play an essential cellular role.

Previously we have shown by RFLP analysis that the rainbow trout genome contains three distinct bands when hybridized with a rainbow trout Mx probe, suggesting the presence of at least three Mx genes (Trobridge and Leong, 1995). We previously reported the cDNA sequence of a Mx mRNA induced in raibow trout gonad cells (RTG-2) designated RBTMx1, and two partial Mx cDNA clones, RBTMx2 and RBTMx3. We have now obtained complete cDNA clones of the two additional rainbow trout RBTMx2 and RBTMx3 transcripts from the rainbow trout cell line RTG-2 and report these sequences here.

In addition, polyclonal antisera was generated against a recombinant RBTMx3 fragment. This antisera detected poly IC dsRNA induced endogenous Mx protein in RTG-2 cells. The cellular localization of the three trout Mx proteins was determined in transfected salmon cells by confocal microscopy. Using a single-cell Mx antiviral assay the anti-viral activity of trout Mx proteins against the fish rhabdovirus IHNV was also investigated.

Materials and Methods

Cloning of the Rainbow Trout Mx2 and Mx3 cDNAs

The RACE method used to obtain these clones has been described in detail (chapter 3). Briefly, after using PCR to obtain both the 5 prime and 3 prime ends of each RBTMx cDNA, primers were directed to the 5 prime and 3 prime noncoding regions to amplify the complete Mx2 (5' RACE primer ME 257 5'-TGCAGTTGCAGTGTATTAACC 3', 3' RACE primer ME 253 5' CCATTAACCAACCTATCTGAAG 3') and Mx3 (5' RACE primer ME 199 3' TTATCAGAGAGCAGGACACT, 3' RACE primer ME 253 5' TGAATATGTTGTTATCCTCC 3') ORF. To improve PCR fidelity PCR reactions were performed with reduced final concentrations of 2.5 mM magnesium, 100mM dNTPs, and 1U of Taq DNA polymerase in a total reaction volume of 50 µl. The denaturing, annealing, and extension times were reduced to 1 min, 1.5 min, and 1.5 min respectively. The number of cycles was reduced from 35 to 30, the minimum necessary to obtain enough product for subsequent cloning.

The PCR products were cloned into the PCRIII TA cloning kit (Invitrogen) following the manufacturers protocols. This vector contains the CMV immediate early promoter with transcription termination and polyadenylation signals from the bovine growth hormone gene. The control beta galactosidase (Beta Gal) construct, with the beta galactosidase gene inserted into the pcDNA3 vector (Invitrogen) was used as a control throughout the experiment. This vector also contains the CMV immediate early promoter and bovine growth hormone polyadenylation and transcriptional termination signals.

Nucleotide Sequence Analysis of Trout Mx cDNAs

Sequencing was performed at the Oregon State University Center for Gene Research and Biotechnology Central Services facility on an Applied Biosystems 373A DNA sequencer using the Taq dideoxy terminator cycle sequencing kit with AmpliTaq (Perkin Elmer). Both strands of each clone were sequenced. The deduced Mx protein sequences were aligned using Clustal V (Higgins *et al.*, 1992) using the Genetics Data Environment (GDE) set of sequence manipulation programs developed by Steven Smith (University of Illinois and Harvard University) with minor adjustment. Sequences were

compared using the Bestfit (Deveraux *et al.*, 1984) program in the Genetics Computer Group set of programs.

In Vitro Transcription and Translation of RBTMx1, 2, and 3

The RBTMx1, 2, and 3 cDNA clones were analyzed using the TnT T7/SP6 coupled reticulocyte lysate translation system (Promega). One μg of each plasmid DNA, along with a negative control of TE buffer used to resuspend the DNA samples, was added to the kit components following the manufacturers protocols. 4 μl of ^{35}S methionine (10 mci/ml) (Amersham) was added to each reaction. The coupled transcription/translation reaction was incubated at 30°C for 2h, mixed with an equal volume of 2X SDS sample buffer, and boiled for 3 min. 15 μl of sample in SDS running buffer was analyzed in a 12.5% SDS-polyacrylamide gel with a 5% stacking gel. The autoradiograph was scanned using a Molecular Dynamics densitometer using Imagequant software.

Preparation of Rainbow Trout Mx Antibodies

(i) Fusion protein construction. Polyclonal antisera was generated to a fragment of the rainbow trout Mx3 protein expressed in *E. coli* using the Qiaexpress (Qiagen) histidine tag system. A trout Mx fragment was generated with SphI and Bgl II ends compatible with the PQE-70 prokaryotic expression vector (Qiagen) using PCR. The forward primer, ME 224, 5'TAGGCATCCTGACCAAGCCTGAC 3' and the reverse primer ME 246 5'ACCATATCTTTCCAGCTCGGCATG 3' ,with engineered Sph I and Bgl II restriction sites respectively, were used to PCR a DNA fragment encoding a 114 amino acid fragment corresponding to amino acids 209 to 323 of RBTMx3 (genbank accession number OMU47946). A standard PCR reaction was run for 25 cycles and the resulting PCR product cloned into the TA cloning kit PCRII vector. The SphI to Bgl II fragment was then subcloned into the pQE-70 expression vector and transformed into *E. coli* M15 cells following the Qiaexpress protocols. Positive clones identified by restriction digest were confirmed by small scale induction of protein following the manufacturers protocol, and by sequencing.

(ii) Production of fusion proteins. Purified rainbow trout Mx fusion protein was prepared following the QIAexpress protocol for large scale purification of insoluble proteins. Briefly, histidine tagged fusion protein from clone PQE-MX7 was grown in large scale and induced with IPTG. The RBTMx fusion was purified using a nickel-NTA column collecting fractions in decreasing pH of guanidine hydrochloride buffer. Fractions containing Mx protein were pooled and dialyzed overnight in PBS. The resulting histidine tag purified fusion protein was analyzed by polyacrylamide gel electrophoresis in a 10% gel followed by coomassie blue staining.

(iii) Immunizations and antisera purification. 2 ml of purified Mx recombinant protein in PBS at 1.4 mg/ml was emulsified 1:1 with Fruends complete adjuvant (FCA) and injected subcutaneously into two New Zealand White female rabbits. Both rabbits were boosted subcutaneously at 4, 6, and 10 weeks with 1ml of 0.7 mg/ml emulsified 1:1 in FIA. Blood was collected, clotted overnight and 5ml of sera was adsorbed sequentially to two CHSE-214 monolayers in 150 cm^2 TCF. The adsorbed sera was IgG purified using a protein A purification kit (Pierce Immunopure IgG purification kit) following the manufacturers protocols.

Western Blot Analysis of Mx Antisera

For *in vitro* analysis of RTG-2 Mx protein, 15 µl of sample were loaded per lane. The membrane was then incubated in 25 ml of polyclonal rabbit ant-RBTMx at a 1:200 dilution of 1mg/ml IgG purified antibody in TBS with 1% BSA and 0.02% Tween 20 for 1h. The membrane was washed 4X with 100 ml of TBS with 1% BSA and 0.02% Tween. The membrane was then incubated with a 1:2000 dilution of 2 mg/ml goat anti rabbit-alkaline phosphatase (ProMega) for 1h in TBS-0.02% tween 20. The membrane was washed 4X in 100ml TBS-0.02% tween 20 and incubated in BCIP/NBT alkaline phosphatase substrate using the one step detection kit (Kierkegaard-Perry Laboratories).

Transfection and Immunofluorescent Detection

(i) Transfection. Tissue cultures were maintained as previously described in Trobridge and Leong, 1995. CHSE-214 cells (Fryer *et al.*, 1965) were plated at approximately 70 to 80% confluency on 8-chamber multiwell slides (Fisher) and allowed

to attach overnight. Cell monolayers were washed twice in Optimem (Gibco laboratories) before transfection. 1 μg of qiagen column purified RBTMx1, 2, 3, or beta galactosidase control plasmid DNA in the PCR III vector was mixed with 200 μl Optimem (Gibco) and then mixed with 200 μl Optimem containing 9 μl of Lipofectamine (Gibco). The 400 μl mixture was then incubated at RT for 1h and then Optimem was added to a final volume of 2 ml. The transfection mixture was then put on the monolayers of CHSE-214 cells at 0.2 ml per 1 cm^2 well and incubated at 17°C overnight. Following transfection the monolayers were washed twice in MEM with 5% fetal bovine serum and 100 IU/ml penicillin and streptomycin, and incubated for 3 or 4 days before challenge with IHNV virus.

(ii) Infection with IHNV and Immunofluorescence staining. The transfected monolayers were infected with IHNV (RB-1) at three moi's of 10, 1, and 0.1. or uninfected, and incubated at 17°C. 20h post-infection the monolayers were washed in 0.2 ml of ice-cold PBS and fixed for 20 min with 0.5 ml of freshly prepared 3% paraformaldehyde in PBS. Cells were then permeabilized with 0.5 ml of 0.1% triton X-100 for 10 min, followed by one wash in PBS. The monolayers were blocked with 5% nonfat dry milk in PBS for 30 min. Primary antibody incubations were carried out for 1h with 0.2 ml/chamber of either 1mg/ml rabbit Anti-RBTMx diluted 1:500, or 2 mg/ml rabbit anti-beta-galactosidase (Cappel laboratories) diluted 1:1000, and mouse anti-IHNV-nucleocapsid monoclonal 14D (Ristow and Arntzen, 1989) at 1:1000. The cells were then washed 3X for 5 min each wash and incubated with secondary antibodies. Secondary antibody concentrations were goat anti-rabbit Texas Red 2 mg/ml diluted 1:2000 (Molecular Probes Inc.), and goat anti-mouse fluorescein, 2 mg/ml diluted 1:1000 (Molecular Probes Inc.). Following staining, the slides were briefly air dried, treated with Slow Fade (Molecular Probes Inc.), and mounted with cytoseal. Slides were visualized on a fluorescent microscope with a triple band pass filter (Omega Optical). Confocal microscope images were captured using a Leica TCS4 confocal microscope.

Table 4.1 Comparison of Rainbow Trout Mx cDNA clones

		RBTMx1	RBTMx2	RBTMx3
Transcript length		2,514	2,089	2,113
Deduced amino acid length		621	636	623
Molecular weight		70.6 kD	72 kD	70.8 kD
% identity	RBTMx1	-	90.9	96.2
nt	RBTMx2	88.7	-	90.6
aa	RBTMx3	96.6	88.2	-

Results

Cloning of the Rainbow Trout Mx2 and Mx3 cDNAs

In order to improve the fidelity of PCR over our original RACE clones (chapter 3), some of which had interrupted open reading frames (ORF), we modified the PCR amplification of the reverse transcribed mRNA . Using the modified PCR procedure, the number of nucleotide differences between PCR clones dropped approximately threefold and we were able to obtain clones with contiguous full length ORFs of RBTMx1 and RBTMx3 suitable for transfection analysis of antiviral activity. Two complete clones for each of the RBTMx2 and RBTMx3 open reading frames (ORFs) were sequenced along with overlapping 5 prime and 3 prime clones to give a consensus cDNA sequence from four clones. The rainbow trout Mx2 and Mx3 sequences including 5 prime and 3 prime noncoding regions (RBTMx2 and RBTMx3) have been deposited in Genbank with the accession numbers OMU47945, and OMU47946 respectively. One full length clone for RBTMx1 called R1B1 and one full length clone for RBTMx3 called R3B5 were chosen for subsequent analyses. Both full length RTGMx2 clones, R2A2, and R2B1 contained interrupted ORFs, presumably from PCR amplification, so a contiguous RBTMx2 clone was constructed by replacing a 1.3 kb AccI to HpaI interrupted ORF fragment from R2B1, with its R2A2 uninterrupted counterpart. The resulting repaired RBTMx2 clone, RBTMx2rep, size was confirmed by restriction digest, and the AccI and HpaI ligation sites were confirmed by sequencing. The RBTMx1 and RBTMx3 full length expression clones differed from the consensus sequences deposited in Genbank at the following locations; the RBTMx1 clone has a serine in place of a glycine at aa 238, and the RBTMx3 expression clone R3B5 has a proline residue in place of a leucine at aa 165. These point mutations were considered to be reverse transcription errors, PCR errors, or allelic differences. Mutational analyses of the murine Mx1 protein has suggested that Mx proteins are refractory to point mutations in these regions (Melen and Julkunen, 1994) and these clones were thus judged suitable for transfection assay.

Figure 4.1 The Amino Acid Sequence of Rainbow Trout Mx1, Mx2 and Mx3. The amino acid ORFs from RBTMx1, RBTMx2 as Mx3 as deduced from the cDNA sequences (Genbank Acc. #U30253-RBTMx1,OMU47945-RBTMx2, OMU47946-RBTMx3) are shown. The tripartite GTP-binding domain of the Mx proteins are underlined. The putative leucine zipper repeats are in bold typeface and the potential N-linked glycosylation sites are marked with an asterisk. Sequence identity is represented by dots and sequence gaps are represented by dashes.

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        *
RBTMx1 MNNTLNQHYE EKVRPCIDLI DSLRSLGVEK DLALPAIAVI GDQSSGKSSV LEALSGVALP
RTGMx2 ..Y..... .....S.... .......... .......... .......... ..........
RTGMx3 .......... .......... .......... .......... .......... ..........

RBTMx1 RGSGIVTRCP LELKMKRKKE GEEWHGKISY QDHEEEIEDP SDVEKKIREA QDEMAGVGVG
RTGMx2 .......... .......... .......... ..R....... ....N...K. ..........
RTGMx3 .......... ........R. .......... .......... .......... ..........

RBTMx1 ISDDLISLEI GSPDVPDLTL IDLPGIARVA VKGQPENIGE QIKRLIRKFI MKQETISLVV
RTGMx2 .......... .......... .......... .......... .......... T.....N...
RTGMx3 .......... .......... .......... .......... .......... ......N...

RBTMx1 VPCNVDIATT EALKMAQEVD PEGERTLGIL TKPDLVDKGT EETVVDIVHN EVIHLTKGYM
RTGMx2 .......... .......... .D........ .......... .......... ...Q......
RTGMx3 .......... ...Q...... .......... .......... .......... ..........

RBTMx1 IVKCRGQKEI MERVSLTEAT EREKAFFKEH AHLSTLYDEG HATIPKLAEK LTLELVHHIE
RTGMx2 .......... .......... .......... .......... .......... ......Q...
RTGMx3 .......... .......... .......... .......... .......... ..........
                                                      *
RBTMx1 KSLPRLEEQI EAKLSETHAE LERYGTGPPE DSAERLYFLI DKVTAFTQDA INLSTGEEMK
RTGMx2 ..M...K... .E..E..RTA ..KC...... .PK....... ....L..... ........L.
RTGMx3 .......... .......... .......... .....I.... .......... ........L.

RBTMx1 SGVRLNVFST LRKEFGKWKL HLERSGEIFN QRIEGEVDDY EKTYRGRELP GFINYKTFEV
RTGMx2 ..-DI..... ..T......A YVD...KN.. KK..K..A.. ..R....... ..........
RTGMx3 .......... ..Q....... ..D....N.. .......SN. .......... ..........
                                                           *
RBTMx1 MVKDQIKQLE GPAVKKLKEI SDAVRKVFLL LAQSSFTGFP NLLKSAKTKI EAIKQVNEST
RTGMx2 I......... E........L ...A..A.I. ...N...... I...T..... .T...EK...
RTGMx3 .......... E......... .......... .......... .......... ..........

RBTMx1 AESMLRTQFK MELIVYTQDS TYSHSLCERK REE--DEDQP LTE------- ------IRST
RTGMx2 ...T...... .......... ...S..KK.. ...EEL.EGE .VKNNLGSWK GLPVVSV...
RTGMx3 .......... ..M....... ......S... .....ED.R. .PT....... ....PK....

RBTMx1 IFSTDNHATL QEMMLHLKSY YWISSQRLAD QIPMVIRYLV LQEFASQLQR EMLQTLQEKD
RTGMx2 VNGL.T.... R......... .H.A...... .......... .......... ..........
RTGMx3 .......... .......... .R........ .......... .......... ..........

RBTMx1 NIEQLLKEDI DIGSKRAALQ SKLKRLMKAR SYLVEF
RTGMx2 .......... .......... .........H N.....
RTGMx3 .........F .......... N......... ......
```

Figure 4.1

Sequence Analysis of the RBTMx2 and RBTMx3 cDNA Clones

The alignment of the ORFs from rainbow trout Mx1, Mx2 and Mx3 are given in Figure 1. All three Mx proteins contain the characteristic tripartite GTP binding domain, common to all Mx proteins. In addition, the putative leucine zipper repeats reported for RBTMx1 were also found in RBTMx2 and RBTMx3. Potential glycosylation signals were found in all three Mx proteins and their positions are shown in figure 4.1. Neither N nor O-linked glycosylation has been reported for murine Mx1 (Melen *et al.*, 1992) despite the presence of two potential N-linked sites. The trout Mx proteins share approximately 50% identity at the amino acid level with other Mx proteins with the highest conservation of sequence in the amino terminal half (chapter 3). Table 4.1 shows the size of the cDNA transcripts without polyadenylation, the deduced lengths, the molecular weights of the proteins, and their % identity at the nucleotide and amino acid level.

In Vitro Transcription/Translation Of Rainbow Trout Mx cDNAs

The size of each trout Mx protein was analyzed using a coupled *in vitro* transcription translation system, figure 4.2. The presence of an approximately 70 kD product in each lane confirmed that each cDNA clone was able to express a full size protein. No proteins were produced in the negative control lane with TE buffer used to resuspend the plasmid DNAs. The *in vitro* translated products ran at approximately 71, 69 and 72 Kd, in close agreement with the predicted sizes of 70.6, 72, and 70.8 Kd for RBTMx1, 2 and 3 respectively.

The Production of Antisera to Rainbow Trout Proteins

Polyclonal antisera to the rainbow trout proteins was generated to a fragment of rainbow trout Mx3 protein. This fragment was chosen in a region where the three RBTMx proteins were highly conserved, but on the carboxyl side of the GTP binding region where Mx proteins share strong homologies with other GTP binding proteins. The 114 aa rainbow trout Mx3 fragment differed by only one aa to RBTMx1 and nine aa to RBTMx2. These differences were clustered near the carboxyl end of the fragment. The protein fragment antigen was thus designed to generate antisera that would react with

all three rainbow trout Mx proteins, minimizing cross-reaction with other GTP-binding proteins. The PCR generated fragment was expressed in the Qiagen PQE-70 histidine tagged vector and purified using a nickel-NTA column. The resulting fusion protein used for injection is shown in figure 4.3a.

Western Blot Analysis of Poly IC Induced Rainbow Trout Mx Proteins.

The specificity of the antisera was tested by inducing the RTG-2 fish cell line with poly IC for 48h and performing a western blot on the induced and control extracts. Figure 4.3b shows the presence of a large unique band of approximately 70kD in the RTG-2 poly IC induced extracts. The sizes of this protein band corresponds to the results of the *in vitro* translation assay.

Transfection Analyses of Trout Mx Protein Localization

The rainbow trout Mx1, 2 and 3 proteins were expressed in CHSE-214 cells to 1) would confer protection against a well characterized fish rhabdovirus, IHNV. We chose to express the RBTMx cDNA clones using the PCRIII expression vector (Invitrogen) under control of the CMV early promoter in a transient assay system. The CMV early promoter is an efficient method of expressing transgenes in fish cells (Hansen *et al.*, 1991; Anderson *et al.*, 1996). Figure 4.4 shows the localization of all three rainbow trout Mx proteins and the control beta galactosidase construct. Confocal microscopy of a section through the nucleus demonstrated the subcellular localization of the trout Mx proteins. Rainbow trout Mx1 was expressed in a large globular pattern in the cytoplasm with most expression surrounding, but not within, the nucleus. Long "string like" expression patterns were sometimes observed. Rainbow trout Mx2 was confined to the nucleus in most cells, but a few cells expressed RBTMx2 in the cytoplasmic and nucleus. In both cases, RBTMx2 stained in a highly punctate pattern. Rainbow trout Mx3 was strictly expressed in the cytoplasm. The distribution of RBTMx3 differed from RBTMx1 in that it was expressed throughout the cytoplasm uniformly in a diffuse staining pattern.

Figure 4.2 Coupled in vitro transcription/translation analyses of full length trout Mx cDNA clones. 1 μg of plasmid DNA for each trout Mx clone was analyzed using a coupled transcription translation system with ^{35}S labelled methionine and analyzed by SDS-PAGE in a 12.5% gel. Lane C is the negative control of TE buffer used to resuspend the plasmid DNA. Lanes 1,2, and 3 are the full length clones RBTMx1, RBTMx2, and RBTMx3 respectively.

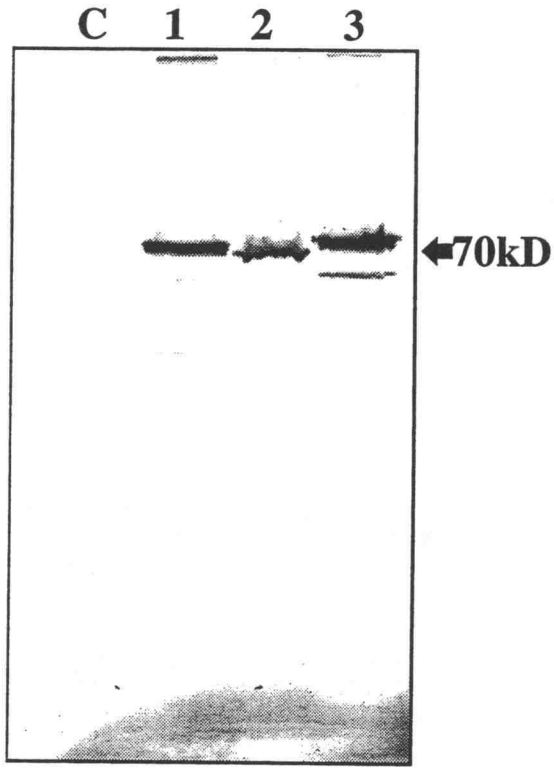

Figure 4.2

Figure 4.3 Detection of trout Mx protein *in vitro* with polyclonal antisera generated to a recombinant trout Mx fragment. A) Coomassie blue stained 10% polyacrylamide gel of purified Mx antigen used for generation of rabbit polyclonal anti trout Mx. Lane M are prestained broad range protein molecular weight markers. Lane MxF is the purified 114 aa fragment used for immunization after histidine tag purification and dialyzation of the *E. coli* lysate. B) Western blot analysis of poly IC dsRNA induction of RTG-2 Mx proteins with IgG purified polyclonal rabbit anti-Mx sera. Cell extracts were electrophoresed in a 10% SDS-PAGE gel, transferred to nitrocellulose and detected with the monospecific polyclonal rabbit anti-trout MX.Lane C-48h is the mock induced control cells. Lane pIC-48h is RTG-2 cells poly IC treated for 48h.

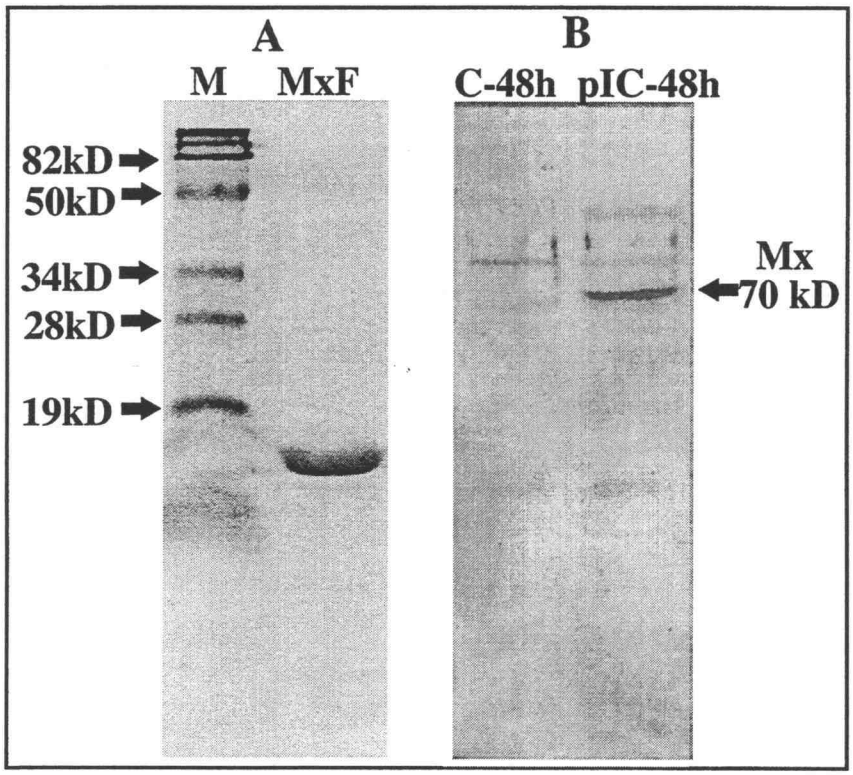

Figure 4.3

Trout Mx Proteins do not Confer Protection Against the Fish Rhabdovirus IHNV

In order to determine if either of the rainbow trout Mx proteins conferred protection against the fish rhabdovirus, IHNV, double label immunofluorescence studies were performed. A beta galactosidase construct was also used as a negative control to demonstrate that any resistance was not due to the transfection protocol. This double label, single-cell assay is similar in design to previous investigators (Staeheli *et al.*, 1986; Bernasconi *et al.*, 1995; Melen and Julkunen, 1994). At 17°C, the IHNV Rb-1 isolate exhibits nucleoprotein (N) expression at 15h post infection increasing to 24h post infection in CHSE-214 cells (data not shown). Transfected CHSE-214 cells were fixed at 18h post infection and double label immunofluorescence stained. At an moi of 10, greater than 90% of the monolayer exhibited strong staining for the IHNV N protein. The number of transfected cells staining either positive or negative for IHNV nucleoprotein were counted. Figure 4.5 shows the typical staining patterns observed. All the transfected cells in the 1 cm^2 transfection were scored as positive or negative for IHNV nucleoprotein expression as evidenced by fluorescein staining. Beta galactosidase transfected cells were used as a negative control for inhibition of viral protein synthesis to eliminate any potential effects of transfection on viral replication. The results are shown in table 4.2. The rainbow trout proteins did not confer a high degree of resistance to IHNV in CHSE-214 cells as evidenced by the accumulation of IHNV nucleoprotein.

Discussion

The rainbow trout Mx proteins are highly homologous to other characterized Mx sequences. In addition, trout Mx proteins are tightly regulated by IFN as demonstrated by their response to poly IC dsRNA and viral infection *in vitro* and *in vivo*. Poly I.C and virus induction of rainbow trout liver induces the expression of two distinct mRNA transcripts (Staeheli *et al.*, 1989; Trobridge and Leong, 1995). The cDNA sequence lengths reported here suggest that the large, approximately 2.9 Kb transcript may be the polyadenylated form of the Mx1 2.6 Kb transcript while the smaller 2.6 Kb transcript size is comprised of the polyadenylated RBTMx2 and RBTMx3 transcripts which are both 2.1 Kb.

Figure 4.4 Localization of transfected trout Mx protein in CHSE-214 cells. CHSE cells were transfected either a CMV-Beta galactosidase construct or CMV-RBTMx1, RBTMx2 or RBTMx3. Transfected cells were stained with either rabbit anti-beta gal (Beta gal) or with rabbit anti-trout Mx (RBTMx1, 2, 3) followed by Texas red goat anti-rabbit sera. Single layer confocal images were captured through the nucleus.

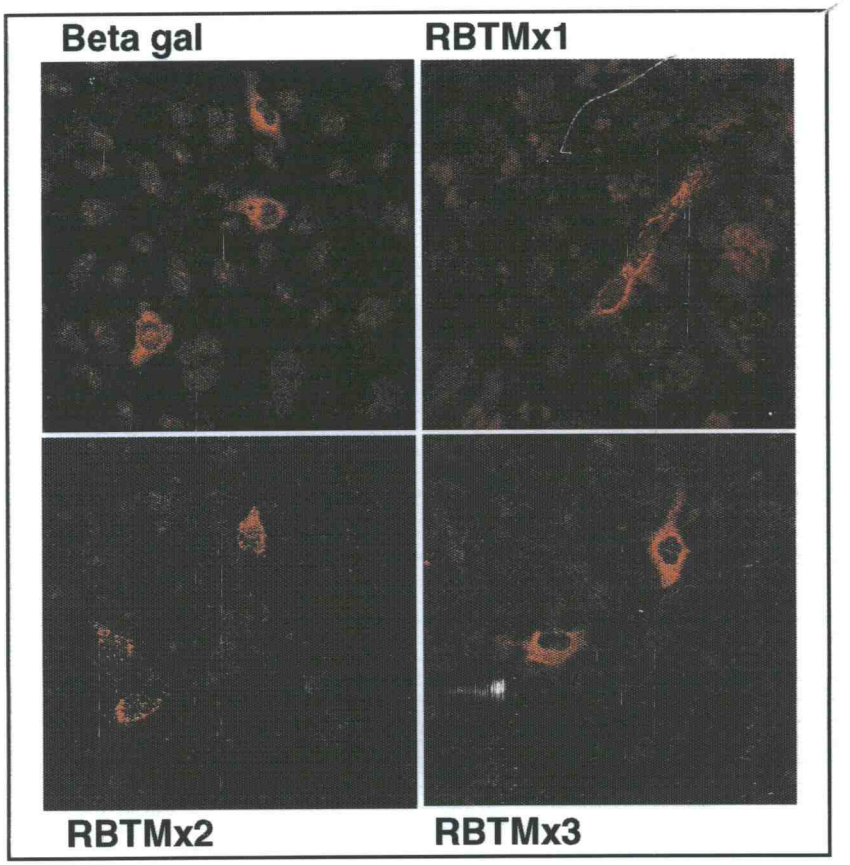

Figure 4.4

Figure 4.5 Double label immunofluorescent detection of Mx transfected cells infected with IHNV. The CHSE-214 cell line was transiently transfected with CMV-RBTMx1, RBTMx2, RBTMx3, or the control CMV-beta gal and infected with IHNV. Transfected cells were detected with rabbit polyclonal antisera and secondary Texas Red antibody. IHNV infected cells were detected by mouse monoclonal anti-nucleocapsid protein and a secondary fluorescein antibody. Photos were taken A) using a single bandpass filter that detects fluorescein (IHNV N protein) alone, and B) using a triple bandpass filter that detects both texas red (Mx protein) and fluorescein (IHNV N protein). Transfected cells expressing IHNV N protein are indicated by arrows.

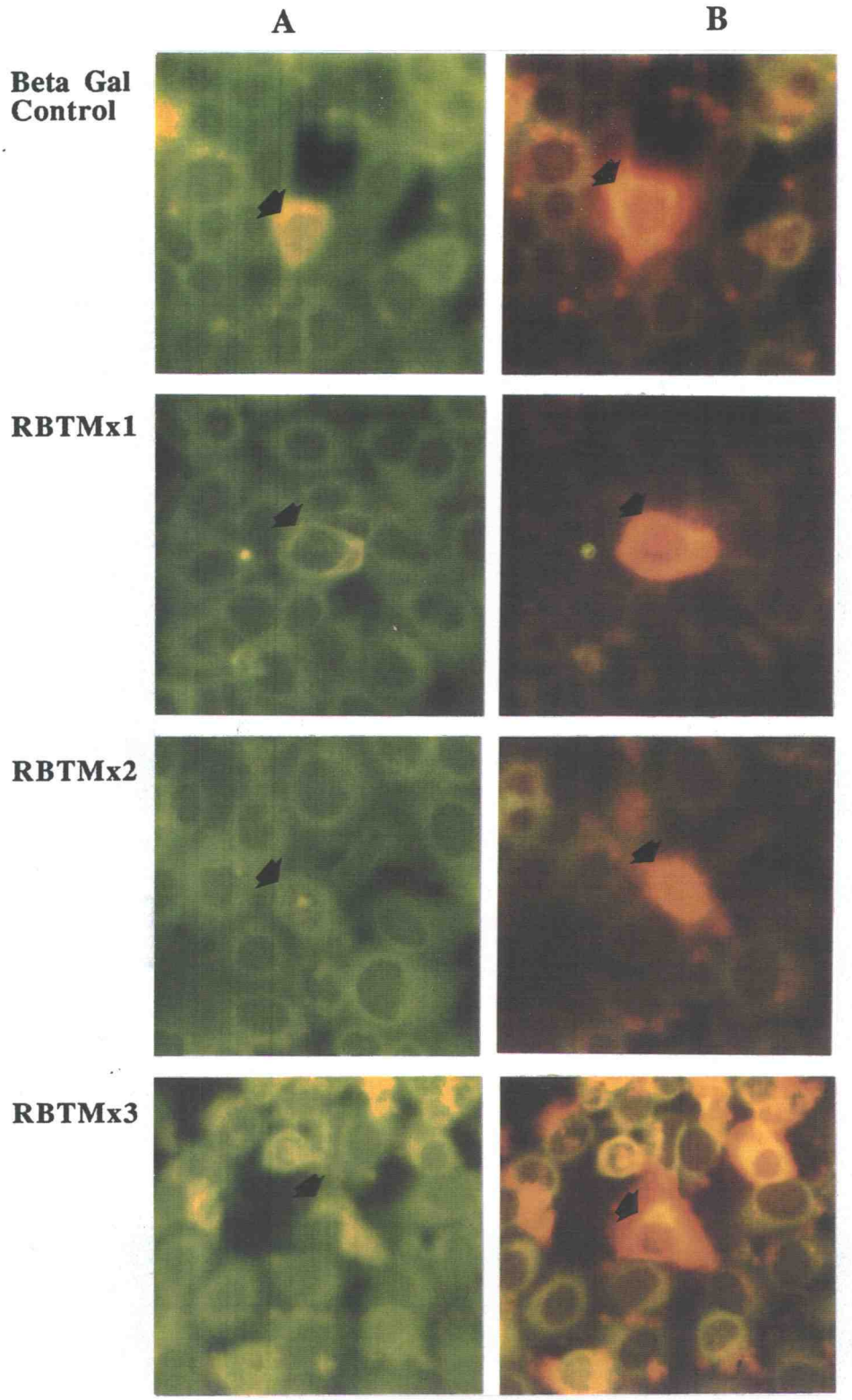

Figure 4.5

The high degree of sequence identity (96.6%) between RBTMx1 and RBTMx3 suggested that these cDNAs may have been derived from different alleles of the same gene. However the divergence of the 5' and 3' non-coding regions, and the difference in amino acid length strongly suggests that these cDNAs are derived from separate genes. In addition, these proteins, while both expressed in the cytoplasm, exhibit consistently different staining patterns; the RBTMx1 is expressed in globular and string-like aggregations surrounding the nucleus, while RBTMx3 is expressed uniformly throughout the cytoplasm in a diffuse staining pattern. Further, RFLP analysis of inbred trout lines using a single exon probe (data not shown) also suggests that there are at least three Mx genes. Similar analysis of inbred lines of rats was used to investigate rat Mx2 and Mx3 proteins which are 98.8% identical but derived from separate genes (Meier *et al.*, 1990).

The *in vitro* translated Mx proteins sizes corresponded to the induced RTG-2 proteins by western blot. The antisera produced to the RBTMx3 fragment can detect all three Mx proteins by Western blot and each trout Mx protein separately by transfection assay.

The production of antisera against the trout Mx proteins has allowed us to determine the cellular localization of the three trout Mx proteins. RBTMx1 is cytoplasmic, RBTMx2 is punctate nuclear and/or cytoplasmic, and RBTMx3 is diffuse cytoplasmic. The RBTMx2 punctate nuclear localization is characteristic of the murine Mx1 protein. However, unlike the nuclear murine Mx1 the RBTMx2 protein was found in the nucleus of some cells and in the cytoplasm of other cells. There were some cells which expressed RBTMx2 in both the cytoplasm and nucleus. The duck Mx proteins are also expressed in the nucleus and cytoplasm (Bazzhiger, 1993). Duck Mx protein concentrated at the nuclear membrane and was not distributed uniformly throughout the cytoplasm, like RBTMx1. However, unlike duck Mx protein, confocal microscopy shows that RBTMx1 is not found inside the nucleus.

We used a single cell transient transfection assay to determine if any of the trout Mx proteins could inhibit the accumulation of the rhabdovirus nucleoprotein, N. There are no known Mx negative fish cells, however we have previously shown that CHSE-214 cell have a delayed response to poly IC induction of Mx mRNA (chapter 3), and other investigators have demonstrated poor induction of antiviral activity by poly IC in CHSE-214 cells (MacDonald and Kennedy, 1979). For these reasons, and because transfection protocols have been established for this cell line (Anderson *et al.*, 1995) we used CHSE-214 cells to examine transient, constitutive expression of Mx rainbow trout

proteins. Mx positive COS cells have been used successfully for transient transfection analyses of the murine Mx1 (Melen and Julkunen, 1994) suggesting that Mx- cell lines are not critical for evaluating constitutive, transiently expressed Mx antiviral activity. Trout Mx protein inhibited IHNV N expression in 7.9, 8.8, and 3.4% of the RBTMx1, 2, and 3 transfected cells respectively, while 2.5% of the beta-galactosidase expressing cells inhibited IHNV N accumulation. Other investigators have seen inhibition of influenza viral protein synthesis in over 90% of transfected cells using similar assays with the murine Mx1 (Staeheli *et al.*, 1986; Melen and Julkunen, 1994). We conclude that the trout Mx proteins are not efficient inhibitors of IHNV replication in salmon cells as evidenced by accumulation of viral nucleoprotein.

Studies analyzing Mx inhibition of the measles paramyxovirus glycoprotein have shown that Mx can inhibit specific viral proteins in a cell-type specific manner. Although the trout Mx proteins do not inhibit IHNV N protein accumulation in CHSE-214 cells, we have not ruled out the possibility that trout Mx proteins might interfere with IHNV replication by specifically reducing other viral proteins in a cell type specific manner.

The RBTMx1, 2, and 3 proteins of rainbow trout are highly homologous to other Mx proteins. In addition they are regulated by poly IC dsRNA or viral infection presumably through IFN induction. Although the trout Mx proteins did not inhibit the rhabdovirus, IHNV, we plan to test other fish viruses for sensitivity to Mx expression. The effect of trout Mx expression on the recently described orthomyxovirus, infectious salmon anemia (ISA) virus (Dannevig *et al.*, 1995), of atlantic salmon (*Salmo salar*) will be of specific interest.

Acknowledgements

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Table 4.2 Double label immunofluorescence analysis of trout Mx inhibition of IHNV

	Number of Transfected Cells	Number of Transfected Cells Expressing IHNV	% IHNV Inhibition
Beta Gal Control	39	38	2.5%
RBTMx1	88	81	7.9%
RBTMx2	41	45	8.8%
RBTMx3	86	83	3.4%

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CHAPTER 5
ANALYSIS OF RAINBOW TROUT MX PROTEIN EXPRESSION AFTER INDUCTION BY POLY IC AND IHNV

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This paper reports a portion of the work described in a thesis to be submitted to Oregon State University Department of Microbiology in partial fulfillment of the requirements for a PhD degree.

Abstract

Rainbow trout (*Oncorhynchus mykiss*) Mx protein was detected by Western blot and by immunohistochemistry *in vitro* and *in vivo*. Poly IC dsRNA treated RTG-2 cells expressed Mx protein detectable by Western blot at 24h post induction with increased expression at 48h, declining at 72h post induction. Infectious hematopoietic necrosis (IHN) virus was not an efficient inducer of Mx protein *in vitro*. Immunohistochemistry was used to detect transfected trout Mx proteins in CHSE-214 cells and production of endogenous RTG-2 Mx protein in poly IC induced cells. Endogenous Mx protein was detected in RTG-2 cells induced with 5, and 50 µg/ml of poly IC dsRNA at 48h post induction. Immersion of rainbow trout in a high m.o.i. of IHNV (Rangen) induced the production of Mx protein in the kidney of 4/4 fish examined by Western blot. Mx protein was detected in the kidney tubules by immunohistochemistry in 3/3 rainbow trout fry challenged with the RB-76 isolate of IHNV. The use of Mx protein as a marker for fish IFN is discussed.

Introduction

The IFNs provide vertebrates with a first line of defense against viral infection. These cytokines are produced by infected cells and stimulate the production of IFN induced proteins in neighboring uninfected cells. Some of these IFN regulated proteins (IRPs) confer a state of resistance to infection by a number of different viruses. The contribution of some of these proteins to the antiviral and cell proliferation control of IFNs are now beginning to be understood. For the P1 kinase, the 2'-5' oligoadenylate synthetase, and the Mx proteins specific intracellular activity has been demonstrated (Koromilas *et al.*, 1992; Meurs *et al.*, 1990; Staeheli *et al.*, 1986).

There have been several reports characterizing IFN-like activity in fish cells *in vitro* and *in vivo*. Gravell and Malsberger (1965) first demonstrated the *in vitro* production of fish IFN in fathead minnow (*Pimephalus promelas*) cells when exposed to infectious pancreatic necrosis virus (IPNV). *In vitro* induction of a fish IFN has since been reported by several investigators (Beasley *et al.*, 1966; Oie and Loh, 1971; DeSena and Rio, 1975; MacDonald and Kennedy, 1979; Sano and Nagakura, 1982; Tengelsen *et al.*, 1989). Several studies have suggested that the IFN system is an important innate immune component of fish. de Kinkelin and Dorson (1973) showed that trout injected with viral hemorrhagic septicemia (VHS) virus produced an IFN-like substance that was

able to confer protection to rainbow trout gonad (RTG-2) cells against IPN, VHS, and IHN virus. Eaton (1990) induced IFN production in chum (*Oncorhynchus keta*) and sockeye (*O. nerka*) salmon. The induced IFN stimulated an antiviral response that decreased IHNV titre, decreased cumulative mortalities, and delayed virus replication in IHN challenged salmon.

The IFN induced mammalian Mx proteins are involved in IFN mediated resistance to influenza viruses (Staeheli *et al.*, 1986a; Frese, 1995; Thimme, 1995) the rhabdovirus vesicular stomatitis virus (VSV) (Meier *et al.*, 1990) and the measles paramyxovirus (Schneider Schaulies *et al.*, 1994). We have previously reported the cloning of three trout Mx cDNA clones from rainbow trout cells (Trobridge and Leong, 1995) and generated antisera to trout Mx protein (Trobridge *et al.*, 1996). We further characterize here the induction of trout Mx protein by poly IC dsRNA and IHN virus. Mx protein has been used as a marker for IFN in human studies (Oh *et al.*, 1994; Towbin *et al.*, 1992). We demonstrate here that the Mx protein is a useful marker for IFN using the artificial IFN inducer, poly IC dsRNA, and the rhabdovirus IHNV to detect Mx protein expression *in vitro* and *in vivo*.

Materials and Methods

Tissue Culture and in Vitro Inductions.

Tissue culture of RTG-2 cells (Wolf and Quimby, 1962) and CHSE-214 cells (Fryer *et al.*, 1965) was performed as previously described in chapter 3. For western blot analysis of poly IC dsRNA induction of salmonid Mx protein, RTG-2 cells were plated in 12 well multiplates. Confluent monolayers were washed with MEM-0 and treated with control, MEM-0, 500µg/ml Poly IC (Pharmacia) in MEM-0, or IHNV at an moi of 0.1 in MEM-0, for 24, 48, and 72h. At each time point, the monolayer was washed twice with PBS and 200µl of 2X SDS loading buffer (recipe) was added to each well. The loading buffer was removed, boiled for 3 min and frozen. For immunohistochemistry, RTG-2 cells were plated in 6 well multiplates at 50% confluency. Cells were washed in MEM-0 and treated with poly IC at 0 (control), 5, and 50 µg/ml for 48h.

Virus Stocks

The Rangen Isolate of IHNV was used for *in vivo* induction of Mx protein detected by Western blot. This virus was isolated from dead rainbow trout fry at the International Aquaculture Research Center (Rangen Research), Hagerman, Idaho. The RB-76 strain of IHNV used for immunohistochemical localization of IHNV in fish tissue was isolated from moribund steelhead (*O. mykiss*) fry from an epizootic at Round Butte Hatchery, Oregon in 1976. To produce stocks used for challenge, virus was grown on 90% confluent CHSE-214 monolayers.

In Vivo Induction of Mx by IHNV.

(i) Induction detected by western blot. Rainbow trout, average weight 2.2g, were held in 5 gallon aquaria at 12°C with a flow rate of 0.25 gallons/minute. 20 fish were challenged with IHNV virus (Rangen) by immersion for 5h in 10^5 $TCID_{50}$ infectious doses. 20 control fish were mock challenged with tissue culture supernate. This isolate at this challenge dose is known to cause high mortality. Approximately 2 to 10 mg of liver and kidney were removed at 0, 2, and 4 days post challenge. The tissues were broken up by maceration with a pipette tip in 200 µl of 2X loading buffer (above). Samples were then boiled for 5 min and frozen until assayed.

(ii) Induction detected by immunohistochemistry. Rainbow trout fry (0.5 g) were used for the waterborne challenge. Fish were exposed to 10^5 PFU/ml of the IHNV strain RB-76. The fish were exposed by static immersion for 6 hours at 13°C. A majority of the fish that would ultimately die began to show clinical signs of disease at 9 days post exposure (d.p.e.). These signs were distended abdomen, petechial hemorrhages, and a whirling form of swimming. Moribund fish were collected and fixed in 10% buffered formalin and embedded in Paraplast paraffin (Oxford Labware, St. Louis, MO). Embedded fish were sliced sagittally and 6 µm sections were fixed at 40°C overnight onto slides coated with 0.1% gelatin and 0.1% chromic potassium sulfate.

Western Blot Analysis

All samples were electrophoresed in 10% polyacrylamide gels and transferred to Optitran supported nitrocellulose membranes (Schleicher and Schuell) using a Biorad Miniprotean transfer apparatus. Blots were blocked overnight in 5% nonfat dry milk in TBS at 4°C. All subsequent steps were performed at room temperature.

(i) For *in vitro* analysis of RTG-2 Mx protein, 15 µl of sample were loaded per lane. After electrophoresis and transfer the membrane was incubated in 25 ml of polyclonal rabbit ant-RBTMx (Trobridge *et al.*, 1996) at 1:200 of 1mg/ml IgG purified antibody in TBS with 1% BSA and 0.02% Tween 20 for 1h. The membrane was washed 4X with 100 ml of TBS with 1% BSA and 0.02% Tween. The membrane was then incubated with a 1:2000 dilution of 2 mg/ml mouse anti rabbit-alkaline phosphatase (ProMega) for 1h in TBS-0.02% tween 20. The membrane was washed 4X in 100ml TBS-0.02% tween 20 and incubated in AP substrate using the one step detection kit (Kierkegaard-Perry).

(ii) For *in vivo* analysis of both Mx protein and IHNV, 10 µl of both kidney and liver in SDS-PAGE loading buffer were loaded. Because of the increased sensitivity, and consequent increased background of chemiluminescent detection, the procedures were modified as follows. Blots were incubated with 25 ml 1:500 of 1mg/ml rabbit anti RBTMx in TBS 1% blotto with 0.05% tween 20 for 1h or 25 ml of 1:500 polyclonal anti-IHNV (Ristow and Arnzen, 1989). Blots were washed 4X in 100 ml of TBS with 0.05% tween. Blots were then incubated in a 1:5000 dilution of mouse antirabbit-HRP (Promega) in TBS-0.05% tween 20 for 1h. The membranes were then washed 4X 10 min in TBS-0.05% tween 20. Membranes were then incubated in 20 ml of luminol substrate (Pierce Laboratories) for 10 min and exposed to Hyperfilm (Amersham) for 1 to 5 minutes.

Transfection of Rainbow Trout Mx cDNA Clones in CHSE-214 Cells

CHSE-214 cells were transfected as previously described (chapter 4). Briefly CHSE-214 cells were plated at 70 to 80% confluency in 8 well multichamber slides. Cells were transfected using with the three RBTMx cDNA clones with lipofectamine

reagent (Gibco laboratories) at a ratio of 1 μg DNA to 9 μl transfectamine. 72h post transfection monolayers were assayed for immunocytochemistry.

Immunocytochemistry of Poly IC Induced RTG-2 Cells

Following induction, monolayers were washed in ice cold PBS and fixed for 20 min with 0.5 ml of freshly prepared 3% paraformaldehyde in PBS. Cells were then permeabilized with 0.5 ml of 0.1% triton X-100 for 10 min, followed by one wash in PBS. The monolayers were blocked with 5% nonfat dry milk in PBS for 30 min, then incubated with 0.5 ml of 1mg/ml Anti-trout Mx diluted 1:1000 for 1h at RT. The monolayer was washed in PBS for 4X 5 min. The Vectastain rabbit ABC-AP kit (Vector laboratories, Burlingame, CA) was used for subsequent incubations according to the manufacturers protocols except all washes were for 4X 5 min in PBS. The alkaline phosphatase substrate kit III (Vector laboratories), blue substrate was used for detection and incubation was carried out for 5 minutes (transfected cells) or 2h (poly IC induced cells) in the dark.

Immunohistochemistry of IHNV Exposed Rainbow Trout

Assays were carried out as described by Drolet *et al.*. (1994). Briefly, the tissue sections were deparaffinized by serial xylene and alcohol treatments and rehydrated for 10 minutes in distilled water. Sections were then equilibrated for 20 minutes in phosphate buffered saline (PBS, pH 7.4). The sections were blocked for one hour with 5% powdered nonfat dry milk in PBS and then incubated with anti-Mx polyclonal antibody for one hour. The Vectastain-ABC rabbit IgG kit and the Vector-Red phosphate substrate (Vector Laboratories) were used to label and develop the sections. Sections were counterstained in hematoxylin for 1 min and 0.2% ammonium hydroxide in 70% ethanol solution for 30 seconds. Sections were covered with Crystal Mount (Biomeda, Foster City, CA) and examined by light microscopy.

Figure 5.1 Induction of RTG-2 Mx protein by poly IC dsRNA. RTG-2 monolayers were mock induced(C), induced with 50 μg/ml poly IC dsRNA (pIC), or treated with IHNV at an moi of 0.1 (V) for 24, 48, and 72h and analyzed by western blot for Mx protein. Molecular weights are indicated on the left as determined by prestained MW markers. The arrow on the right side of the blot indicates the triplet band of expressed Mx protein at approximately 70 kD.

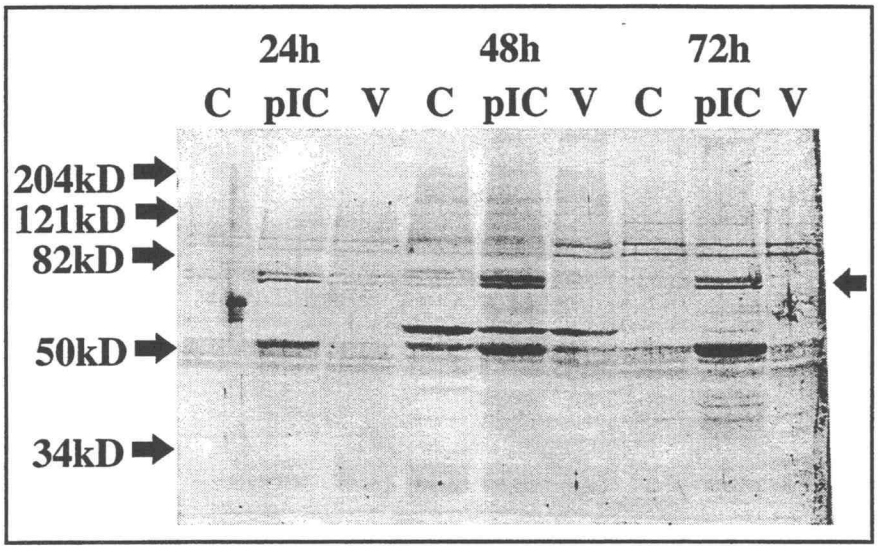

Figure 5.1

Results

Time Course of Mx Protein Induction in RTG-2 Cells.

Experiments with Mx mRNA had previously shown that RTG-2 cells produce two transcripts upon poly IC dsRNA induction first detectable at 24h, peaking at 48h and decreasing until 72h (Trobridge and Leong, 1995). We used polyclonal rabbit antisera generated to RBTMx3 that has previously been shown to detect all three RBT Mx proteins by transfection assay and detect RBT Mx protein in RTG-2 cells by Western blot (chapter 4). RTG-2 cells were treated with poly IC, infected with IHNV Round Butte isolate 4 (RB-4) at an m.o.i. of 0.1, or mock induced with tissue culture supernatant. Cells were lysed and analyzed by western blot at 24, 48 and 72h. Three bands at approximately 70 kD were present in RTG-2 cells induced with poly IC dsRNA but not in mock induced controls or in IHNV infected monolayers, see figure 5.1. These sizes correspond to the *in vitro* translation products of RBTMx1, 2 and 3 (chapter 4). Mx protein was detected at 24h post induction, peaked at 48h post induction, and was still detectable at 72h. Smaller bands at approximately 48 kD were also detected in induced cells. Also a band of approximately 50 kD was found in the poly IC induced cells only. Arnheiter *et al.* (1990) reported a breakdown product of murine Mx1 in western blots of poly IC induced A2G mice and transgenic mice. The predominant breakdown product from the figure in that paper was also approximately 50 kD. We suggest that the 50 kD band in induced RTG-2 cells may also be a breakdown product as it accumulates over time and is expressed only in induced cells. Whether or not this putative breakdown product is related to the 50 kD protein reported by Arnheiter *et al.* is unknown.

Immunocytochemistry of Transfected Mx Protein

In order to establish an immunohistochemistry protocol to detect Mx protein in fish cells we used CHSE-214 cells transfected with the three rainbow trout Mx proteins and a control beta galactosidase construct. Trout Mx transfected cells were readily detected by immunohistochemistry as evidenced by blue staining (figure 5.2). The subcellular localization of these Mx proteins has been determined using confocal

Figure 5.2. Detection of Transfected Mx Protein by Immunocytochemistry. CHSE-214 cells were transfected with a control CMV-beta gal construct, CMV-RBTMx1, CMV-RBTMx2, or CMV-RBTMx3. Transfected cells were detected by immunohistochemistry using either rabbit anti-beta gal (control), or rabbit anti-Mx (RBTMx1, 2, and 3) as the primary antibody. Blue-purple coloring indicates Mx expression.

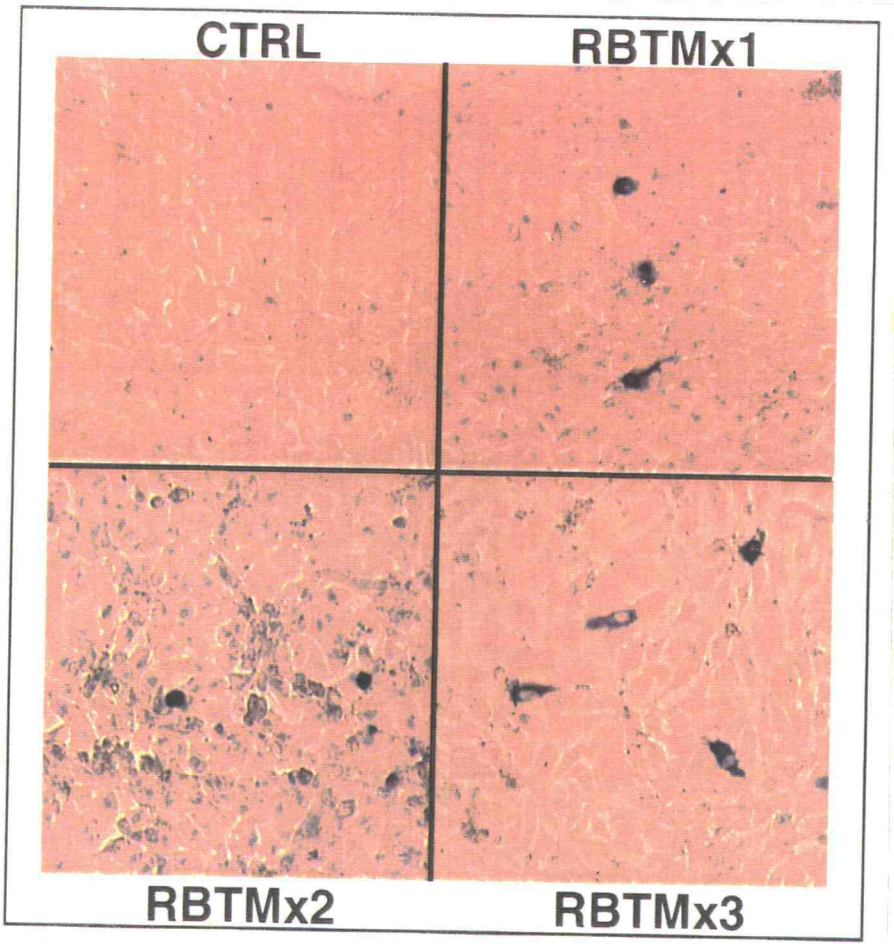

Figure 5.2

Figure 5.3. Induction of RTG-2 endogenous Mx by poly IC dsRNA. RTG-2 cells were mock induced or induced with 5μg/ml or 50μg/ml poly IC dsRNA for 48h and analyzed for Mx protein in duplicate using immunohistochemistry. Panel A shows a photograph of one row of the multiwell plate, Panel B shows a magnification of the RTG-2 cells of panel A; 0=mock induced with MEM-0, 5=5μg/ml poly IC, 50=50μg/ml poly IC. Blue-purple coloring indicates Mx expression.

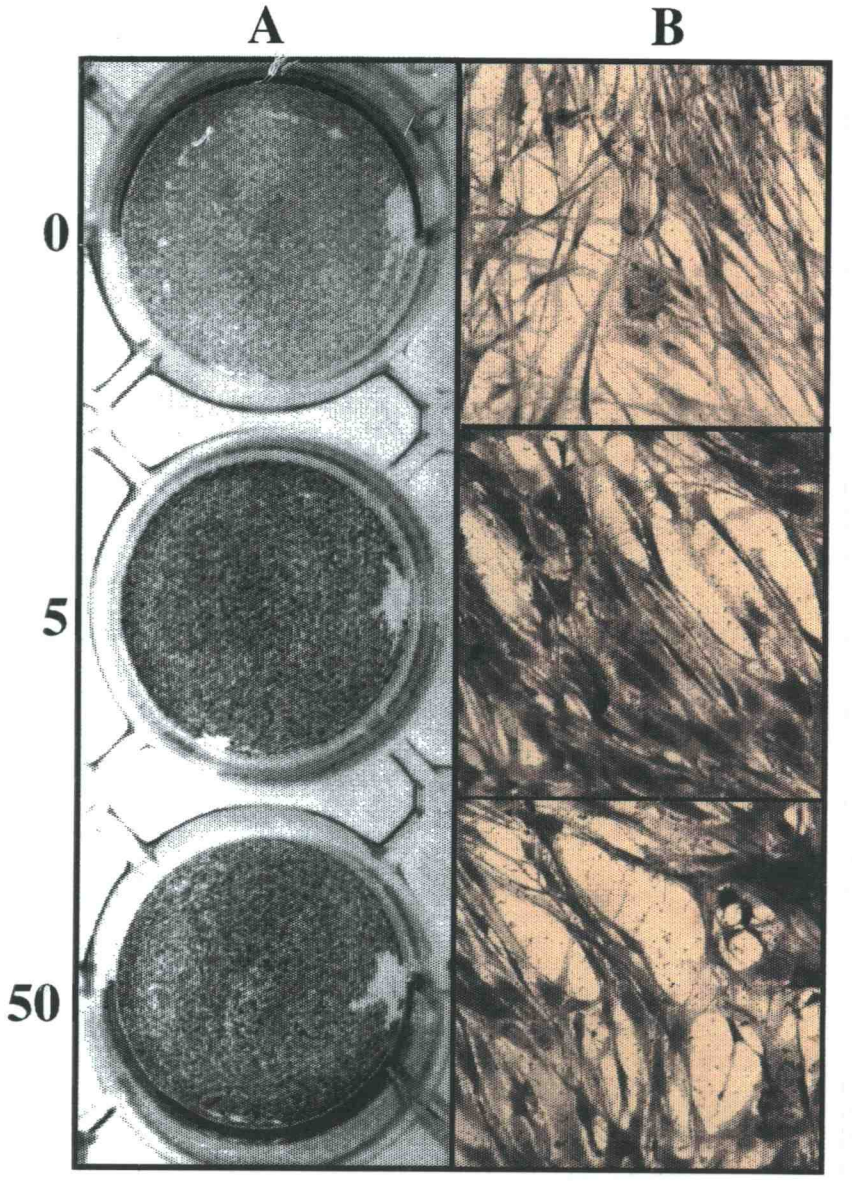

Figure 5.3

microscopy (chapter 4) and the immunohistochemistry detection corroborates these findings. The RBTMx1 construct appears to stain the nucleus and some areas surrounding the nucleus of CHSE-214 transfected cells. Confocal imaging has shown that the RBTMx1 is actually localized surrounding the nucleus but not within the nucleus. The RBTMx2 exhibits a nuclear staining confined within the nucleus. The RBTMx3 protein is clearly restricted to the cytoplasm.

Immunocytochemistry of Mx Protein Expression in RTG-2 Cells

In order to characterize *in situ* expression of Mx protein, RTG-2 cells were treated in duplicate with 5μg/ml, 50μg/ml of poly IC dsRNA and control mock-induced and analyzed at 24 and 48 hours by immunocytochemistry with anti-Mx polyclonal sera. Figure 2 shows control and poly IC dsRNA induced RTG-2 cells after detection by immunocytochemistry with the anti-Mx antibody. 5 μg/ml poly IC dsRNA was sufficient to induce detectable differences in Mx protein expression. 50 μg/ml poly IC also resulted in Mx induction, but at this concentration toxicity was evident after 24h. To detect Mx protein in poly IC induced RTG-2 cells using this method, long substrate incubation times of up to two hours were required that resulted in some background. However, clear differences are seen between controls and poly IC induced cells.

Induction of Mx Protein In Vivo

We had previously shown IHNV to be a potent inducer of Mx mRNA (chapter 3). With a highly virulent strain of IHNV, this induction of Mx mRNA occurs in up to 100 percent of fish injected with virus (Trobridge *et al.*, 1996). We chose to examine IHNV induction of Mx protein by immersion at a high moi with a viral isolate that causes up to 100% mortality. Rainbow trout were immersed in IHNV and controls in MEM-0. At time 0, 2 days, and 4 days fish were collected and kidneys and livers were analyzed by western blot for Mx induction and for IHNV proteins. At 2 days post-infection some infected fish were moribund and at 4 days the remaining infected fish were either moribund (5 fish) or dead (9 fish). 4 of the remaining 5 moribund fish were sampled and the experiment was terminated. All livers and kidneys were analyzed by western blot for Mx protein and for IHNV proteins using polyclonal antisera. Table 5.1 shows the

Figure 5.4 Induction of Mx protein in rainbow trout liver and kidney. Rainbow trout were infected by immersion with IHNV (Rangen) or by mock immersion in tissue culture supernatant (control). At time 0, day 2, and day 4 post injection liver and kidney tissue was analyzed by western blot for expression of Mx protein. These blots show the day 4 results of a) liver, b) kidney and c) fish #4 liver and kidney electrophoresed and transferred on the same blot. Molecular weights were determined by running prestained markers. The arrows on the right show the 70 kD Mx protein and the putative breakdown product.

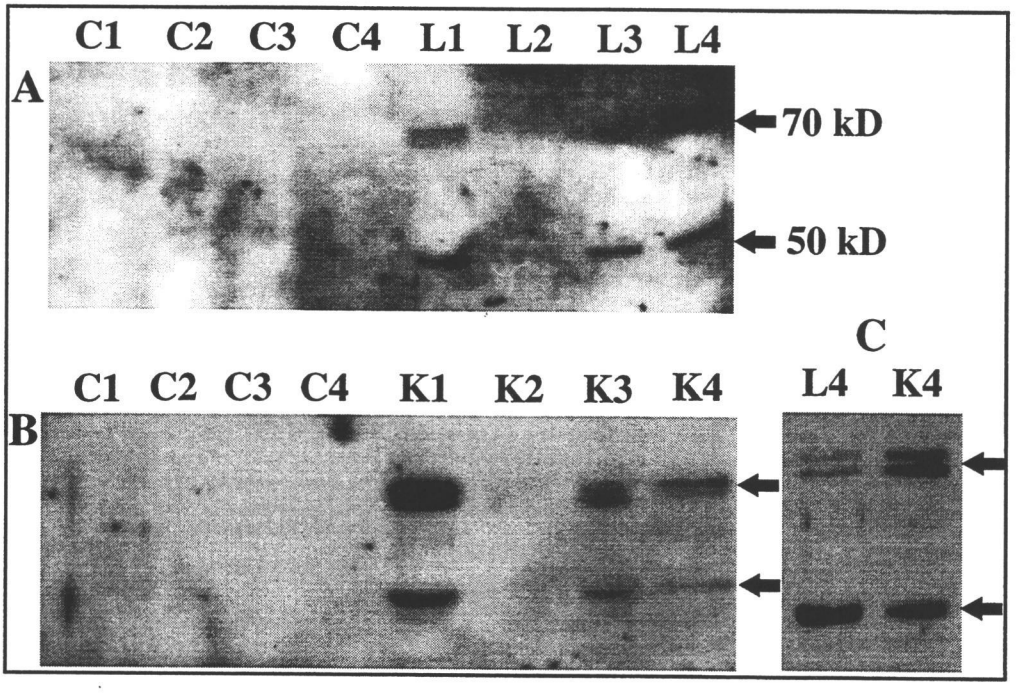

Figure 5.4

results of Mx protein induction. Figure 5.4 shows the blots of tissues examined on day 4. At day 4, all four IHNV treated fish examined contained Mx protein bands in the kidney at approximately 70 kD, and a smaller band at approximately 50 kD. The fish #2 kidney (K2) was positive as determined by overexposing the blot (data not shown. Mx protein was detected in the livers of 3 of the 4 fish examined. Day 4 control fish on the same blot contained no detectable bands at 70 kD or 50 kD. IHNV challenged fish #4 liver and kidney samples were subjected to electrophoresis on the same blot to compare sizes and amounts of Mx protein. Both kidney and liver contained three Mx bands of approximately 70 kD.

Immunohistochemistry of Mx Protein in Trout

The immunohistochemical staining for the Mx protein in IHNV RB-76 infected fish showed positive red staining in the kidney tubules (Figure 5.5). Kidney tissues of the negative control fish showed no red staining. The fish examined for this study were collected 2 days post exposure. At least three whole-body serial sections from each fish were stained for Mx protein. The tissues of the liver, stomach, intestine, and pyloric caecum were positively stained for Mx in some, but not all fish examined (data not shown).

Discussion

Rainbow trout Mx mRNA is rapidly and transiently induced by the IFN inducer poly IC dsRNA and by injection of virus *in vivo* (Staeheli *et al.*, 1989; Trobridge and Leong, 1995). We characterize here the induction of the trout Mx proteins *in vitro* by poly IC dsRNA and *in vivo* by immersion challenge with IHNV. The data presented here establishes the use of trout Mx protein as a marker for IFN induction in fish.

Trout Mx protein was detected by Western blot in RTG-2 cells induced by poly IC dsRNA treatment. Three protein bands of approximately 70 kD were first seen 24h post induction with 50 μg/ml poly IC dsRNA. The Mx protein peaked at 48h, and declined at 72h post induction with 50 μg/ml poly IC dsRNA. The induced Mx protein sizes correspond to the sizes of the cloned RTG-2 Mx proteins as determined by *in vitro*

Table 5.1 Induction of Mx in rainbow trout challenged by IHNV. Rainbow trout, average weight 2.2 g were immersed in 10^5 TCID50 units of IHNV (Rangen) and assayed for Mx protein by western blot at the indicated time points. The number of fish expressing Mx over number of fish examined are represented for each time point, and for both kidney and liver tissues.

	Control mock injected fish		IHNV infected Fish	
	Kidney	Liver	Kidney	Liver
Time 0 :	0/4	0/4	0/4	0/4
Day 2 :	0/4	0/4	2/4	0/4
Day 4 :	0/4	0/4	4/4	3/4

Figure 5.5 Immunohistochemical staining of infected fish tissue for Mx. The tissues of fish infected with RB-76 were stained with a polyclonal antibody directed against Mx. The red staining in the tissues indicates the presence of Mx protein. Kidney tissues were examined with 20X (200X total magnification) and 40X (400X total magnification) objective lenses. Kidney tissue from a fish that was not exposed to virus served as a negative control.

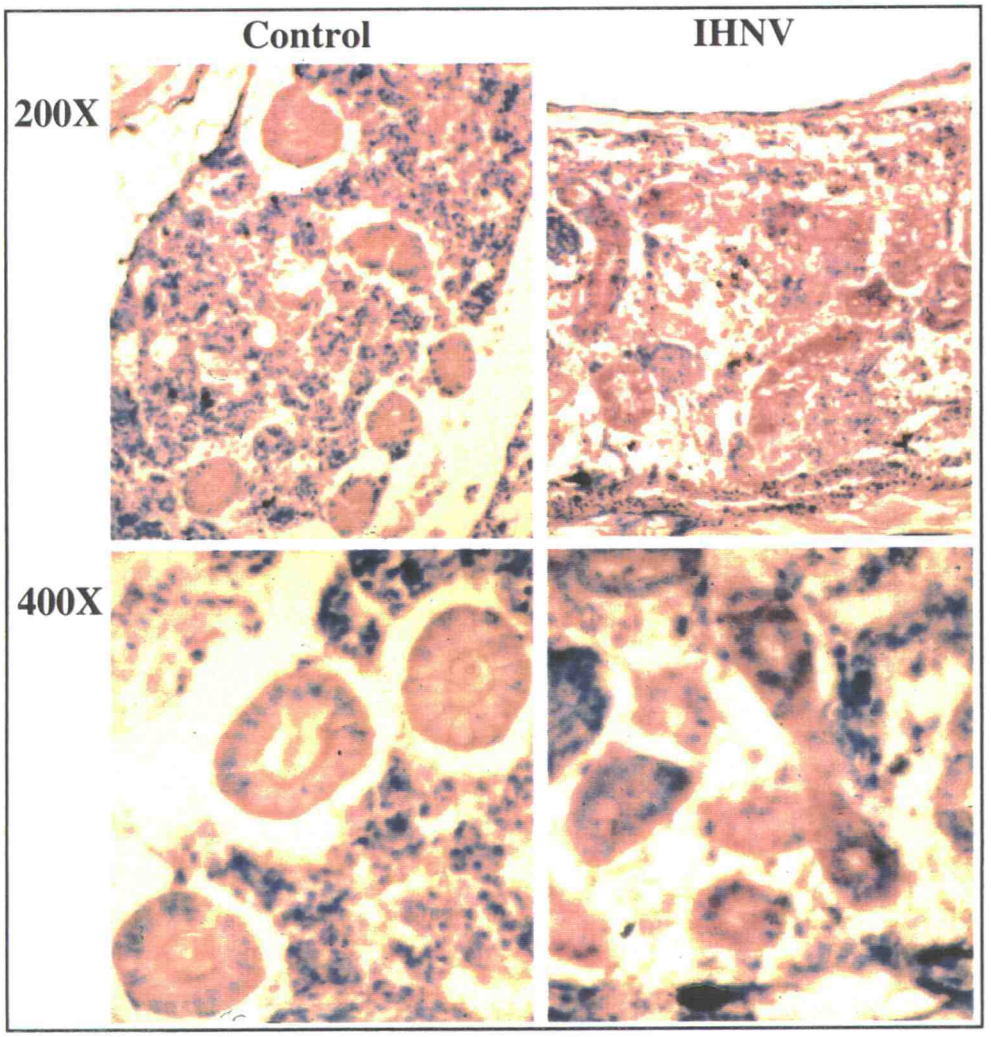

Figure 5.5

transcription/translation (RBTMx1-71 kD, RBTMx2-69 kD,RBTMx3-72 kD) (chapter 4). Interestingly IHNV infection did not produce significant amounts of Mx protein in RTG-2 cells. We have previously been unable to induce Mx mRNA *in vitro* by IHNV infection (unpublished observations) while IHNV injection induces Mx mRNA *in vivo* at up to 99% efficiency (Trobridge *et al.*, 1996). We reason that the induction of IFN in RTG-2 cells is not as efficient as in fish tissues where the presence and interaction of several cell types might lead to more potent induction of IFN. Using RBTMx1 clones we established immunohistochemistry protocols for detection of transgenic Mx protein in CHSE-214 cells. Mx protein expressed under control of the constitutive CMV promoter was readily detected. The CMV promoter has previously been shown to express transgenes efficiently in fish cells (Anderson *et al.*, 1996; Hansen *et al.*, 1991). Endogenous Mx protein of RTG-2 cells was detected by immunohistochemistry after induction by poly IC dsRNA. Immunohistochemical staining revealed Mx protein in the cytoplasm and nucleus. Mx protein was induced at both 5 and 50 μg/ml poly IC dsRNA after 48h. Some cytotoxicity was evident in the 50μg/ml poly IC dsRNA induced wells. The amount of Mx endogenously produced was lower than that of transfection expressed Mx protein in CHSE-214 cells as evidenced by the long incubation times of substrate required to see a difference between control and poly IC treated wells and the resulting higher background.

The induction of Mx protein *in vivo* was examined using western blotting by challenge with IHNV via immersion. Mx protein was induced in all fish examined in the kidney 4 days post immersion with a high m.o.i. of virulent IHNV. No Mx was detected in uninfected controls. This preliminary experiment suggests that Mx protein may be a consistent marker for infection with IHNV. It is possible that Mx protein may be useful marker for other fish viral infections and an aid to classifying fish diseases of unknown etiology as viral. Future studies will be needed to demonstrate that Mx protein induction is specific to viral infections.

To establish the sites of Mx protein expression we challenged rainbow trout fry by exposure to the RB-76 isolate of IHNV. In a study by Drolet et. al. the progression of IHNV through the body of an infected fish was determined by immunohistochemistry. They found that the gill epithelium was the first tissue to be infected, followed by the lamellar and filament endothelium. IHNV was later found in the dorsal aorta, and finally the kidney tissue via the renal arteries. Strong positive staining for viral antigen in the kidney tubules was seen. In addition, Drolet *et al.* were able to detect viral antigen in the tissues of the gill, kidney, thymus, spleen, pyloric caecum, liver, and intestine.

Mx protein was detected consistently in the kidney tubules. Mx was also detected in the gill, kidney, thymus, spleen, pyloric caecum, liver, and intestine of some fish. Future immunohistochemical studies will be needed to address the expression of Mx in these additional tissues. The expression of Mx in the kidney suggests IHNV replication was occurring in the tubules inducing IFN, and thus Mx protein. Double label experiments with virus antisera and anti-Mx sera may help us to find out where and when virus is replicating, and where IFN is being produced. This type of study may help us understand how IFN is expressed during viral infections of fish. To date no antibodies to fish IFN are available to detect IFN directly.

Several characteristics make the Mx protein an ideal marker for IFN. Mx proteins are rapidly and transiently induced specifically by type I IFNs in mammals. More importantly, unlike IFN, Mx is produced in large amounts and has a long half-life. Mx in induced cells can comprise up to 1% of the total cytoplasmic protein (Horisberger, 1992) with a biological half-life of 2.5 days (Ronni *et al.*, 1993). Moreover, cellular induction of Mx protein is not subject to feed-back inhibition even at high doses of IFN therapy (von Wussow *et al.*, 1990). Immunoassays have been developed to detect human Mx in blood (Oh *et al.*, 1994; Towbin *et al.*, 1992) and Mx has been used as a marker for IFN therapy (Jakschies *et al.*, 1989). Mx has been used in several studies as a marker for type I IFN (Kraus *et al.*, 1992; Yamada *et al.*, 1994; Abrams *et al.*, 1995).

Roers *et al.* (1994) used the presence of Mx, and therefore type I IFN, to assess vaccination efficacy of attenuated yellow fever virus in volunteers. In this study unvaccinated volunteers produced less Mx protein than vaccinated volunteers upon subsequent challenge with virus. This suggested that successfully vaccinated individuals have enough circulating antibody to clear virus before IFN is induced, and therefore Mx is not produced. Such an inventive use of the trout Mx as an IFN marker may provide a powerful method to evaluate fish viral vaccine efficacy.

The Mx protein should be a useful marker to further characterize the importance of the IFN induced defense to viral infection in fishes.

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CHAPTER 6
THESIS SUMMARY

The IFN inducible Mx proteins of mammals are a component of the innate immune system. These proteins are induced by viral infection and some members of this protein family are responsible for resistance to viral infection by some negative sense RNA viruses. We present here a molecular study of the Mx proteins of rainbow trout.

cDNA Cloning and Analysis

Three trout cDNA sequences were identified and made available in gene banks. These are the first complete Mx cDNA clones of fish. Sequence analysis of the clones demonstrated that trout Mx proteins share approximately 50% sequence identity with avian and mammalian Mx proteins. The trout Mx proteins contain the GTP binding motif, common to all Mx proteins, in the amino terminal third of the protein. This region is highly conserved with other Mx proteins. In addition the trout Mx proteins contain a leucine zipper motif, near the amino terminal which is found in all Mx proteins.

Identification of conserved regions between mammalian, avian, and now complete piscine Mx sequences may help us to understand the importance of protein domains conserved between these taxa.

Analysis of Trout Mx Genes Using a Trout Mx DNA Probe

A trout Mx gene probe was developed to study Mx mRNA expression and to identify Mx genes in salmonid fishes. The DNA probe generated from a single exon of rainbow trout genomic DNA demonstrated the presence of Mx genes in other salmonid fishes. Northern blot analysis showed that rainbow trout Mx mRNA is expressed by IFN inducers *in vitro* and *in vivo*. The similarity of avian, mammalian and piscine control of Mx gene expression further suggests these genes have similar functions in their hosts.

Production of Antisera to Trout Mx Proteins

Polyclonal antisera generated to a recombinant trout Mx protein fragment was used to characterize Mx protein expression. The antisera developed is able to detect all three trout Mx proteins. Trout Mx protein was detected *in vitro* and *in vivo* using western blotting techniques. Immunohistochemistry protocols were developed for detecting Mx protein *in vitro*. Trout Mx proteins are expressed *in vitro* and *in vivo* under conditions where IFN is induced.

Analysis of Mx Expression by Transient Transfection

Full length expression clones were developed to study trout Mx protein expression by transient transfection assay. The subcellular localization of these three proteins was discovered. The expression patterns of the three Mx proteins suggest that they may have distinct roles within the cell. A double label immunofluorescence assay was developed to study the antiviral potential of the trout Mx proteins. A double label immunofluorescence study of transfected cells indicated the trout Mx proteins do not inhibit IHNV replication as evidenced by accumulation of the IHNV nucleoprotein, N.

Future Studies

This initial characterization of the trout Mx system has provided several tools for the investigation of the IFN system of fish. We have already used the Mx gene probe for restriction fragment length polymorphism (RFLP) analysis of rainbow trout stocks in an attempt to correlate Mx RFLP patterns with disease resistance. The transfection clones will be available to investigate the antiviral potential of the trout Mx proteins against other viral pathogens of fish. Of specific interest will be the atlantic salmon orthomyxovirus, infectious salmon anemia (ISA). The trout Mx cDNA sequences have already provided PCR primers to isolate and sequence three Mx cDNA clones of atlantic salmon. The 5' trout Mx cDNA sequences could be used to identify and clone the trout Mx promoter. The tight regulation of the trout Mx promoter make it an ideal candidate for a fish derived inducible promoter. Finally, the trout Mx antibody should be a useful tool to further

characterize the trout IFN system. No IFN antibody is presently available to detect trout IFN so the Mx protein should provide a convenient marker for IFN.

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