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Molecular Characterization of Zebrafish Interferon, MX, and MX Promoter

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MOLECULAR CHARACTERIZATION OF ZEBRAFISH INTERFERON, MX, AND MX PROMOTER

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Type I interferons (IFNs) represent a family of biological molecules whose antiviral, antitumor, and immunomodulatory role is well known. IFNs were first identified in the 1950's and have since been used extensively for the treatment of various cancers, and viral infections. In order to more fully characterize the IFN response, it is often necessary to use animal models. Although the mouse has been used extensively for IFN studies, a lower order vertebrate model is also desirable, as it would provide information about the structure and function of a more ancestral IFN. To this end, herein is described the cloning and characterization of an IFN gene from the zebrafish, *Danio rerio,* as well as the IFN-inducible gene Mx. Zebrafish IFN (zfIFN) has a nucleotide sequence of 558 bases in length, with a deduced amino acid sequence 185 residues in length. Alignment with known IFN sequences reveals a low but significant similarity at the amino acid level, indicating the distant evolutionary relationship of zfIFN to mammalian IFNs. To further characterize zfIFN, zebrafish liver cells in culture were treated with the synthetic double-stranded RNA molecule polyinosinic:polycytidylic acid (Poly IC), which acts as a viral mimetic and thus an IFN-inducer. Analysis of messenger RNA (mRNA) levels at various times post-induction revealed maximal expression of zfIFN mRNA at six and 12 hours post-induction, with a dramatic decrease to basal expression levels by 24 hours. This expression profile fits the pattern of early induction and rapid degradation of mRNA that is a hallmark of

higher order vertebrate IFNs, and thus lends further support to the role of zfIFN as an evolutionary precursor to mammalian IFN. To demonstrate the antiviral activity of zfIFN, zebrafish cells were transiently transfected with an expression construct containing zfIFN DNA and subsequently infected with virus. Cells transfected with zfIFN showed a 36% reduction in the number of plaques formed, compared to cells that were not treated with zfIFN. Having determined the validity of zfIFN as a true member of the IFN family the next step was to characterize the regulatory effect of zfIFN on the zebrafish antiviral gene Mx (zfMx). Zebrafish liver cells produced high levels of zfMx mRNA in response to induction by Poly IC, with peak expression at 24 hours post-induction, indicating upregulation of zfMx by zfIFN. To further characterize this regulation, the zfMx promoter region was cloned and inserted upstream of a reporter gene. Addition of zfIFN to cells transfected with the zfMx promoter resulted in high level expression of the reporter gene. Examination of the zfMx promoter revealed the presence of two DNA elements known to bind IFN-inducible transcription factors. Deletion of these elements from the zfMx promoter led to a marked reduction in reporter gene expression, demonstrating the importance of these elements in zfIFN-induced upregulation of zfMx. Together, these data definitively prove the existence of IFN in a lower order vertebrate, as well as provide a mechanism for the regulation of zfMx by zfIFN. Conservation of this pathway throughout evolution indicates its success in dealing with viral invasion.

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Chapter 1

LITERATURE REVIEW

Overview of Interferon

The type I interferons (IFN) are a superfamily of cytokines with several subfamilies, including IFN alpha, IFN beta, IFN omega, and IFN tau. The IFN alpha/beta subfamilies have been best characterized, with the IFN alpha subfamily consisting of at least 14 separate genes and IFN beta represented by a single gene. Activation of type I IFNs occurs mainly by induction with double stranded RNA, a common product of virally infected cells but not of uninfected cells. Induction by dsRNA causes IFN genes, normally under strict negative regulation, to be activated through release of a repressor protein. Once synthesized, IFN proteins are secreted into the extracellular environment where they bind their cognate receptors on neighboring cells. The binding of IFN to its receptor initiates a signal transduction cascade in which a complex of signal transducers and activators of transcription (STAT) molecules forms. This complex then translocates to the nucleus and binds to a specific cis-acting DNA element, termed the interferon-stimulated response element (ISRE), on the promoter region of a variety of genes, collectively comprising the suite of IFN-effector molecules [1, 2].

Structure of Interferon

IFN proteins are relatively simple molecules, composed of five major alpha helices (A to E), four of which make up the conserved four helix bundle (A, B, C, E) that places IFN within the family of class II helical cytokines [3]. The four helix bundle is arranged in up-up-down-down topology, meaning that, when reading from N terminus to C terminus, two of the helices run in one direction, and two run in the antiparallel direction. These four helices are joined together through two overhead connections. Of the five major alpha helices, helix A is the shortest, being composed of only 11 amino acids. The other four helices are each about 20 amino acids in length. Another important feature of IFN structure is a long stretch of about 30 amino acids

connecting helix A and B, termed the AB loop. The first 11 residues of the AB loop, composed of Arg22 to Asp32, is oriented roughly perpendicular to the helix bundle axis and exists in multiple conformations. The second region of the AB loop consists of residues Arg33 to Gly44 and runs parallel to the axis bundle in an extended conformation. Within the second region, Gln40 to Phe43 are regularly observed to exist in a 310 helix conformation. The third region of the AB loop, corresponding to residues Gly44 to Gly50, is highly flexible and consequently accurate structure determination has been difficult. Dihedral angles of residues 49 and 50 within the third region are observed in the left-handed alpha helix region of the Ramachandran plot, hinting at a possible structure for this segment [3].

The Type I Interferon Receptor

The type I IFN receptor is a multi-subunit complex in the class II helical cytokine receptor family. Subunit ifnar2 is composed of two subdomains, sd100a and sd100b, with the dominant feature of these subdomains being an immunoglobulin-like fold [4]. Subunit ifnar1 has undergone duplication of the two external subdomains, exposing two identical surfaces on either side of the duplicated subdomains. Although the ifnar1 and 2 subunits exist separately on the cell surface, they are believed to dimerize when bound by ligand. It is this dimerization that leads to the activation of downstream kinases [4].

The interaction of IFN with its receptor takes place via a two-step process. In the first step, IFN binds to ifnar2 by way of an electrostatic interaction [5]. This interaction is very rapid and acts at relatively large distances. Consequently, the kinetics of IFN binding to its receptor reveals a high association constant. The second step involves close-range hydrophobic interactions that act to secure the ligand into the binding pocket of the receptor [5]. This reaction occurs much more slowly, but leads to a highly stable complex with a low dissociation constant.

Receptor-Kinase Interaction

Upon ligand binding, receptor-associated tyrosine and janus kinases become activated by tyrosine phosphorylation followed by subsequent phosphorylation of downstream signaling molecules. Specifically, tyrosine kinase 2 (Tyk2), associated with the ifnar1 subunit, and janus kinase 1 (Jak1), associated with the ifnar2 subunit, are activated by receptor dimerization. Activated Jaks become intra and intermolecularly phosphorylated, after which they phosphorylate both the IFN receptor subunits, as well as the STAT molecules [1]. Although phosphorylation of the receptor subunits is necessary in some systems, its role in the type I IFN signaling pathway is not entirely clear.

Structurally, both Jak1 and Tyk2 share the same set of conserved domains common to all members of the Jak family. In all, there are seven conserved Jak Homology (JH) domains [6]. JH1, the most C-terminal domain, is the tyrosine kinase domain. Immediately N-terminal to this is JH2, the kinase-like domain, whose function is unknown. The remaining five domains, JH3-JH7, all towards the N-terminal end of the protein, are not quite as conserved, and their function also remains unclear.

The Role of STATs in Interferon Signaling

As mentioned above, activated Jaks attract STAT proteins, which are then phosphorylated by the Jak-receptor complex [7]. STATs 1 and 2 then dimerize via the Src homology 2 (SH2) domains of these proteins. The mechanism of dimerization and subsequent nuclear translocation and DNA binding has been uncovered, due in large part to solving the crystal structure of a phosphorylated STAT1 dimer bound to DNA [8].

A critical tyrosine residue, Tyr701, was determined to be crucial for STAT activity. Tyr701 is just C-terminal to the SH2 domain and it is believed that the phosphotyrosine of one STAT molecule binds to another STAT molecule through the formation of an antiparallel beta

sheet. The reciprocal binding of phosphotyrosines to SH2 domains of each STAT monomer results in a tight interaction that will allow for subsequent DNA binding [8].

The DNA binding domain is immediately N-terminal to the SH2 domain, containing an immunoglobulin-like fold shared by other DNA binding proteins such as NFkB and p53. SH2-mediated STAT dimerization leads to the formation of a V-shaped structure, with DNA binding occurring as a clamp formed by the dimer. In general, there is not a highly conserved consensus sequence recognized by the DNA binding domain, which suggests that specificity of DNA binding is determined more by the SH2-mediated dimer. In other words, the shape of the molecule, as defined by the dimerization, limits DNA binding to a fairly narrow range of sequences.

In addition to the interaction between two STAT1 molecules, another interaction can occur between a molecule of STAT 1 and a molecule of STAT2. While superficially very similar, STAT2 possesses the ability to bind a third molecule, interferon regulatory factor 9 (IRF9). In this instance, IRF9 is the molecule that binds to DNA as opposed to the STAT molecules in the STAT1 homodimer [9]. It is now well known that formation of STAT1 homodimers leads to activation of IFN gamma-responsive genes, while the STAT1/2/IRF9 complex leads to activation of IFNalpha/beta responsive genes.

Physiological Effects of Interferon

The end result of ISGF3 binding to an ISRE on the promoter of an ISG is the upregulation of a large suite of genes exhibiting a variety of biological effects. The most well characterized of these effects can fall into three categories, antiproliferative, immunomodulatory, and antiviral. Current advances in microarray technology have placed the number of ISGs at over 300 [10, 11], suggesting as yet unidentified mechanisms through which IFN regulates the activity of a cell.

Antiproliferative Activity of Interferon

There are several mechanisms by which IFN exerts an antiproliferative effect on cells. One such mechanism involves the induction of apoptosis, or programmed cell death. A recently identified gene that initiates a pro-apoptotic signal is the TNF-related apoptosis inducing ligand (TRAIL) [12]. This transmembrane protein binds to its cognate receptor on target cells, inducing a pro-apoptotic signal through activation of downstream effectors. One popular effector of apoptosis is caspase 8 [13], a cysteine protease responsible for many of the characteristic signs of apoptosis, including chromatin condensation, DNA fragmentation, and membrane blebbing.

IFNs are also able to inhibit cell growth by regulation of genes directly involved in cell cycle control. For example, down regulation of c-myc by IFN results in cell cycle arrest at the G0/G1 phase [14]. Additionally, the down regulation of c-myc has been correlated with a decrease in the ability of the transcription factor E2F to bind DNA [15]. Other cell cycle regulatory genes targeted by IFN include cyclin A, cyclin-dependent kinase 2, and the cyclin-dependent kinase inhibitors [16].

Immunomodulatory Activity of Interferon

Although classified as a component of the innate immune response, IFN does play a role in adaptive immunity. One well known gene upregulated by IFN is major histocompatibility complex I (MHC I), a crucial component in CD8+ T cell recognition of presented antigen [17]. IFNs are also responsible for upregulating cytokines involved in augmenting T cell activity, such as interleukin 12 (IL12), IL15, and IFN gamma [18]. This suggests a definite link between the innate and adaptive immune responses, in terms of regulation by IFN.

Another avenue of immune regulation by IFN is through the regulation of development and cytocidal activity of several immune cell types. For instance, upregulation of perforin by IFN leads to increased cytotoxicity in natural killer (NK) cells [19]. IFN has also been shown to aid in the maturation and terminal differentiation of dendritic cells, one of the primary antigen

presenting cells [20]. IFN has even been shown to regulate the differentiation of Th1 cells [21], as well as enhance the survival of activated T cells [22].

Antiviral Activity of Interferon

IFN is perhaps most well known for its role in the defense against viruses. Thus far, three key players have been identified as important in antiviral defense, the protein kinase PKR, 2',5'oligoadenylate synthetase (2,5OAS), and the myxovirus resistance gene Mx.

Protein Kinase PKR. PKR is a serine/threonine kinase that contains two double stranded RNA (dsRNA) binding domains [23]. Binding of dsRNA (an intermediate in viral replication) to PKR creates a conformational change in the enzyme which allows it to autophosphorylate. The activated PKR is then able to phosphorylate its target, the alpha subunit of the eukaryotic translation initiation factor (eIF2alpha). Phosphorylation of eIF2alpha inhibits the de novo synthesis of proteins, including all viral proteins. In this regard, PKR can also be classified as antiproliferative.

Oligoadenylate Synthetase. Another enzyme activated by dsRNA is oligoadenylate synthetase (OAS) [24]. When activated, this enzyme generates 2',5' linked oligoadenylate molecules from ATP. These oligoadenylates then bind with high specificity to a latent ribonuclease, RnaseL. RnaseL is responsible for cleaving both cellular and viral single stranded RNA, thus inhibiting protein synthesis. As with PKR, the ability to inhibit both cellular and viral protein synthesis makes the OAS pathway antiproliferative as well.

Mx

The third major antiviral protein induced by IFN is Mx. The gene for Mx was first discovered in mice that contained an inborn resistance to orthomyxovirus infection [25] and has since been found to exist, in one form or another, in a variety of vertebrate and invertebrate organisms. The activity of Mx has been directed largely against RNA viruses such as

orthomyxoviruses, rhabdoviruses, paramyxoviruses, and bunyaviruses [26-28], however, the mechanism of action remains to be fully elucidated.

Mx proteins contain a highly conserved tripartite GTP-binding domain in the amino terminal half of the protein that is believed to play a role in antiviral activity [29], a leucine zipper motif near the carboxy terminus that functions in oligomerization [30], and a dynamin family signature . Although the exact mechanism of Mx action is not completely understood, it has been shown that, in human MxA, oligomerization mediated by intramolecular backfolding is necessary for antiviral activity [31]. Further, the intrinsic GTPase activity of Mx has also been shown to be vital for antiviral activity against certain viruses [32] .

Mx genes have been cloned from a variety of mammalian [33, 34], avian [35], and fish species [36-40], as well as in yeast [41], where a GTP-binding protein similar to Mx has been cloned. Mx proteins localize to either the cytoplasm or the nucleus, with a nuclear localization signal in the carboxy terminus being correlated with nuclear localization as in mouse Mx1 [42], human MxB [43], and Atlantic halibut Mx[36]. Mx proteins that localize in the nucleus obviously inhibit different steps in the viral life cycle than their cytoplasmic counterparts [44].

Mx Promoter

Production of Mx gene transcripts comes about through the activity of the well characterized Mx promoter. All Mx promoters cloned to date have been found to contain between one and three ISREs, with human MxA containing three ISREs, fugu Mx containing two ISREs, and mouse, chicken, and trout Mx containing only one ISRE. There are numerous reports in the literature in which mutation or deletion of an ISRE has either attenuated or completely abolished Mx gene transcription upon induction with IFN.

The ISRE consensus sequence 5' AGTTTCNNTTTCNC/T 3', as defined by Darnell [45], has been found in all known IFN-inducible genes to date. Moreover, the necessity of a functional ISRE for proper IFN-induced signaling has been demonstrated repeatedly in

mammalian, avian, and fish systems through the use of mutagenesis. Nakade et al. [46], generated a series of mutant constructs of the human MxA promoter to determine the minimum promoter region necessary to drive the transcription of a luciferase reporter gene. They found that only the ISRE closest to the transcription start site was essential to confer IFN-inducibility. Deletion of ISRE1 resulted in a construct that showed neither IFN-inducible or basal transcription of luciferase. However, the activity of mutant constructs containing ISRE1 was still significantly diminished compared to the wild type Mx promoter. Mutational analysis of the human MxA promoter has also been carried out by Ronni et al. [47]. This group also showed that deletion of ISRE1 completely abolished MxA promoter activity while deletion of ISRE2 severely reduced, but did not completely abolish, MxA promoter activity. Deletion of ISRE3, which lies further upstream and does not conform entirely to the ISRE consensus, did not appear to effect MxA promoter activity.

Analysis of the chicken Mx promoter has revealed only one ISRE, beginning 68 nucleotides upstream from the transcription start site. Deletion of this ISRE by Shumacher et al. [48] resulted in total abrogation of Mx promoter activity compared to the wild type chicken Mx promoter, again demonstrating the functional necessity of this element in conferring IFN-inducibility.

More recently, several fish Mx promoters have also been cloned and characterized [40, 49]. Collet et al. [49] have characterized the rainbow trout Mx1 promoter and found it to contain only one ISRE, beginning 101 nucleotides upstream of the transcription start site. Although no mutagenesis studies were performed on this promoter, its inducibility by poly IC, and hence IFN, was clearly established. The Mx promoter from fugu has also recently been cloned and characterized [40]. The fugu Mx promoter was found to contain two ISREs in close proximity, separated by only 34 nucleotides, with the proximal most ISRE beginning 51 nucleotides from the transcription start site. Deletion analysis of this promoter has demonstrated a pattern similar to that seen in human and chicken Mx promoters, in that deletion of the upstream ISRE resulted

in significantly reduced promoter activity, while deletion of both ISREs completely abolished promoter activity. This similarity in Mx promoter function among such evolutionarily distinct vertebrates as humans, chickens, and fish suggests a highly conserved mechanism of IFN activation of Mx through the Mx promoter.

Fish Immune System

Adaptive Immune Response

The adaptive immune response of teleost fish has several commonalities with that of higher order vertebrates, including demonstrated B-cell activity [50] and T-cell activity [51]. The predominant antibody isotype found in fish is the pentameric IgM [52], although recently an isotype with homology to IgD has been characterized [53]. Recombination of heavy/light chains and variable/constant regions of fish Igs also occurs [54], through the action of recombination activating gene (RAG) 1 and 2 [55], whose function is similar to that seen in mammals. In terms of cell-mediated immunity, T-cell receptor (TCR) isotypes have been found in fish, with TCR alpha, beta, gamma, and delta homologues being identified thus far [56]. Other evidence for a T-cell response in fish includes proliferation of T-cells by T-cell mitogens [57], function as helper cells in the production of antibodies directed against thymus-dependent antigens [58], and secretion of lymphokines [59].

Innate Immune Response

Like the adaptive immune response, the innate immune response of fish has many similarities with the mammalian innate response. Some of these similarities, which will be discussed below, include chemokines and cytokines, the complement pathway, the Toll pathway, and an oxidative burst.

Chemokines and cytokines. Chemokines are a group of chemoattractant proteins that function in the activation of specific immune cells during an inflammatory response. There are two classes of chemokines, CC and CXC, where the CC class has no spacing between the first set of cysteines and the CXC class has a one amino acid separation. Fish chemokines of the CC class have been cloned in trout [60] and carp [61], while the only CXC-like chemokine found to date comes from zebrafish [62].

Cytokines are another important group of proteins involved in the innate immune response. Typically secreted by phagocytic cells at the site of injury, cytokines play a role in the induction of acute phase proteins, increasing vascular permeability, and inducing a proinflammatory state, to name a few. Several important cytokines found in mammals have had their homologues uncovered in fish. Tumor necrosis factor alpha (TNF alpha), a proinflammatory cytokine with additional cytotoxic activity, has been cloned in rainbow trout [63] and Japanese flounder [64], and determined to be TNF alpha homologues through both sequence conservation and functional assays [63, 64]. The cytokine interleukin (IL)-1 beta, a member of the IL-1 family that, along with TNF alpha, is responsible for incurring a proinflammatory state, has been cloned in both rainbow trout [65, 66] and carp [67]. Like mammalian IL-1 beta, exposure of fish cells to LPS efficiently induced IL-1 beta expression [68].

Complement Pathway. The complement pathway involves a host of proteins whose sequential activation leads to the eventual opsonization and/or lysis of target microorganisms. There are several ways in which complement can be activated, with the alternative pathway and lectin pathway not requiring specific antibodies, and thus being classified as part of the innate immune response. Several components of the complement pathway have been found in fish, including C3 [69], C4 [70], C8B and C9 [71]. Although not all complement components have been cloned in fish, the evidence for a functional pathway is strong [72].

Toll Signaling Pathway. The Toll signaling pathway is composed of a group of cell surface receptors that function in nonspecific pathogen associated pattern recognition [73]. Induction of

the Toll pathway leads ultimately to the induction of a variety of immune effector molecules. Initially identified in Drosophila, Toll homologues have since been found in humans [74], mouse [75] and chicken [76]. Only recently has the search for Toll homologues in fish begun in earnest. To date, there is only one published report of a Toll-like receptor being cloned in fish [77].

Oxidative Burst. The oxidative burst is one of the most ancient defense mechanisms used by eukaryotic organisms. In this mechanism, activated macrophages and neutrophils produce toxic molecules known as reactive oxygen species (ROS), sequestered in specialized intracellular compartments called lysosomes. Macrophages engulf microbes into a phagosome, which fuses with an ROS filled lysosome to form a phagolysosome. Exposure of the microbe to ROS results in its rapid destruction. There is considerable evidence showing an oxidative burst in fish, especially when induced by soluble factors such as cytokines [78-80].

Mx Genes in Fish

An important component of the fish antiviral response is the IFN-inducible protein Mx. Genes for Mx have now been cloned in a number of fish, with a fairly high percent identity between different fish species. In rainbow trout, Trobridge et al. Found three Mx genes, all with high identity to each other (between 88.7 and 96.9%), but were unable to demonstrate antiviral activity [39, 81]. Three Mx genes have also been cloned in Atlantic salmon, although two of these genes may be classified as alleles of the same gene [38]. As with the rainbow trout Mx gene, the poly IC-, and thus IFN-inducible nature of Mx gene expression in these fish is consistent with that seen in mammalian Mx. An Mx gene from Atlantic halibut has been cloned, and was shown to be upregulated in response to IPNV infection, although direct antiviral effects were not determined [36]. Mx genes have also been cloned from Japanese flounder and pufferfish, with IFN-inducible expression, but not antiviral activity, being established [37, 40].

The Zebrafish Immune System

The immune system of zebrafish shares many components of its mammalian counterparts. Genes for rag1 and rag2 have been cloned, indicating that zebrafish are capable of somatic gene rearrangement [55]. A gene for TCR alpha has been cloned in zebrafish, although genes for TCR beta, gamma, and delta remain to be found [56]. The structure of IgM in zebrafish has been characterized, along with evidence for sequence diversity in the IgM heavy chain variable region [52]. Other important immune genes characterized in zebrafish include beta 2 microglobulin and MHC II [82].

Zebrafish as a Model of Human Disease

The zebrafish is emerging as an outstanding model of both development and genetics. From a developmental perspective, zebrafish embryos are fertilized externally, and, along with a transparent chorion, allows for clear visualization of developmental processes [83]. Genetically, the high yield of embryos per mating (>200) combined with a short generation time (3 months) greatly facilitates genetic studies by enabling large scale genetic screens for mutant phenotypes [83]. In addition, the conservation of synteny across the zebrafish and human genomes has led to the positional cloning of several zebrafish genes whose human homologues are responsible for such diseases as Huntingtons [84] and Alzheimers [85].

Although not as well established as current murine models, molecular techniques that utilize zebrafish are advancing rapidly. The construction of yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) libraries in zebrafish have been instrumental in positional cloning endeavors [86]. Whole mount *in situ* hybridizations of zebrafish embryos has allowed unique insight into the timing and pattern of gene expression [77]. The use of antisense RNA has proven invaluable as a tool to attenuate gene expression where the ability to completely abolish gene expression (gene knockout) is possible only in mouse models.

Fish Viruses and the Interferon Response

Among the most prevalent viruses affecting fish are members of the family Rhabdoviridae, which include infectious hematopoietic necrosis virus (IHNV) [87], spring viremia of carp virus (SVCV) [88], and snakehead rhabdovirus (SHRV) [89]. Other important viruses with an impact on aquaculture include infectious pancreatic necrosis virus (IPNV) [90], a member of the Birnaviridae fa mily, and infectious salmon anemia virus (ISAV) [91], which belongs to the family Orthomyxoviridae.

The Rhabdoviridae, of which IHNV, SVCV, and SHRV are all members, are a class of negative sense single stranded RNA viruses with a bullet shaped morphology. IHNV infects primarily trout and salmon, with clinical disease occurring between 8-15° C under natural conditions. IHNV has been classified into the new genus *Novirhabdoviridae* based on the presence of a unique Non-virion gene found between the glycoprotein and polymerase genes on the IHNV genome [87].

Another member of the rhabdovirus family, SVCV, does not contain the non virion gene found in IHNV. The predominant pathological effect of SVCV infection is an impairment of the salt-water balance stemming from edema and hemorrhages [92]. Affecting mainly cyprinids, SVCV infection occurs at temperatures of 10-17° C under natural conditions, while *in vitro* studies show an optimal infection temperature of 20° C. The SVCV genome is composed of a linear molecule of negative sense, single stranded RNA coding for five proteins; nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and polymerase [88].

A third member of the family Rhabdoviridae known to infect fish is SHRV. Isolated from the snakehead fish *Ophicephalus striatus*, SHRV has also been shown to infect catfish spleen cells and channel catfish ovary cells. Replication in chinook salmon embryo cells was minimal, while epithelioma papulosum cyprini and rainbow trout gonad cells were completely refractory to infection. Infection with SHRV causes necrotic ulcerations, both in wild and pond-

cultured fish. Phylogenetic data places SHRV within the Novirhabdovirus genus, although a definitive classification must await identification of the non virion gene that is characteristic of this genus [89].

Among the aquatic birnaviruses, IPNV is probably the most prevalent, with its nonenveloped icosahedral morphology and bi-segmented double stranded RNA genome. IPNV has a broad host range, including salmonids, tropical fish, eels, sea bass, and lamprey. IPN-like viruses have even been isolated from mollusks and crustaceans [92].

One of the most notorious fish viruses in recent history is ISAV, an orthomyxovirus responsible for the death of large numbers of farmed Atlantic salmon over the past several years. The ISAV genome, like that of other orthomyxoviruses, is composed of eight segments of single stranded RNA ranging in size from 1.0 to 2.3 kb. ISAV has yet to be isolated from any fish other than Atlantic salmon, with clinical symptoms including anemia, enlarged liver and spleen, and hemorrhaging [92]. The negative economic impact of these viruses on the aquaculture industry has led researchers to investigate the innate immune response of fish to such pathogens. This has meant primarily characterizing the type I IFN pathway, as IFN has been shown to provide the front line of defense against viruses in higher order vertebrates.

The type I IFN signaling pathway, although well elucidated in humans, is less clear in fish, although, with the advent of genome sequencing, this puzzle is rapidly being put together. The type I IFN receptor has only recently been cloned in fish, and was shown to be lacking in IFNAR1, although an IFNAR2 receptor was clearly present (Lutfalla et al., manuscript in print). Several other components of the IFN pathway have been identified in fish, including STAT1 and STAT3 in zebrafish [93], a putative STAT molecule in catfish [94], STAT5 from the pufferfish, Jak1 from catfish, and IRF1 and IRF2 in rainbow trout [95].

Zebrafish STAT1 and STAT3. The cloning of STATs 1 and 3 in zebrafish suggests conservation of the interferon signaling components. Zebrafish STAT3 displays a conservation of synteny with the human and mouse genomes and is expressed in a tissue restricted manner

during embryogenesis. Zebrafish STAT1, although not sharing synteny with mammalian chromosomes, was able to rescue the interferon signaling pathway in a STAT1-deficient human cell line [93].

Catfish STAT. A STAT protein with antibody reactivity to human STAT6 has been identified from channel catfish. The investigators demonstrated nuclear translocation, a prerequisite for proper STAT function, as well as binding to an IFN-response element on the catfish genome, a trait common to mammalian STAT proteins [94].

Pufferfish STAT5. A gene with homology to mammalian STAT5 was isolated from a pufferfish genomic library, showing 76% amino acid identity to mouse STAT5a, and less than 35% identity to other mammalian STATs. The pufferfish STAT5 protein was able to bind a specific DNA-binding element on an artificially constructed fish promoter, thus demonstrating its functional classification as a STAT protein.

Catfish Jak. Miller et al. were able to demonstrate the presence of a putative Jak molecule in catfish by using antibodies to human Jak1. Catfish Jak was shown to be constitutively phosphorylated in channel catfish longterm leukocyte cell lines. The investigators were also able to demonstrate coimmunoprecipitation of catfish Jak with the previously cloned catfish STAT.

Rainbow Trout IRF1 and 2. Genes for IRF 1 and 2 have been cloned from rainbow trout. *In vivo* constitutive expression of IRF1 and IRF2 was seen in head kidney, gill and spleen, but not liver. As expected, expression of the positive interferon regulator IRF1 was demonstrated one week after injection with a DNA vaccine against viral hemorrhagic septicemia virus [95].

In chapter two of this thesis is described the first IFN gene to be cloned in any fish species. Since the publishing of this manuscript, an IFN gene from catfish has also been cloned and characterized (Miller et al., manuscript in print). The data thus compiled points to a conserved evolutionary pathway, with many key proteins in the mammalian pathway finding their homologues in fish. This situation is ideal, in that it allows for more direct comparisons to be

made between mammalian and fish immune systems, and serves to reinforce the use of zebrafish as a model for the innate immune response.

Chapter 2

MOLECULAR AND FUNCTIONAL ANALYSIS OF AN INTERFERON GENE FROM THE ZEBRAFISH, *DANIO RERIO*

Chapter Abstract

The type I interferon (IFN) family consists of a large group of cytokines involved in the innate immune response against various microorganisms. Genes for IFN have been cloned from a variety of mammalian and avian species, however, IFN genes from lower-order vertebrates have not been forthcoming. Here, we report the cloning and characterization of IFN from the zebrafish, *Danio rerio*. Zebrafish IFN (zfIFN) is 185 amino acids in length with the first 22 amino acids representing a putative signal peptide. Treatment with the known IFN-inducer, polyinosinic:polycytidylic acid (PolyIC), resulted in an increase in zfIFN mRNA transcripts. ZfIFN was also able to activate the IFN-inducible Mx promoter when cotransfected with a plasmid containing the zebrafish Mx promoter upstream of a luciferase reporter gene. To demonstrate antiviral activity, zebrafish cells were transfected with zfIFN and challenged with a fish rhabdovirus. A 36% reduction in plaque number was seen in zfIFN transfected cells, as compared to cells transfected with a control vector. Phylogenetic analysis has shown zfIFN to be approximately equally divergent from avian and mammalian IFN, consistent with its origin from an IFN present in the most recent common ancestor of these divergent lineages. A putative IFN from pufferfish, *Fugu rubripes,* was also found when zfIFN was used to search the fugu genome database, demonstrating that zfIFN can be used to find additional fish IFN genes. These results demonstrate that zebrafish can be used as an effective model for studying innate immunity and immune response to infectious disease.

Introduction

The interferons (IFNs) represent a large family of soluble cytokines with biological and antiviral activity [96]. IFN was first discovered by Isaacs and Lindenmann in 1957 as an agent capable of inhibiting the growth of influenza virus in embryonated chicken eggs [97]. When induced by viral infection or the synthetic double stranded RNA molecule PolyIC [98], IFN is synthesized and secreted into the extracellular environment, where it binds to its cognate receptor on neighboring cells [99]. This results in a signal transduction cascade, ultimately leading to the stimulation of an array of antiviral proteins, among them 2'-5' oligoadenylate synthetase, protein kinase p68, and Mx. These proteins are responsible for the degradation, or prevention of synthesis, of viral RNA [100]. The Mx gene promoter contains an interferon-stimulated response element (ISRE) that is recognized by an IFN-induced transcription factor composed of signal transducer and activator of transcription (STAT) 1 and 2, bound to interferon regulatory factor (IRF) 9 (see review by Taniguchi and Takaoka [101]). By linking the ISRE of the Mx promoter to a reporter gene, the presence of IFN may be detected [48, 49, 102]. In addition to antiviral activity, the IFNs have been implicated in cell proliferation and differentiation, as well as in the suppression of some forms of cancer [103, 104]. IFN β knockout mice have been shown to exhibit diminished antiviral response to vaccinia virus [105], demonstrating the importance of a functional IFN system.

IFNs are grouped into type I, consisting of IFNα, β, ω, δ, κ and τ, and type II, consisting of IFNγ [100]. Primary IFN peptides are 185 to 190 amino acids in length, the first 23 to 30 amino acids representing a hydrophobic signal peptide necessary for secretion. The signal peptide is cleaved prior to extracellular translocation of IFN to yield the mature peptide.

Genes for type I IFN have been cloned from a variety of mammals, including human [106, 107], mouse [108, 109], pig [110], and several avian species such as duck [111], chicken [112], and turkey [113]. To date, there have been no reports of an IFN gene being cloned from any fish species, although IFN-like antiviral activity has been demonstrated [114]. A sequence for flatfish

IFN has been reported [115], however, Magor et al. [116] found that it had no similarity to other IFNs. Furthermore, analysis with the basic local alignment search tool (BLAST) revealed >60% amino acid identity of two-thirds of this sequence to sequences from filamentous phage [116]. There are several reasons why fish IFN has not previously been cloned, among them the relatively low homology among species of IFN genes, which increases the difficulty of designing effective primers for use in PCR as well as in designing probes for library screening. Also, the lability of IFN transcripts renders isolation of sufficient quantities of IFN mRNA for amplification purposes very difficult. Researchers have thus used downstream effectors of IFN to demonstrate IFN activity indirectly in fish [39, 117, 118].

The zebrafish, *Danio rerio*, has recently emerged as an ideal model for the study of development and genetics. Genetically, the conservation of synteny between various chromosomal loci of humans and zebrafish has allowed positional cloning of several zebrafish genes [119]. Increasingly, these syntenic relationships have been used to find the homologs of genes responsible for human diseases such as Huntington's [84] and familial Alzheimer's [85]. Further, the similarity between humans and zebrafish, in terms of disease, extends beyond gene homology to actual similarities in the proteins involved in pathogenesis [119].

The innate immune response of zebrafish to infectious pathogens is currently not well characterized. However, due to its relative hardiness and resistance to infection and its evolutionary lineage, the zebrafish is likely to provide clues to the early evolution of the innate immune system. We report here the cloning and characterization of an IFN gene from zebrafish. Expression of this gene was inducible by PolyIC and was able to stimulate upregulation of the IFN-inducible Mx promoter. ZfIFN was also shown to exhibit antiviral activity, as demonstrated by a reduction in plaque formation in zebrafish cell monolayers. Phylogenetic analysis has shown zfIFN to be equally related to mammalian and avian IFN. A putative fugu IFN was also found by using zfIFN to search the fugu genome database (www.jgi.doe.gov/fugu). ZfIFN represents the lowest order vertebrate IFN cloned to date. Future studies of zfIFN will enable a deeper

understanding of the IFN system and its importance to innate immunity, both in zebrafish and in humans.

Materials and Methods

Cell Culture and Viruses

Zebrafish liver cells (ZFL), ATCC number CRL-2643 [120], were grown in a medium containing a combination of 50% L-15, 35% Dulbecco's minimum essential medium (DMEM), 15% F-12 with sodium selenite, and supplemented with 5% v/v fetal bovine serum (FBS), 0.5% v/v trout serum, 0.5% v/v insulin, and 50 μg/mL each of penicillin, streptomycin, and ampicillin. The embryo fibroblast cell line ZF4, ATCC number CRL-2050 [121], was grown in a 1:1 ratio of F-12 and DMEM supplemented with 10% FBS. Spring viremia of carp virus (SVCV) and snakehead rhabdovirus (SHRV) were propagated in ZFL cells. Cells were infected at a multiplicity of infection of 0.1. The supernatant was then collected and filtered to obtain purified virus at a titer of 1.25×10^7 tissue culture infectious dose 50% ($TCID_{50}$)/mL.

Generation of cDNA

To obtain total RNA, ZFL cells were grown to confluence in 75 cm^2 flasks and were treated with either 50 μg/mL PolyIC (Sigma, St. Louis, MO), 10^4 $TCID_{50}$/mL SVCV, or not treated. Cells were harvested at 6, 12, 24, 36, and 48 h post-treatment and total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA). A reverse-transcription reaction was then performed to convert total RNA into cDNA. Briefly, 400 ng of RNA and 0.5 μg of random hexamers were combined and incubated at 70°C for 5 min, followed by a quick chill on ice for 5 min. To this mixture was added ImpromII 5× reaction buffer, 25 m*M* $MgCl_2$, 10 m*M* dNTP, 0.5U

RNase inhibitor, and 1 μL IMpromII reverse transcriptase (Promega, Madison, WI). The reaction mixture was incubated for 1 h at 37°C followed by a 15 min heat inactivation step at 70°C.

RACE-PCR

RACE-ready cDNA was prepared using the GeneRacer kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Upstream RACE-PCR was performed using a gene-specific primer designed from an EST (GenBank accession number BI708494). This primer, designated 66rev, with the sequence 5' GCTCTGCGTCTACTTGCGAATGGC 3', lies at base pairs 43 to 66 of the EST and primes upstream amplification when used with the downstream GeneRacer primer. This reaction yielded an amplification product containing a region of the 5' untranslated region (UTR) of zebrafish IFN, which was subsequently used as template to design primers for downstream RACE.

IFN-specific oligonucleotides were then designed to anneal to the 5' UTR of zebrafish IFN and prime downstream amplification. The sequence of the primary IFN primer, which sits at position –73 to –49 relative to the start site and is designated –73fwd, is 5' CCAGCACTCTCCATCATGTCTCTG 3' and the sequence for the nested IFN primer, sitting at position –7 to +18 relative to the start site and designated –7fwd, is 5' CGCAAAGATGAGAACTCAAATGTGG 3'. These primers were used with GeneRacer 3' primary and nested primers, respectively, in two rounds of PCR. PCR amplification reactions were carried out according to the manufacturer's instructions (Sigma, St. Louis, MO). Cycling parameters were 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min for a total of 30 cycles. Nested PCR reactions used the nested primers and first round product as template.

The amplified product was subcloned into a pGEM-T Easy vector (Promega, Madison, WI) and submitted for sequencing at the University of Maine Sequencing Facility with an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA).

Plasmid Construction

Using the cloned IFN product as template, PCR was performed with the upstream primer 5' CAGCCGGGTACCTAAGGAGGCCACCATGAGAACTCAAATGTGGACC 3', which anneals to base pairs 1–21 and contains a *Kpn*I site, and the downstream primer 5'CAGGAATTCTACGAATGCTATTACACTCGAGGATTGAC 3', which anneals to base pairs 557–540 and contains an *Eco*RI restriction site. The modified IFN gene was then subcloned into an FRM expression vector suited for zebrafish, kindly provided by Dr. Pat Gibbs, Rosenstiel School of Marine and Atmospheric Sciences, Miami, FL [122], and digested with *Kpn*I and *Eco*RI, thus generating the plasmid pFRMIFN.

Mx Promoter Transfections

Transfections were carried out using the cationic liposome reagent Transfast (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 1.0 μg of DNA was added to serum-free ZF4 media, after which 2.0 μg of TransFast was added and the resulting mixture vortexed. The DNA:liposome complex was added to cells and allowed to incubate at normal growth temperature (28°C) for 1 h, at which time 1.0 mL of ZF4 media supplemented with 10% FBS was added to each well. The Mx promoter construct contains a region of the zebrafish Mx promoter (GenBank accession number AF532732) linked to a luciferase reporter gene in a pGL3 series vector (Promega, Madison, WI). One treatment group of ZF4 cells was cotransfected with zfIFN and the Mx promoter construct, while another treatment group received the Mx promoter construct with a control pcDNA3 vector in place of zfIFN. A third treatment group was

transfected with the Mx promoter construct alone, then induced with 25 μg/mL PolyIC to serve as the positive control. A fourth treatment group was transfected with the pGL3 basic vector, containing the luciferase reporter gene but lacking the Mx promoter, then induced with 25 μg/mL PolyIC. Cells were then treated with BrightGlo luciferase assay reagent (Promega, Madison, WI) and incubated for 5 min. Luciferase activity was measured in relative light units (RLU) in a Fusion Universal Microplate analyzer (Packard, Meriden, CT) with a 10 sec read time for each well.

Quantitative PCR

Quantitation of IFN cDNA was carried out using the fluorescent SYBR Green nucleic acid stain [123, 124]. The primers 66rev and –73fwd were used to amplify a 139-bp fragment of zfIFN. A standard curve was constructed by serially diluting a linearized plasmid containing the open reading frame of zfIFN. Zebrafish 18S ribosomal primers (PE Applied Biosystems, Foster City, CA) were used to normalize for starting quantity of RNA. Reactions were performed in an iCycler iQ Real-Time PCR detection system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Reactions were carried out in a volume of 25 μL containing 12.5 μL of 2× SYBR Green PCR Master Mix (Qiagen, Valencia, CA), 1.0 μL of 5.0 μ*M* primers, 10.5 μL of nuclease-free water, and 1.0 μL of cDNA. Cycling parameters were 94°C for 15 min to activate the polymerase followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Fluorescence measurements were taken at each cycle during the 55°C step. RNA levels are expressed as copy number based on the standard curve, after which these values were normalized to the corresponding 18S values to generate the relative copy number.

Antiviral Assay

ZF4 cells were seeded in 12-well plates prior to transfection. One group of cells was transfected with pFRMIFN and one with the control vector pcDNA3. A third group of cells,

representing the positive control, received 25 μg of PolyIC plus 1 μg of liposome, and a fourth group of cells, representing the negati received 1 μg of liposome only. After 16 h, medium was removed and all cells were infected with 400 μL of SHRV at a titer of 1.25 x 10^3 $TCID_{50}$/mL. After 24 h, the antiviral assay was terminated by fixing and staining overnight in a solution of 1% crystal violet, 0.9% NaCl, and 40% formaldehyde to allow visualization of plaques.

Phylogenetic Reconstruction

Phylogenetic analyses were performed using algorithms contained in PAUP (version 4.0) [125]. Maximum parsimony and evolutionary distance methods were used to infer tree topology. In all analyses, branch swapping was by tree-bisection-reconnection (TBR) with characters weighted equally (weight = 1) and gaps treated as missing data. Bootstrap analyses were performed using the full heuristic search option with 100 replicates for maximum parsimony and evolutionary distance analyses. Sequences were aligned using Clustal W, employing the BLOSUM series matrix. The final alignment contained 212 amino acid positions, 185 of which are parsimony informative.

Results

Cloning of Zebrafish IFN

The Zebrafish Information Network expressed sequence tag (EST) database was searched using BLAST with a gene for chicken IFN. Only one EST was found (accession number BI708494), containing all but the very 5' end of zfIFN. Upstream RACE-PCR was performed to obtain the 5' end of zfIFN using a primer designed from this EST. PCR amplification yielded a 198-bp product that contained the 5' end of zfIFN, including the ATG and a portion of the 5'

UTR. To ensure that the amplified product was IFN, 3' RACE was performed using primers designed from the 5'UTR, thus spanning the entire open reading frame. The amplified product yielded full-length zfIFN, in addition to approximately 400 bp of 3'UTR. This gene codes for a protein 185 amino acids in length (Fig. 2.1), with the first 22 amino acids representing a putative signal peptide that was characterized by analysis with the SignalP program (version 1.1; Center for Biological Sequence Analysis [http://www.cbs.dtu.dk/services/SignalP/]). Amino acid 23 represents the putative first residue of the mature peptide. The N-linked glycosylation motif NXS/T, which is typically seen in other IFNs, was not observed. In addition, zfIFN is alkaline in nature, as opposed to other IFNs, which have an acidic isoelectric point.

Induction of Mx Promoter Activity by zfIFN

To determine whether zfIFN could induce the Mx gene, cotransfections were performed on ZF4 cells using the Mx promoter-pGL3 luciferase construct and zfIFN (Fig. 2.2). As a positive control, the Mx promoter construct was transfected alone, and then stimulated with the known IFN-inducer PolyIC. The Mx promoter construct was also cotransfected with control pcDNA3 vector to demonstrate that random DNA does not significantly induce the Mx gene. In a previous experiment, the FRM and pcDNA3 vector backbones were cotransfected with the Mx promoter to rule out the possibility of differences in CpG content causing increased induction of luciferase

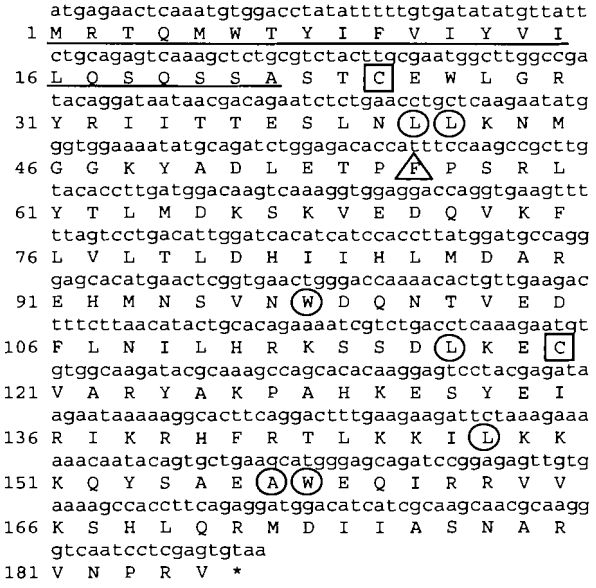


Figure 2.1 Nucleotide and deduced amino sequence of zfIFN. The nucleotide sequence is shown in lower-case letters and the amino acid sequence in capital letters. The two cysteine residues are boxed, the highly conserved residues are circled, Phe56 is marked by a triangle, and the putative signal peptide is underlined.

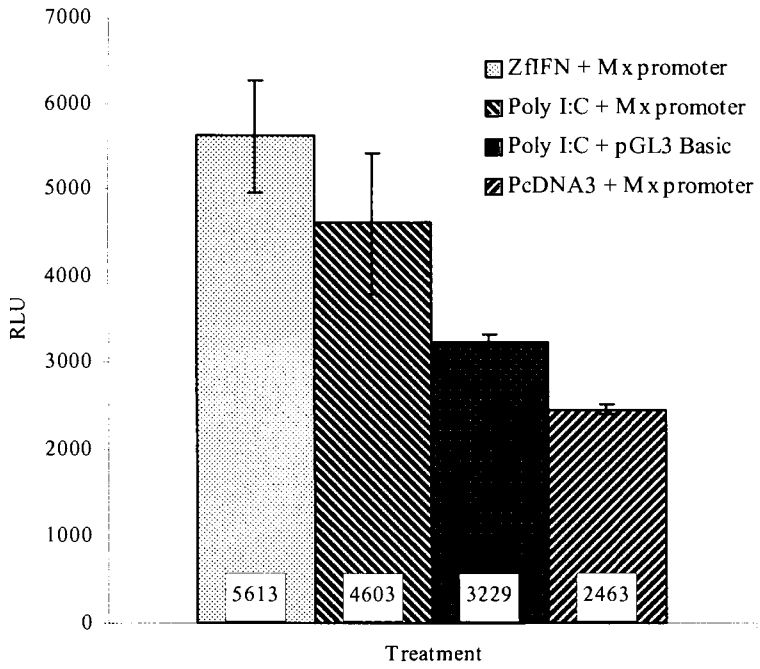


Figure 2.2 Induction of Mx promoter construct by zfIFN expression. ZF4 cells were either induced with 25 μg/mL PolyIC, transfected with zfIFN or empty vector, or untreated. After 24 h, cells were lysed and luciferase activity measured. The experiment was performed in triplicate, with each bar representing the mean of three samples. Error bars = ± SD.

(data not shown). As shown in Fig. 2, zfIFN induction of ZF4 cells averaged 5.6×10^3 RLU, while PolyIC induction yielded an average of 4.6×10^3 RLU. A 42% increase in RLU was seen in zfIFN-transfected cells compared to cells transfected with the Mx promoter only, and a 56% increase was seen compared to cells induced with PolyIC but lacking the Mx promoter. Background luminescence, as determined by reading wells containing media only, averaged 2500 +/- 100 RLU. The fact that the construct lacking the Mx promoter showed diminished luciferase activity compared to the Mx promoter (+) construct demonstrates that the Mx promoter is necessary to drive luciferase expression when induced with IFN, making the Mx promoter a useful diagnostic tool for the presence of IFN.

Induction of zfIFN by PolyIC

To determine the extent to which zfIFN could be induced by PolyIC in vitro, ZFL cells were treated with 25 μg/mL of PolyIC and RNA was extracted at time points of 6, 12, 24, 36, and 48 h. Quantitative PCR using the dye SYBR green (Fig. 2.3) revealed an increase in PolyIC induced samples of zfIFN mRNA at 6 h to 1.4×10^4 copies versus 3.6×10^2 copies for the 6 h control group, and a further increase at 12 h to 3.0×10^4 copies versus 1.1×10^3 copies for the 12 h control group. At 24 h, zfIFN mRNA levels in the induced sample had decreased almost to basal levels ($3.0 \times 10^2 - 3.0 \times 10^3$ copies), demonstrating the labile nature of IFN transcripts. A low level of constitutive expression was seen, consistent with previous results [126]. Control reactions, in which cells were not induced with PolyIC, yielded no significant increase in expression levels.

zfIFN Protects ZF4 Cells from Viral Infection

To determine whether zfIFN has antiviral properties, ZF4 cells, chosen for their ability to form plaques when infected with SHRV, were transfected with zfIFN and 16 h later exposed to SHRV for 1 h. The assay was terminated by addition of a crystal violet fixing and staining solution, allowing visualization of plaques (Fig. 2.4a–d). At the 10^2 $TCID_{50}$/mL titer, a 31% reduction in plaques was seen in zfIFN transfected cells compared to untransfected cells, and a 36% reduction compared to cells transfected with control pcDNA3 vector. At the 10^3 $TCID_{50}$/mL virus titer, reductions of 28% and 24% from untransfected and pcDNA3 transfected cells, respectively, were seen (Fig. 2.4e). The size of the plaques was not significantly reduced in zfIFN-transfected cells. Cells treated with PolyIC alone prior to infection with SHRV, however, demonstrated a substantial reduction in both plaque size and number. The percent reduction of plaques at the 10^2 $TCID_{50}$/mL virus titer for PolyIC treated cells was 55% and 59% that of untransfected and pcDNA3 transfected cells, respectively, and 57% and 55% at the 10^3 $TCID_{50}$/mL virus titer of untransfected and pcDNA3 transfected cells, respectively (Fig. 2.4e). These results demonstrate that zfIFN can confer a protective effect on ZF4 cells when administered 16 h prior to viral challenge, and that this effect can be seen as a reduction in plaque number.

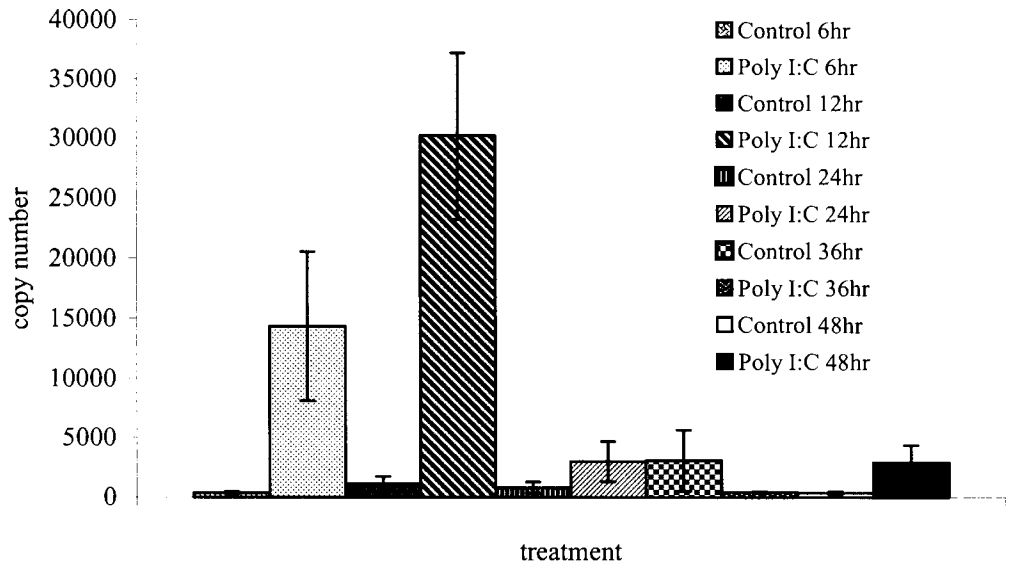


Figure 2.3 Quantitation of zfIFN mRNA upon stimulation with PolyIC. ZFL cells were either induced with 25 μg/mL PolyIC or uninduced, and total cellular RNA was harvested at selected time points. Each bar represents the mean of three replicates. Error bars = ± SD.

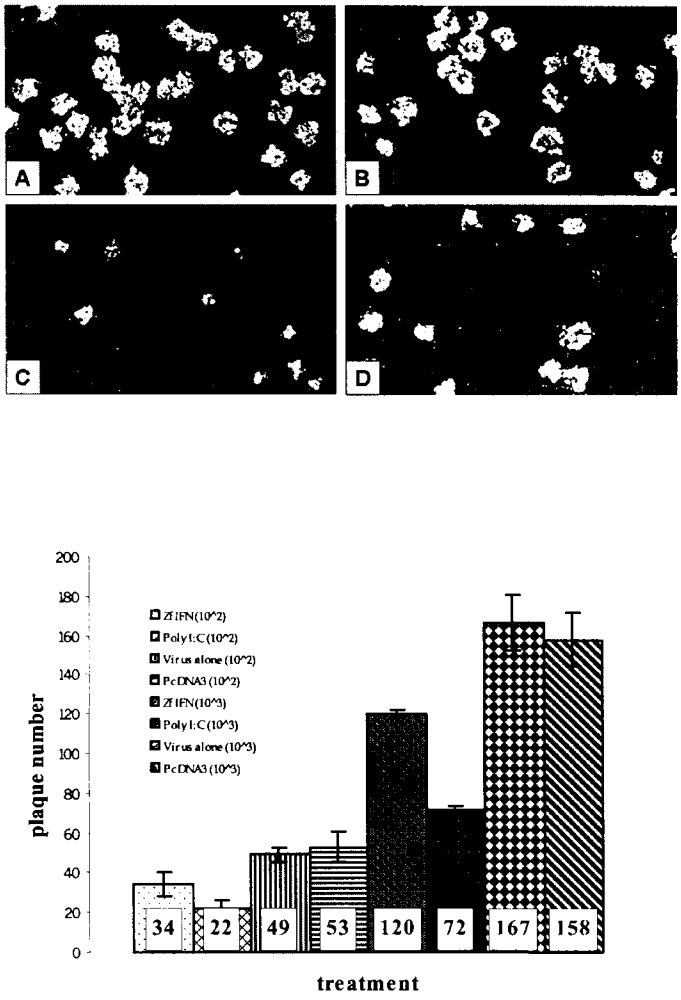


Figure 2.4 Plaque reduction in cells transfected with zfIFN and infected with SHRV. ZF4 cells were either induced with 25 μg/mL PolyIC, transfected with zfIFN or empty pcDNA3 vector, or untreated. All cells received equal amounts of liposome. After 16 h, all cells were challenged with SHRV for 24 h before termination by staining in crystal violet and ethanol; a) pcDNA3 transfected; b) liposome only; c) PolyIC induced + liposome; d) zfIFN transfected; e) graph of plaque reduction numbers. The first four bars represent experiments done with the 10^2 virus titer; the second four bars represent the 10^3 virus titer. The experiment was performed in triplicate. Bars represent the means of three replicates. Error bars = ± SD.

Alignment of zfIFN

A CLUSTAL W alignment was performed on a variety of type I IFN genes from mammals, birds, and zebrafish using the BLOSUM matrix (Fig. 2.5). Among the most conserved residues are Leu41, Leu42, Trp98, Leu117, Leu148, Ala157, and Trp158 (positions relative to the zfIFN start codon). Two zfIFN Cys residues, at positions 25 and 120, are conserved among all IFN sequences aligned except mammalian IFNβ while two additional Cys residues are absent in zfIFN but are conserved across all other species aligned. Previous studies have shown that, in human IFN, three residues, Leu30, Arg33, and Phe36 (positions in reference to the mature peptide of human IFN), may be involved in binding to the type I IFN receptor [127]. Leu30 and Arg33 do not appear in avian IFN or zfIFN, while Phe36 (corresponding to residue 56 in zfIFN), shown to be important for biological activity [127], is identical in zfIFN but not conserved in chicken α, turkey, or duck IFN.

Phylogeny Reconstruction

A single best tree with a minimum evolution score of 5.625 was inferred by evolutionary distance analysis (Fig. 2.6). Maximum parsimony analysis found 18 equally parsimonious best trees of 1,574 steps. All best trees inferred by both methods were identical with respect to all nodes receiving significant bootstrap support (bp >70%). Results indicate that all mammalian IFNs form a single well supported clade as do all avian IFN, and that these clades exclude zfIFN. Pairwise comparisons of amino acid sequence identity and similarity indicate a similar degree of divergence of zfIFN from mammalian and avian α and β IFN subtypes (Table 2.1). ZfIFN has 15% and 14% identity, over the entire sequence, to human IFNα and β, respectively, but 25% and 24% identity to human IFNα and β, respectively, over the conserved domain. When zfIFN was compared to chicken IFNα and β, an increase of only 4 to 5% was seen when aligning the

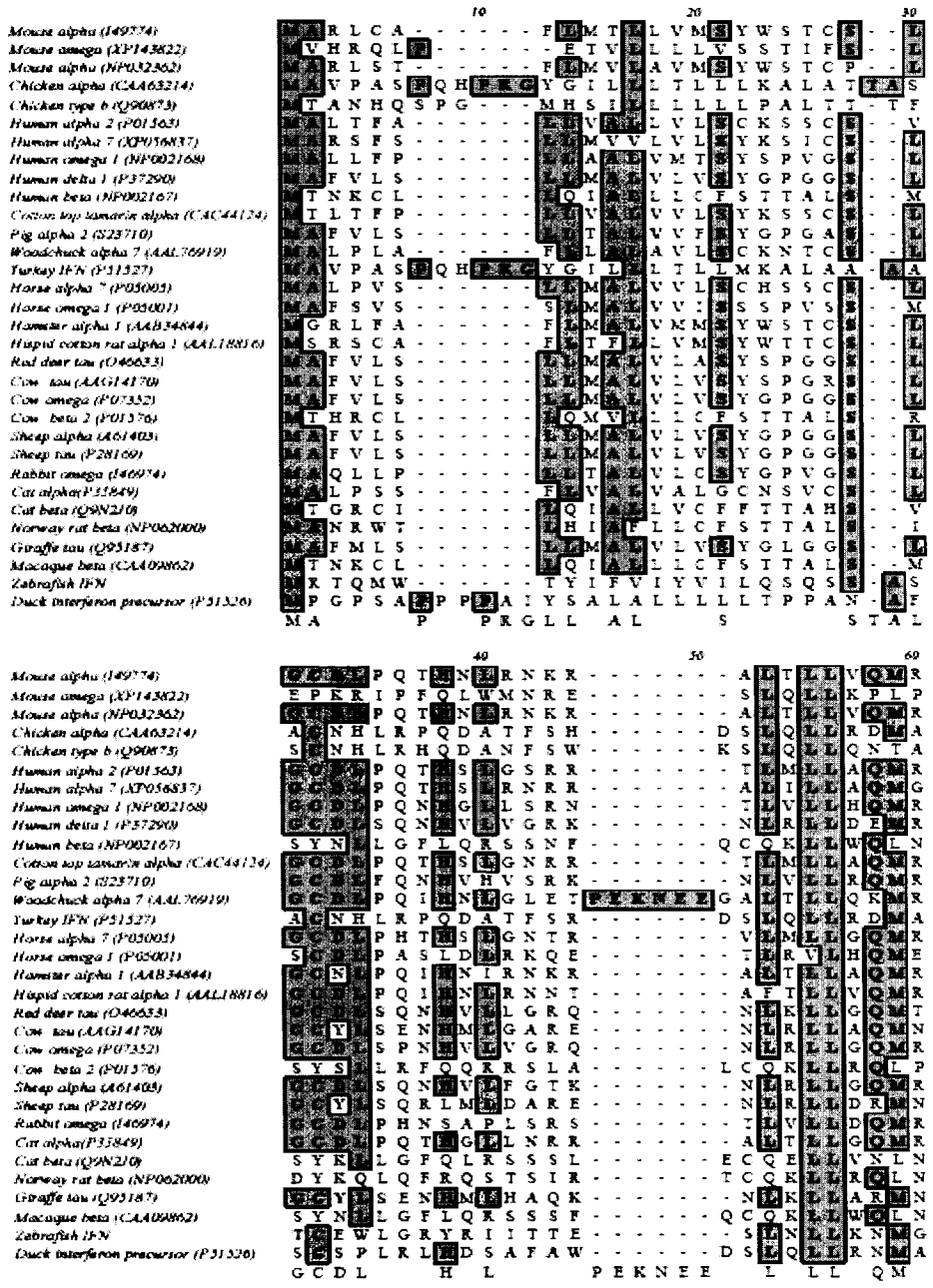


Figure 2.5. Alignment of zfIFN with other vertebrate IFNs. Dashes represent gaps inserted into the alignment. Shaded residues represent identities between species. Accession numbers are shown in parentheses.

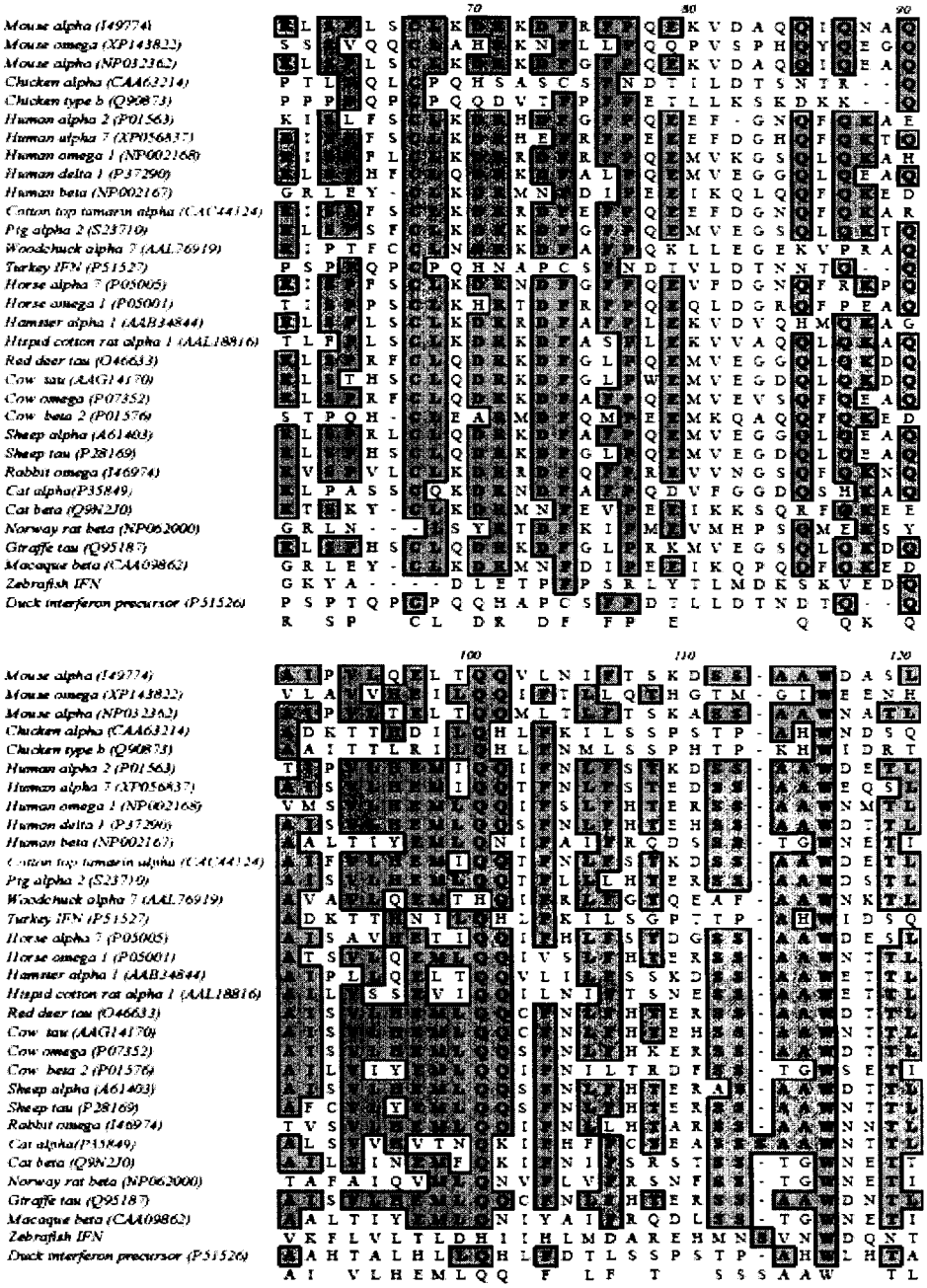
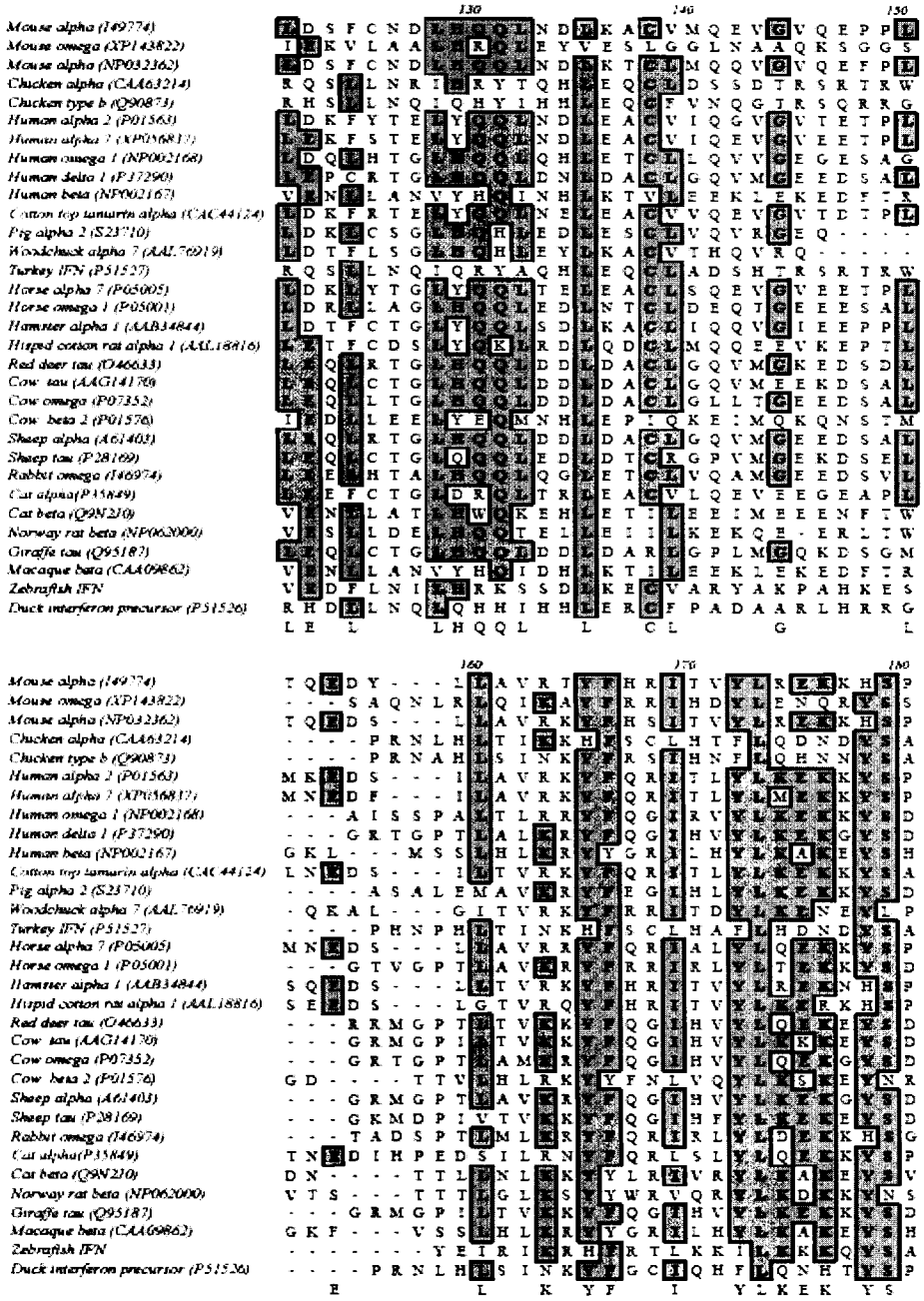


Figure 2.5 continued.



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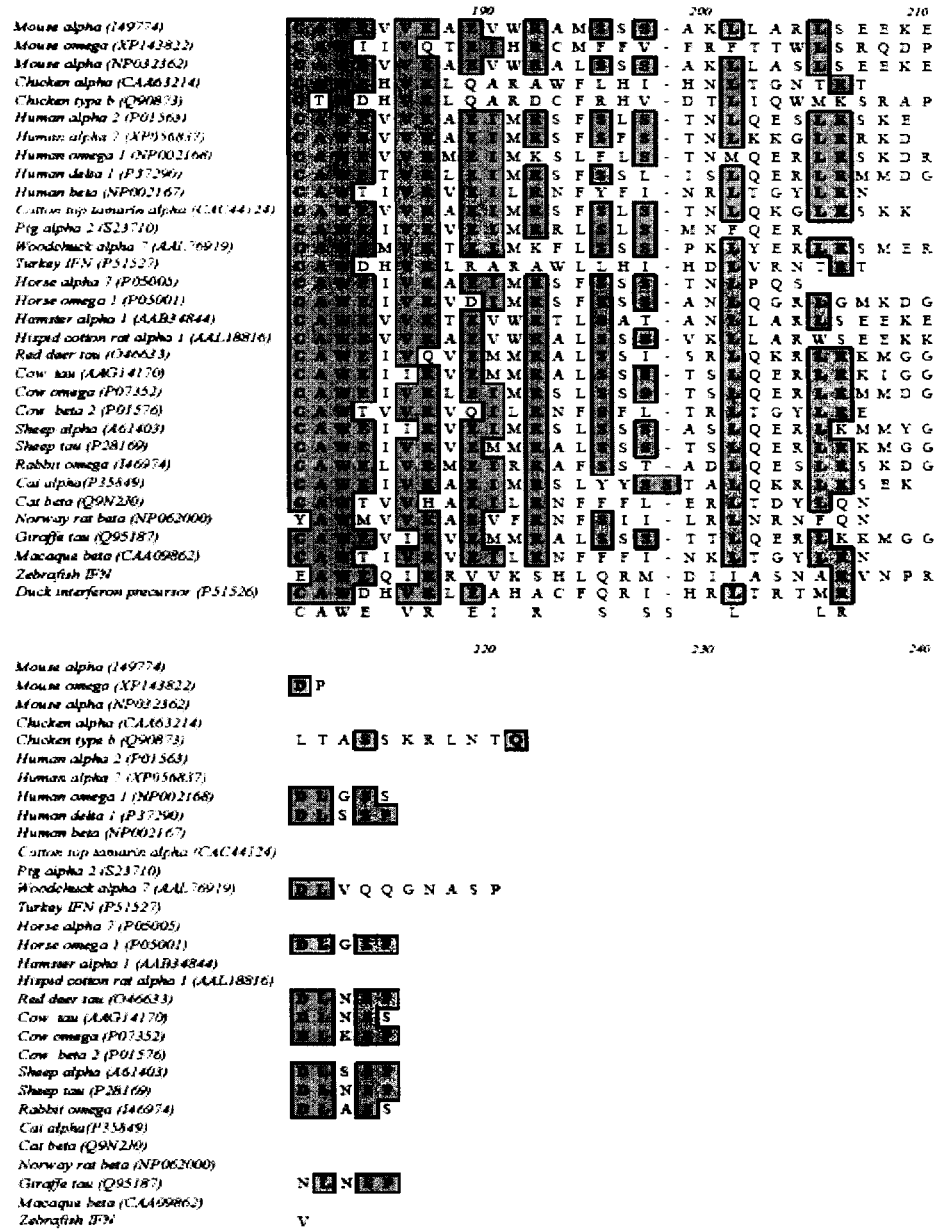

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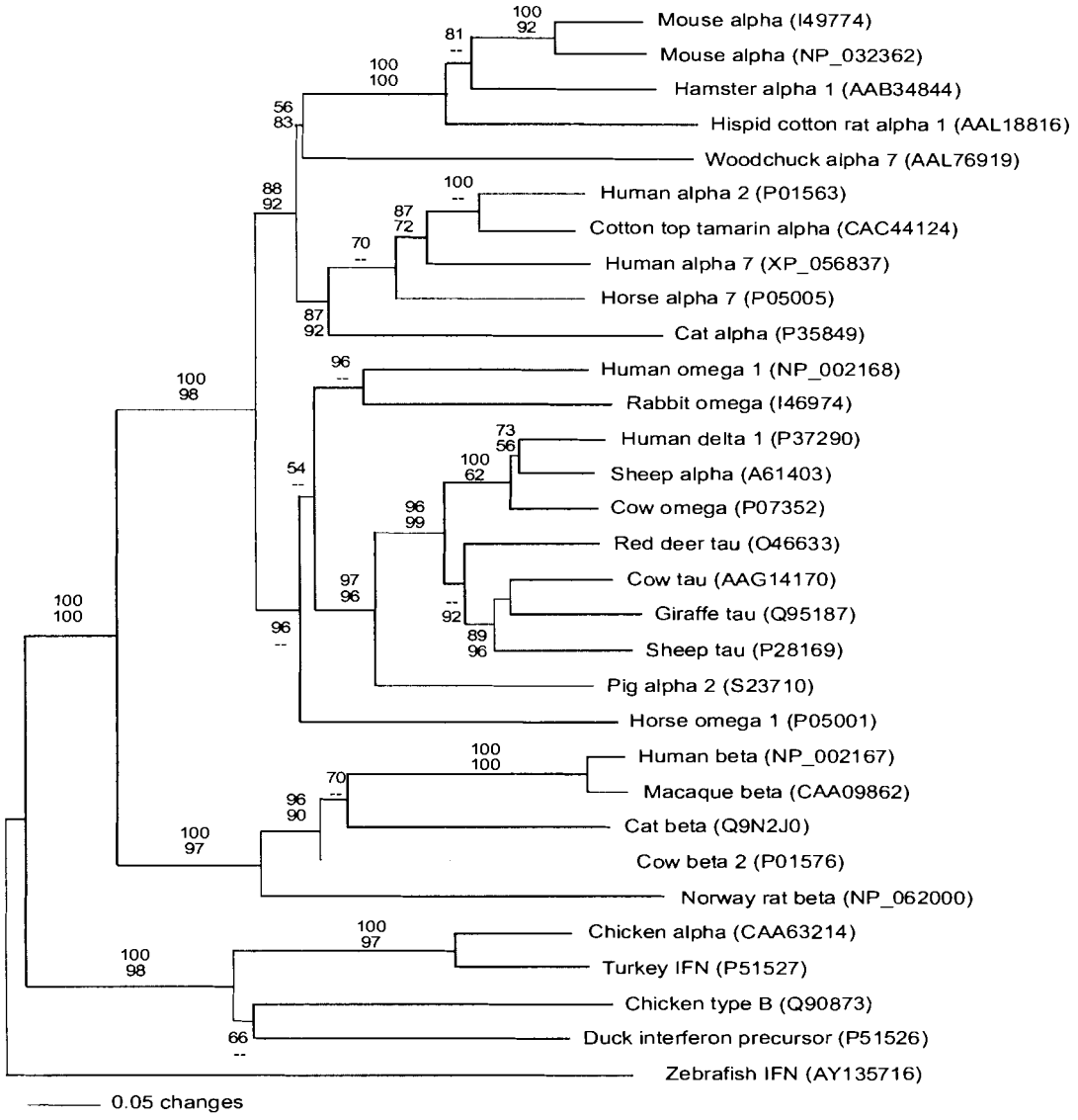

Figure 2.6. Phylogenetic relationships among zfIFN and representative alpha, beta, delta, omega and tau IFN from mammals and birds. Phylogenetic analyses were performed using evolutionary distance (shown) and maximum parsimony algorithms. Bootstrap proportions (bp) are presented as the fractional percentage of 100 replicates (top, evolutionary distance; bottom maximum parsimony). Dashes = bp <50%. Best trees recovered by both algorithms were identical with respect to all well supported nodes (bp >70%). The aligned sequence set included 31 sequences of 212 equally weighted positions (185 parsimony informative). Alignment gaps are treated as missing data. GenBank accession numbers are shown in parentheses.

entire sequence versus the truncated sequence, with identities of 18% and 16% to full-length chicken IFNα and β, respectively, and 22% and 21% to truncated chicken IFNα and β, respectively. A pairwise alignment was also performed on a putative fugu IFN found by searching the fugu genome database with zfIFN. This sequence, containing only 154 amino acids, yielded a 27% identity when aligned with zfIFN. Furthermore, BLAST analysis of the putative fugu IFN revealed a similarity closest to IFNτ from sheep, with an identity of 27% over the entire fugu sequence. Inclusion of the putative fugu IFN sequence in the phylogenetic analysis showed that putative fugu IFN forms a branch—independent of the mammalian, avian, and zebrafish branches—that is equidistant from these three lineages. However, these data are not included in Fig. 6 due to the lack of biological support for the putative fugu IFN sequence.

	Human A	Human B	Mouse A	Chicken A	Chicken B	Zebrafish
Human A		38/54	59/75	24/49	22/44	25/53
Human B	32/52		28/49	28/51	24/47	24/44
Mouse A	59/75	25/44		20/38	20/41	32/55
Chicken A	20/40	21/40	19/37		55/67	22/43
Chicken B	17/41	19/38	16/36	47/61		21/41
Zebrafish	15/34	14/36	16/34	18/38	16/39	

Table 2.1. Pairwise alignment of representative sequences of avian and mammalian IFN with zfIFN. No. below the diagonal represent percent identity/similarity over the entire sequence, and no. above the diagonal represent alignments in which the N-terminal 70 amino acids have been removed from each sequence. The region from amino acids 70 to 170 of all type I IFN sequences represents a conserved domain.

Discussion

In this paper we report the cloning of an interferon gene from zebrafish, coding for a protein 185 amino acids in length. Several lines of evidence support the characterization of this protein as an IFN, including sequence similarity to known IFNs, ability to induce the Mx promoter, upregulation in response to PolyIC, and the ability to reduce viral plaque formation in a zebrafish cell line. The first 22 amino acids of zfIFN, which are highly hydrophobic, represent a putative signal peptide. Although zfIFN contains eight Asn residues, none are part of the NXS/T motif needed for N-linked glycosylation, suggesting that zfIFN is not glycosylated. Since the antiviral activity of zfIFN has been demonstrated, glycosylation does not appear to be necessary for zfIFN to exert its antiviral effect. The low degree of similarity between zfIFN and mammalian and avian IFNs is also evident in zfIFN being highly basic whereas mammalian and avian IFNs are typically more acidic. The pH stability exhibited by most IFNs has made purification of these proteins more efficient, and has also allowed the inactivation of complement by treatment with low pH. If the basic nature of zfIFN is a characteristic shared by other fish IFNs, it may help to explain why the purification of IFN in this lineage has proven so difficult.

Real-time PCR analysis showed that when ZFL cells were induced with PolyIC a significant increase in IFN transcripts was seen at 6 and 12 h, followed by a rapid decrease to basal levels by 24 h, consistent with previous results [113]. The copy number of IFN transcripts in both the control and PolyIC-treated groups ranged from several hundred to several thousand per sample due to low levels of constitutive IFN expression [126]. The labile nature of IFN transcripts is evidenced by the rapid decline in copy number between 12 and 24 h in the PolyIC treated group. These results also demonstrate that ZFL cells are capable of producing IFN.

To demonstrate the antiviral nature of zfIFN, ZF4 cells were transfected with a zfIFN expression plasmid and were later challenged with virus. When compared with cells transfected with control vector, a 36% reduction in plaque number was seen. Cells induced with PolyIC and

then challenged saw the greatest reduction in plaque number, as well as a significant reduction in plaque size. This result confirms that ZF4 cells do indeed produce and respond to biologically active IFN. These results also demonstrate that zfIFN, when administered prior to viral infection, confers an antiviral effect on ZF4 cells.

One important limitation of fish cells is the relatively low transfection efficiencies afforded by these cell lines, as compared to mammalian cell lines (C. Kim, personal comm). The ZF4 cells used in these experiments have a low transfection efficiency, ranging from 1 to 5%, as determined by transfection with a plasmid encoding a constitutively expressed green fluorescent protein (data not shown). Because of this limitation, the difference between the zfIFN treatment versus the pcDNA3 treatment in Fig. 2.2 was necessarily reduced by the inability of the ZF4 cells to take up the plasmids effectively. The reason for this is that the negative control will remain constant regardless of the transfection efficiency, whereas the zfIFN-transfected cells will produce more product in proportion to the number of cells transfected. Thus, the approximately two-fold difference in cells cotransfected with zfIFN and the Mx promoter construct, as opposed to control pcDNA3 vector and Mx promoter construct, would be greater if the general efficiency of transfection could be increased.

The poor transfectability of zebrafish cells can also help to explain the higher luciferase induction by IFNFRM but the greater protection of ZF4 cells by PolyIC. In the plaque assay, methylcellulose was added 16 hr after induction with either IFNFRM or PolyIC. Due to the low transfection efficiency of plasmid DNA, the number of cells overexpressing IFNFRM after 16 hr would most likely be less than the number of cells induced by PolyIC. The addition of methylcellulose would then effectively inhibit further dissemination of extracellular IFN to neighboring cells. Thus, a comparable group of cells induced with PolyIC would show greater coverage of cells with IFN after 16 hr. The Mx promoter assay was allowed to incubate for 24 hr because the expressed protein was not IFN but the more stable intracellular protein luciferase, allowing for maximal accumulation of protein with minimal degradation.

Phylogenetic analyses indicate that mammalian and avian IFNs form distinct clades to the exclusion of zfIFN. Furthermore, pairwise comparisons of amino acid sequence identity and similarity indicate a similar degree of divergence between zfIFN and all mammalian and avian IFN subtypes. These results indicate that the divergence of zfIFN from an ancestral IFN occurred prior to the divergence of mammalian and avian IFN, and that these divergences, in turn, preceded the divergence of extant IFN subtypes in birds and mammals. These results are consistent with the origin of all IFN in a single common ancestral IFN present in the most recent common ancestor of fish, birds, and mammals. This extends the minimum estimated time of origin of IFN from the divergence of the ancestors of birds and mammals in the Carboniferous Period, approximately 310 million years ago (mya), to the fish-tetrapod transition, estimated to have occurred at least 360 to 370 mya.

Assuming a single common ancestor of IFNs, it is possible to infer potential structural and functional features of ancestral and modern IFNs based on conserved features of the extant IFN types. For example, four Cys residues are widely conserved among IFNs. One pair (in positions 51 and 151 of zfIFN) is conserved throughout all lineages, but is absent in zebrafish. A second pair (Cys 25 and Cys 120 in zfIFN) is conserved in all lineages except mammalian β IFNs. This pattern can be explained most parsimoniously in one of two ways given the known phylogeny of the host organisms. Both pairs may have been present in the ancestral IFN with subsequent loss of Cys 51 and Cys 151 in the branch leading to zebrafish and loss of Cys 25 and Cys 120 in the branch leading to mammalian β IFN. Alternatively, Cys 25 and Cys 120 may have been present in the ancestral IFN and subsequently lost in the branch leading to mammalian β IFN, while Cys 51 and Cys 151 were acquired in the branch leading to mammalian and avian IFNs. In either case, these Cys residues have been gained or lost in pairs and have been broadly conserved in IFN evolution, indicating that they are likely involved in disulfide bond formation and may be critical to correct conformation and function of IFN. Similarly, other highly or universally conserved

residues and motifs are likely to be critical to IFN function and should become targets of more intense scrutiny.

It is also clear from these data that proliferation of this gene family is relatively recent and has occurred independently in birds and mammals [128]. Although the known diversity of tissue-specific IFN types is greatest in mammals, followed by birds and fish, it is not yet clear whether this is a sampling artifact or an actual evolutionary trend. Experiments are underway to determine if zebrafish IFN is a multigene family with distinct subtypes in bony fish. In addition, the discovery of a single putative fugu IFN sequence, by searching the completed fugu genome database with zfIFN, suggests that teleost fish, such as fugu and zebrafish, contain only one form of IFN. Interestingly, the putative fugu IFN shares only a 27% identity with zebrafish, and does not cluster with zebrafish when included in the phylogenetic analysis.

All organisms, vertebrate and invertebrate, possess an innate immune response. Adaptive immunity, complete with antigen-specific memory cells, is believed to have developed around the time when jawed and jawless vertebrates diverged, some 450 mya [56]. The zebrafish, representing one of the lowest orders of jawed vertebrates, is well suited for studying the evolution of the innate immune response and the link to adaptive immunity. As more IFN sequences from fish and other lower order vertebrates are identified and characterized, we can begin to construct a clearer picture of the evolution of this diverse gene family.

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Chapter 3

CLONING AND CHARACTERIZATION OF AN MX GENE AND ITS CORRESPONDING PROMOTER FROM THE ZEBRAFISH, *DANIO RERIO*

Chapter Abstract

Type I interferons (IFNs) represent a crucial component of the innate immune response to viruses. An important downstream effector of IFN is the Mx gene, which is activated solely through this pathway. Mx proteins are characterized by a tripartite GTP-binding domain, dynamin family signature, and leucine zipper motif. Mx genes are transcribed upon activation of an interferon-stimulated response element (ISRE) located in the Mx promoter region. In this paper, we describe the cloning and analysis of an Mx gene and its corresponding promoter from the zebrafish (*Danio rerio*). The deduced amino acid sequence of zebrafish Mx contains the conserved GTP-binding domain, dynamin family signature, and leucine zipper motif common to Mx proteins, and shows a 50% identity to human MxA and 69% identity both to rainbow trout and to Atlantic salmon. Zebrafish liver cells produced high levels of Mx mRNA in response to induction by the known IFN-inducer polyinosinic-polycytidylic acid (Poly IC). The zebrafish Mx promoter contains two ISREs homologous to those found in the promoter regions of many IFN-inducible genes, and was able to drive transcription of a luciferase reporter gene when induced by either purified zebrafish IFN or Poly IC.

Introduction

The vertebrate innate immune system contains an effective antiviral response mechanism, mediated by type I IFN. This response is due in part to the action of the IFN-induced protein Mx. Mx was first discovered in mice that contained an inborn resistance to orthomyxovirus infection [25]. The antiviral activity of Mx against a broad range of negative sense RNA viruses has been

well documented [28, 129-131]. Structurally, Mx proteins contain a highly conserved tripartite GTP-binding domain in the amino terminal half of the protein [29] that is involved in antiviral activity, a leucine zipper motif near the carboxy terminus that functions in oligomerization [30], and a dynamin family signature [132]. Mx proteins localize either to the cytoplasm or to the nucleus, with a nuclear localization signal in the carboxy terminus being correlated with nuclear localization as in mouse Mx1 [42], human MxB [43], and Atlantic halibut (*Hippoglossus hippoglossus*) Mx [36]. Mx proteins that localize in the nucleus necessarily inhibit different steps in the viral life cycle than their cytoplasmic counterparts [44].

Expression of Mx is regulated by the Mx promoter, which contains between one and three interferon-stimulated response elements (ISRE) responsible for binding the IFN-induced complex IFN-stimulated gene factor 3 (ISGF3)[133]. ISGF3 is composed of the heterodimeric STAT1:STAT2 complex coupled to IFN regulatory factor 9 (IRF9), which together migrate to the nucleus to activate gene transcription [133]. ISRE elements have been characterized in the promoters of human MxA [46, 47], mouse Mx1 [134], and chicken Mx [48]. To date, the only fish Mx promoters to be characterized are those of rainbow trout and fugu [40, 49]. Mutation or deletion of the ISRE has been shown to reduce the capacity of IFN to induce Mx gene transcription [46, 47, 133-135], demonstrating the importance of this element in the IFN signaling pathway.

It is well known that the synthetic double-stranded molecule Poly IC is a potent inducer of type I IFN [114], initiating a signal transduction cascade that leads to the upregulation of a number of genes, including Mx. Due to the relatively low primary sequence homology between IFN genes from mammals and fish, 20% identity between human IFN alpha and zebrafish IFN [136], the cloning of IFN genes from lower-order vertebrates has proved difficult at best. Thus, the Mx gene and its promoter have played an important role as a diagnostic tool to assess IFN activity [117, 137, 138].

Mx genes have been cloned from a variety of mammalian [33, 34], avian [35], and fish species [36-40], as well as from yeast [41], where a GTP-binding protein similar to Mx has been cloned. Here we report the cloning and analysis of an Mx gene and promoter from zebrafish. Zebrafish Mx codes for a protein 646 amino acids in length containing the highly conserved motifs common to previously cloned Mx proteins. Expression of zebrafish Mx mRNA was upregulated in response to Poly IC induction. The corresponding zebrafish Mx promoter was found to contain two ISREs thirteen nucleotides apart, which upregulated the expression of a luciferase reporter gene in response to either Poly IC or zebrafish IFN in a transient transfection assay. The structure of the zebrafish Mx promoter is more similar to that of fugu, which also contains two ISREs in close proximity, than to that of the rainbow trout, which has only one ISRE. However, the zebrafish Mx promoter is different from that of fugu in that deletion of either ISRE from zebrafish yielded a more drastic reduction in promoter activity than a similar deletion of the fugu Mx promoter.

Materials and Methods

Cell Culture

Zebrafish liver cells (ZFL), ATCC number CRL-2643 [120], were grown in a medium containing a combination of 50% L-15, 35% Dulbecco's minimum essential medium (DMEM), 15% F-12 with sodium selenite, and supplemented with 5% v/v fetal bovine serum (FBS), 0.5% v/v trout serum, and 0.5% v/v insulin.

Generation of cDNA

To obtain total RNA, ZFL cells were grown to confluence in 75 cm^2 flasks and were treated with either 50 μg/mL Poly IC (Sigma, St. Louis, MO) or not treated. Cells were harvested at 6, 12, 24, 36, and 48 h post-treatment and total RNA was extracted using the RNeasy mini kit

(Qiagen, Valencia, CA). A reverse-transcription reaction was then performed to convert total RNA into cDNA. Briefly, 400 ng of RNA and 0.5 μg of random hexamers were combined and incubated at 70°C for 5 min, followed by a quick chill on ice for 5 min. To this mixture was added IMpromII 5× reaction buffer, 25 m*M* $MgCl_2$, 10 m*M* dNTP, 0.5U RNase inhibitor, and 1 μL IMpromII reverse transcriptase (Promega, Madison, WI). The reaction mixture was incubated for 1 h at 37°C followed by a 15 min heat inactivation step at 70°C.

Cloning of the Zebrafish Mx Promoter

The Universal Genome Walker kit (Clontech, Palo Alto, CA) was used to clone the zebrafish Mx promoter. Genome Walker-ready DNA was kindly provided by Dr. Keith Hutchison, University of Maine. Two primers, MxGWprimary and MxGWnested, were designed from the 5' end of zebrafish Mx, priming upstream amplification through two rounds of PCR when used with the Genome Walker primers AP1 and AP2 (Table 3.1). Thermocycler conditions were set according to the manufacturers instructions. The resulting PCR product was subcloned into a pGEM-T Easy vector (Promega) and sequenced at the University of Maine sequencing facility using an ABI 373 DNA sequencer (Applied Biosystems, Foster City CA).

Two new primers, MxproDown and MxproUp, were designed to verify the newly amplified sequence (Table 3.1). MxproDown is complementary to the 5' end of the new sequence and primes downstream amplification in conjunction with MxproUp, which anneals to base pairs (bp) 18 to 42 of the zebrafish Mx open reading frame (ORF). PCR was performed using zebrafish genomic DNA. Thermocycler conditions were 95° C for 5 min, followed by 35 cycles of 95° C for 1 min, 65° C for 1 min, and 72° C for 1 min. The amplified product was purified over a Qiaquick PCR purification column (Qiagen) and sequenced as described above.

Cloning of Zebrafish Mx

Primers for PCR amplification were designed from an expressed sequence tag (EST) homologous to human Mx found on the Washington University Zebrafish Genome Resource (WUZGR) website http://zfish.wustl.edu (EST accession no. fj23d12.y1). The upstream primer Mx1 was used in a 5' rapid amplification of cDNA ends (RACE) PCR reaction with the GeneRacer 5' primer (Invitrogen, Carlsbad CA). The downstream primer Mx2 was used in a 3' RACE PCR reaction with the GeneRacer 3' primer. Thermocycler conditions were designed according to the protocol set forth in the GeneRacer kit. These reactions yielded two separate clones that overlap in the region between the Mx1 and Mx2 primers. Sequence analysis of this region from both clones showed they were identical, demonstrating that both clones are from the same gene. This sequence is identical to that of GenBank accession no. AJ544823, deposited by G. Lutfalla. Zebrafish cDNA (see Generation of cDNA) was used as template. Thermocycler conditions were as recommended by the manufacturer (Invitrogen). DNA was sequenced as described under Cloning of the Zebrafish Mx Promoter. The zebrafish Mx gene can be located on GenBank (accession no. AF533769).

Protein Expression

To express zebrafish IFN, a baculovirus expression system was used (Invitrogen). Briefly, full-length zebrafish IFN cDNA was inserted into the baculovirus transfer vector pFastBac. This construct was then inserted into the baculovirus genome and used to produce infectious recombinant baculovirus particles, which were recovered and used for insect cell infection. Spinner cultures of Sf-9 cells were infected with the recombinant baculovirus at a low MOI, and supernatant from infected cells was harvested 3 days post-infection [139].

Plasmid Construction

The MxproDown/MxproUp-amplified product was subsequently ligated into a pGL3 Basic luciferase reporter vector (Promega) that had been previously digested with *Sma* I to generate a linearized vector with blunt ends. The linearized pGL3 Basic vector was also incubated at 70° C for 10 min with a mixture of 1 U Taq polymerase, 5 μL 10× PCR buffer (Promega), 10 μL 5 m*M* dTTP, 5 μL 25m*M* $MgCl_2$, and 24 μL nuclease-free water to generate T-overhangs for increased ligation efficiency. Ligation of the treated pGL3 Basic vector to the cloned zebrafish Mx promoter yielded the vector hereby designated as Mxpro-luc.

Generation of Mx promoter deletion mutants was done by PCR using the Mxpro-luc construct as template. Mutants MxflnoISRE and MxflISRE1 were generated using the 5' primer MxproDownKpn with the 3' primers MxflnoISRErevXho and MxflISRE1revXho, respectively. Mutant MxflISRE2 was generated by first round amplification using the primers MxproDownKpn and MxflISRE2revXho in one reaction and MxflISRE2fwd and MxproUp in a second reaction. The products of these two reactions were then combined in a third reaction and used as template with the primers MxproDownKpn and MxflISRE2revXho. Mutants Mx187, Mx153, and Mx128 were generated using the 3' primer Mxunivrev with the 5' primers Mx187Kpn, Mx153Kpn, and Mx128Kpn, respectively. Mutant MxISRE2del was generated by first round amplification using the primers MxISRE2delrev and MxproUp. The product of this reaction was combined with the synthesized template MxISRE2delfwd and amplified using the primers Mx187Kpn and Mxunivrev. For all reactions, thermocycler conditions were: 1 cycle of 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec. Amplified products were electrophoresed in a 1% agarose gel and subsequently gel extracted. Purified products were then subjected to restriction digestion with the enzymes *Kpn* I and *Xho* I (New England Biolabs, Beverly, MA) followed by ligation into pGL3 Basic vector. All constructs were verified by sequencing.

Mx Promoter Transfections

Transfections were carried out using the liposome reagent Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. ZFL cells were seeded to 90% confluency in clear, flat-bottomed well plates (Corning, Corning, NY) and allowed to adhere overnight. For all transfections, 0.5 μg of DNA and 1.0 μg of liposome were combined in serum-free ZFL medium. After a 20 min incubation, medium was removed from cells in a 24-well plate and 200 μL of the DNA:liposome mixture was added to each well and incubated for 1 h. After 1 h, 0.5 mL of supplemented ZFL medium was added back to each well. Upon completion of transfection, cells were either induced with 10 μg/mL of Poly IC or uninduced, and allowed to incubate. After 24 h, cells were analyzed for luciferase expression using the Bright Glo luciferase assay system (Promega). Briefly, a 1:1 ratio of Bright Glo and serum-free RPMI1640 medium (Life Technologies, Gaithersburg, MD) was prepared, and 200 μL of this mixture was added per well to cells from which all medium had previously been removed. Cells were lysed for 2 min, after which the resulting lysate was transferred to a 96-well Packard Optiplate and measured in relative light units (RLU) in a Fusion Universal Microplate analyzer (Packard, Meriden CT) with a 10 sec read time for each well.

Quantitative PCR

Quantitation of IFN cDNA was carried out using the fluorescent SYBR Green nucleic acid stain [123, 124, 140] . The primers MxproUp and Mx 5'Down were used to amplify a 106 bp fragment of zebrafish Mx. A standard curve was constructed by serially diluting a linearized plasmid containing the open reading frame of zebrafish Mx. Zebrafish beta actin primers (PE Applied Biosystems, Foster City, CA) were used to normalize for starting quantity of RNA. Reactions were performed in an iCycler iQ Real-Time PCR detection system (Bio-Rad, Hercules,

CA) according to the manufacturer's instructions. Reactions were carried out in a volume of 25 μL containing 12.5 μL of 2× SYBR Green PCR Master Mix (Qiagen), 1.0 μL of 5.0 μ*M* primers, 10.5 μL of nuclease-free water, and 1.0 μL of cDNA. Cycling parameters were 94°C for 15 min to activate the polymerase followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Fluorescence measurements were taken at each cycle during the 55°C step. RNA levels are expressed as copy number based on the standard curve, after which these values were normalized to the corresponding beta actin values to generate the relative copy number.

Results

Cloning of Zebrafish Mx

A full-length zebrafish Mx gene was cloned from Poly IC-induced zebrafish mRNA, with a nucleotide length of 1941 bp encoding a protein of 646 amino acids (Fig. 3.1). Zebrafish Mx contained the highly conserved tripartite GTP-binding domain found in all Mx proteins, located at amino acid positions 39 to 56, 145 to 148, and 214 to 217. In addition, zebrafish Mx exhibited a dynamin family signature at positions 62 to 71 that is common to all Mx proteins. A putative leucine zipper motif was found near the carboxy terminus, represented by a stretch of Leu residues spaced seven amino acids apart. Zebrafish Mx does not contain a nuclear localization signal (NLS), characterized by a short stretch of positively charged residues (K/R) near the carboxy terminus [42], suggesting that it remains localized within the cytoplasm. The zebrafish Mx sequence can be located on GenBank (accession no. AF533769). An alignment of zebrafish Mx with various fish Mx genes, as well as human MxA and chicken Mx, reveals a highly conserved N terminus, most notably in the GTP-binding domain (Fig. 3.2).

Pairwise alignments were also performed using the deduced amino acid sequence of zebrafish Mx with Mx sequences from human, mouse, duck, chicken, rainbow trout, halibut, and Atlantic salmon, as well as yeast VPS1 and a fugu Mx that was found by searching the pufferfish

genome database with zebrafish Mx (Table 3.2). As expected, the highest homology was seen to other fish Mx proteins, with a more distant relationship to mammalian and avian Mx. The low but significant similarity to yeast VPS1, a known GTP-binding protein involved in vacuolar protein sorting [41], implies that the GTP-binding domain of Mx proteins has remained highly conserved throughout evolution. It is known that human MxA and mouse Mx2 are localized in the cytoplasm, and that nuclear retention requires an NLS in the form of a basic motif near the carboxy terminus of a protein. The fact that zebrafish Mx bears a slightly greater identity both to MxA and to Mx2, compared to their counterparts MxB in human and Mx1 in mouse, respectively, and that zebrafish Mx does not contain an NLS, suggest that zebrafish Mx is localized in the cytoplasm. Zebrafish Mx also shows a higher percentage of identity to the cytoplasmic rainbow trout Mx1 protein (69%) than to the nuclear Atlantic halibut Mx (65%).

```
  1 atggagaagttgagttacacgttcagtcagcaatatgaggagaaa
    M  E  K  L  S  Y  T  F  S  Q  Q  Y  E  E  K
 46 attcgcccttgtatcgacactatcgataatttacggtctctggga
    I  R  P  C  I  D  T  I  D  N  L  R  S  L  G
 91 gtcgaaaaggacctcgcgttgcctgccatcgccgtcataggagac
    V  E  K  D  L  A  L  P  A  I  A  V  I  G  D
136 caaagctcgggaaagagttcggttctggaggcgctgtcaggagtt
    Q  S  S  S  G  K  S  S  V  L  E  A  L  S  G  V
181 ccgttacccaggggcagtgggattgttacacggtgccctctcgag
    P  L  P  R  G  S  G  I  V  T  R  C  P  L  E
226 ctcaagatgataagaactaaagaccaagataggtggcatgggaga
    L  K  M  I  R  T  K  D  Q  D  R  W  H  G  R
271 atcagttacaaaacctgtgaggaggactttgatgacccagcggaa
    I  S  Y  K  T  C  E  E  D  F  D  D  P  A  E
316 gtggagaaaaaaatccgccaagcccaagatgagatggctggagca
    V  E  K  K  I  R  Q  A  Q  D  E  M  A  G  A
361 ggtgttggtatcagtgaagaactcatcagtctgcagatcacctct
    G  V  G  I  S  E  E  L  I  S  L  Q  I  T  S
406 gccgatgttcctgacctcactctcattgacctccctggcattgca
    A  D  V  P  D  L  T  L  I  D  L  P  G  I  A
451 cgagtggccgtcaagggtcaacctgagaacattggagatcagatt
    R  V  A  V  K  G  Q  P  E  N  I  G  D  Q  I
496 aaaagactaatcaggaagtttgttacaaggcaagagacaatcaac
    K  R  L  I  R  K  F  V  T  R  Q  E  T  I  N
541 ctggtcgtggtgccatgcaatgttgacatcgccaccacagaagca
    L  V  V  V  P  C  N  V  D  I  A  T  T  E  A
586 ttgcagatggctcaggcagaggatcctgatggcgaaaggacttta
    L  Q  M  A  Q  A  E  D  P  D  G  E  R  T  L
631 ggcatcttaacaaagccagacctggtggacaagggcactgaaggg
    G  I  L  T  K  P  D  L  V  D  K  G  T  E  G
676 acagttgtagacattgtccacaatgaggtcattcacctcactaaa
    T  V  V  D  I  V  H  N  E  V  I  H  L  T  K
721 ggctacatgattgtaaggtgcagagggcaaaaagagataatggat
    G  Y  M  I  V  R  C  R  G  Q  K  E  I  M  D
766 caggtcactctaaatgaggctacagaaacagagagtgccttcttc
    Q  V  T  L  N  E  A  T  E  T  E  S  A  F  F
811 aaagaccatcctcatttcagcaaactctatgaagagggttttgct
    K  D  H  P  H  F  S  K  L  Y  E  E  G  F  A
856 actattcccaagttggcagagaaactaacaattgaattggttcat
    T  I  P  K  L  A  E  K  L  T  I  E  L  V  H
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 901 cacattcagaaatctctacctcgcctagaagagcaaatagagaca
     H  I  Q  K  S  L  P  R  L  E  E  Q  I  E  T
 946 aagcttgctgaaacacagaaggaactagaggcatatggcaatggg
     K  L  A  E  T  Q  K  E  L  E  A  Y  G  N  G
 991 cctccatcagaaccagcagcaagactcagctttttcattgacaaa
     P  P  S  E  P  A  A  R  L  S  F  F  I  D  K
1036 gttacagctttcaatcaagatatgctsaacctgacgactggggag
     V  T  A  F  N  Q  D  M  L  N  L  T  T  G  E
1081 gaygttaaatgtactacagatctactgcttttcccagaacttcgg
     D  V  K  C  T  T  D  L  L  L  F  P  E  L  R
1126 caagaatttgcaaaatggagtcacatcttggatcgttcaggggat
     Q  E  F  A  K  W  S  H  I  L  D  R  S  G  D
1171 tcattcaacaaaaagattgagaaagaagttgataactacgaagtc
     S  F  N  K  K  I  E  K  E  V  D  N  Y  E  V
1216 aagtaccggggaagagagctaccaggattcatcaactacaagacc
     K  Y  R  G  R  E  L  P  G  F  I  N  Y  K  T
1261 tttgaggggcttgtcagggaccagatcaagctgttggaggaacct
     F  E  G  L  V  R  D  Q  I  K  L  L  E  E  P
1306 gcgttaaagacactgaagaccgtctctgatgtggttagaaagaag
     A  L  K  T  L  K  T  V  S  D  V  V  R  K  K
1351 ttcatccaactggcccagtgcagtttcattggattccctaatctt
     F  I  Q  L  A  Q  C  S  F  I  G  F  P  N  L
1396 ctgaaaatagcaaagactaagattgaaggcattaagctaaacaaa
     L  K  I  A  K  T  K  I  E  G  I  K  L  N  K
1441 gaatccctggcagaatctatgctgaaaactcaattcaagatggag
     E  S  L  A  E  S  M  L  K  T  Q  F  K  M  E
1486 ctcattgtttacagccaagatggcacatacagccagagtctaaag
     L  I  V  Y  S  Q  D  G  T  Y  S  Q  S  L  K
1531 catgcaaaggataaattggaggagatggaaaaagaaaggccacaa
     H  A  K  D  K  L  E  E  M  E  K  E  R  P  Q
1576 ccgaaaataaaactgcctttactttcgagtttcgaccttggcaca
     P  K  I  K  L  P  L  L  S  S  F  D  L  G  T
1621 gacaatcatgccaccttgcgtgagatgaggttgcaccttaaatcc
     D  N  H  A  T  L  R  E  M  R  L  H  L  K  S
1666 tactacacaattgcaagcaagcgtctagctgaccagatcccaatg
     Y  Y  T  I  A  S  K  R  L  A  D  Q  I  P  M
1711 gtgatccgctacatgctgctgcaggaagctgctctggagctgcaa
     V  I  R  Y  M  L  L  Q  E  A  A  L  E  L  Q
1756 aggaacatgttacagctgctgcaagataaagacggtgtagacaac
     R  N  M  L  Q  L  L  Q  D  K  D  G  V  D  N
1801 ctgcttaaagaggactgtgacattgggcaaaagcgggaaaactta
     L  L  K  E  D  C  D  I  G  Q  K  R  E  N  L
1846 ctgagccgccagacacgtttaattgaaggcacgcagcctcttggt
     L  S  R  Q  T  R  L  I  E  G  T  Q  P  L  G
1891 cacctttagaagttactttcatagattactgcaacattttaatg
     H  L  L  E  V  T  F  I  D  Y  C  N  I  L  M
1936 caatga
     Q  *
```

Figure 3.1. Full-length nucleotide sequence of zebrafish Mx with deduced amino acid sequence below. Numbers in the left margin correspond to nucleotide sequence. The tripartite GTP-binding domain is boxed, the dynamin family signature is underlined, and the C-terminal leucine zipper motif is in black and italics.

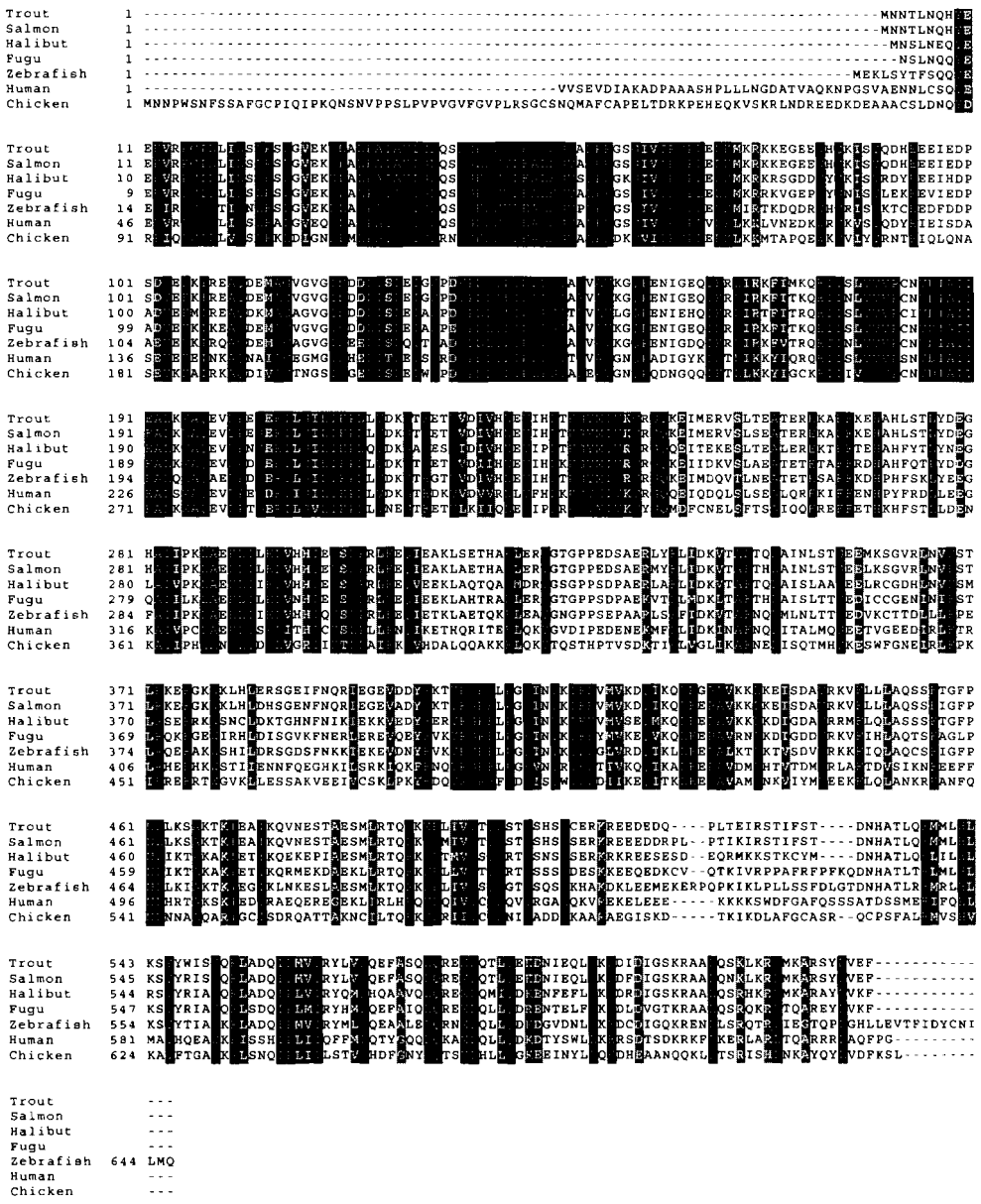

Figure 3.2. Alignment of zebrafish Mx with selected mammalian, avian, and fish Mx genes. Residues shaded in black are completely conserved across all species aligned. Residues shaded in gray are similar with respect to side chains. Accession nos. are: rainbow trout Mx (Q91192); Atlantic Salmon Mx (AAB40994); Atlantic halibut Mx (AAF66055); fugu Mx (AF525215); human MxA (P20591); chicken Mx (Q90597).

Name	Sequence 5' to 3'	Direction
MxGWprimary	CCTTAATAATGTCCAAAGACGAATC	Reverse
MxGWnested	AAAGTTCAGATTACCCGACTGTCT	Reverse
MxprodownKpn	GGTGGTGGTACCAGTCATCATACAGTAAGCATC	Forward
GeneRacer3'	GCTGTCAACGATACGCTACGTAACG	Reverse
GeneRacer5'	CGACTGGAGCACGAGGACACTGA	Forward
MxproUp	CTCCTCATATTGCTGACTGAACGTG	Reverse
MxproDown	AGTCATCATACAGTAAGCATCCGCC	Forward
AP1	GTAATACGACTCACTATAGGGC	Forward
AP2	ACTATAGGGCACGCGTGGT	Forward
Mx1	CATAGATTCTGCCAGGGATTCTT	Reverse
Mx2	CAGGGGATTCATTCAACAAAAA	Forward
Mxunivrev	ACCACCCTCGAGCTCCTCATATTGCTGACT	Reverse
Mx187Kpn	GGTGGTGGTACCAATTCTTACTGAATAACA	Forward
Mx153Kpn	GGTGGTGGTACCCCATTGTGCTTTGCAGGA	Forward
Mx128Kpn	GGTGGTGGTACCTACTGATCAATACGCGCT	Forward
MxflnoISRErevXho	ACCACCCTCGAGCCAATGTTATTCAGTAAGAATT	Reverse
MxflISRE1revXho	ACCACCCTCGAGTGCAAAGCACAATGGTTT	Reverse
MxflISRE2revXho	ACCACCCTCGAGGATCAGTAAGTTTCATTTCC	Reverse
MxflISRE2fwd	ATTGTGCTTTGCAGGAAA	Forward
MxISRE2delrev	ATTGTGCTTTGCATACTGATCCATACGCGCTCCTC	Reverse
MxISRE2delfwd	AATTCTTACTGAATAACATTGGAGAAACGAAACCATTGTGCTTTGCA	Forward

Table 3.1. Table of primers used.

Organism	% Identity	% Similarity
Human MxA	50	65
Human MxB	43	58
Mouse Mx1	47	65
Mouse Mx2	49	67
Duck Mx	43	59
Chicken Mx	40	56
Rainbow Trout Mx 1	69	79
Altantic Halibut Mx	65	77
Atlantic Salmon Mx 1	69	80
Fugu Mx	66	78
Yeast VSP1	27	44

Table 3.2. Pairwise identity/similarity of zebrafish Mx with other Mx proteins. Accession no.: human MxB (B33481); mouse Mx2 (BAA82593); duck Mx (P33238); yeast VSP1 (AAA35216).

Induction of Zebrafish Mx by PolyIC

To determine the level to which zebrafish Mx mRNA is generated in response to IFN, ZFL cells were treated with Poly IC and total RNA extracted at 6, 12, 24, 36, and 48 h post-induction. Quantitative PCR using the dye SYBR Green showed high levels of Mx expression at the earliest time point (6 h), followed by a fairly constant increase in expression through the latest time point (48 h). The results of this assay are depicted in Figure 3.3. At all time points, control samples remained at basal levels, indicating the highly inducible nature of Mx in response to IFN induction by Poly IC.

Cloning of Zebrafish Mx Promoter

PCR amplification of the upstream portion of zebrafish Mx by the method of gene walking yielded a PCR product 1218 bp in length. To verify this sequence, a downstream primer was designed from within the PCR product and was used with an upstream primer designed from the 5' end of the zebrafish Mx ORF. Amplification of this product using genomic DNA yielded an 1113 bp product (Fig. 3.4). Analysis of this sequence revealed two potential ISREs, one at position −165 to −154 (ISRE 1, relative to the ATG) and the other at position −140 to −129 (ISRE 2). ISRE 1 has the sequence AGAAACGAAACC, which is identical to an ISRE from human MxA [49]. ISRE 2, with the sequence GGAAATGAAACT, also contains the highly conserved GAAANGAAA motif, though the remaining nucleotides do not align with either the chicken, mouse, human, trout, or fugu ISREs. However, both ISREs do conform to the consensus sequence of (G/A/T)GAAAN(1-2)GAAA(G/C)(A/T/C) described by Collet and Secombes [49].

Activity of Zebrafish Mx Promoter

To examine the IFN-inducible nature of the Mx promoter, a construct was made in which the zebrafish Mx promoter was linked to a luciferase reporter gene. This construct was transiently transfected into ZFL cells, followed by induction with either zebrafish IFN or the IFN-inducer

Poly IC. After 24 h, cells were assayed for luciferase activity (Fig. 3.5). As expected, cells transfected with the Mxpro-Luc construct and induced with either IFN or Poly IC demonstrated a high level of luciferase activity. Cells transfected with the pGL3 Basic vector, whether induced or uninduced, showed virtually no detectable luciferase activity once luminescence was corrected for background (~2000 RLU). When Mxpro-Luc was transfected with no subsequent Poly IC stimulation, a low level of luciferase activity was detected, indicative of constitutive low level expression of IFN. Together, these results demonstrate the highly inducible nature of Mx in response both to IFN and to Poly IC.

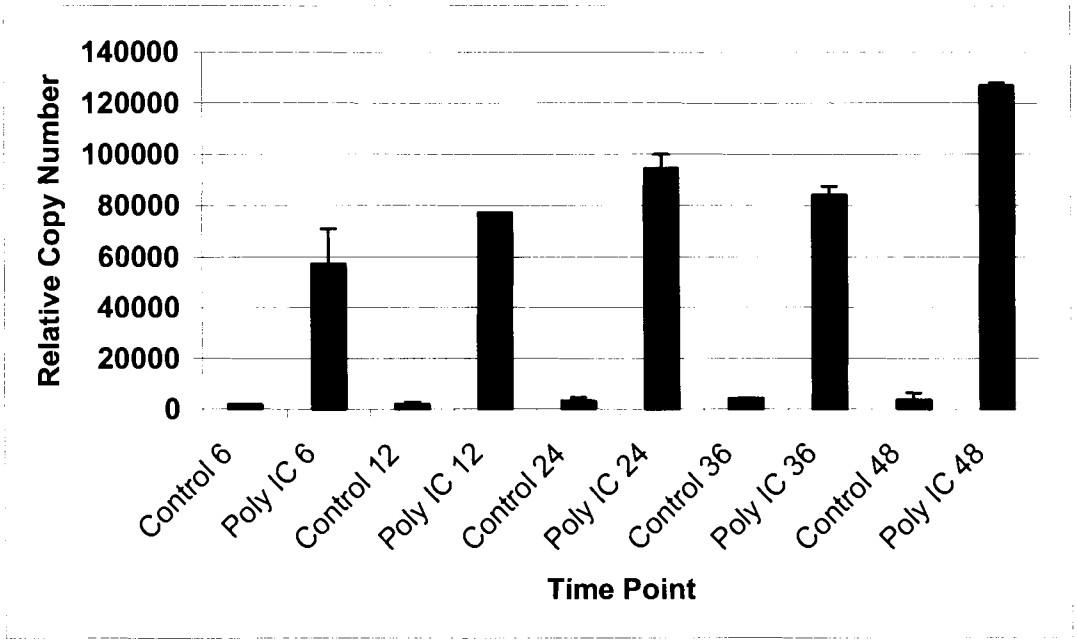

Figure 3.3. Zebrafish Mx induction in response to Poly IC stimulation. ZFL cells were induced with Poly IC, and total RNA harvested at designated time points and reverse transcribed into cDNA. The resultant cDNA was used as template in a quantitative PCR reaction using primers specific for zebrafish Mx. Relative copy number at each time point was determined as described in Materials and Methods. Each time point was assayed in triplicate, with error bars as mean ± S.D. These results are representative of two independent experiments.

```
1     GTCATCATACAGTAAGCATCCGCCACCATCCTCTCACCAGTGACATGCAG
51    TCTAAACGGTCCCTGGATCATTGTGTGTGAAGATTTTAGACATCAAGTGG
101   TTTGCTGCATTTGTCTTGAGGTTGTTTAGTATGATGCGATTTACTGTCCA
151   TGTGTGATTTGATTGGACAGGAATAACAGAACTGATTTTTCCACTCAGCC
201   AATCTCCATAGACAAGAAAAAATAAAGTTATGACTGCAGTTTGAGGGAAA
251   ATAGTGGAGTAAAAGTACATACAGCACTCAAGCTTAAGTGCACATTTTTA
301   AAGCTACTTAGTAAATTACAATTGCTGGGAAAAAGTACTGAATTACAGTA
351   ATTTGAGTATTTTGTTACTTTACACCAATGAACAACAGAAACGGTCAGAA
401   GACCTGCAAGTCAACAGTGATGTTCTGCATGGTCACCGCATCATCTCGGT
451   ATCTGATTATGGATAAACAGCCCCCCTAGAGGGTGATACGTGATATAAAG
501   AAAACGACTTAACAGAAACTACTGACCAGTCTTTGGCATTGACTCAGACA
551   TCTGTGCGTTTACAAAGAAAATTGAATTGAGTCGTCAGTGTAAAAAGTTT
601   AAAAAATAATATAGTAAATCTCATTTTTAAATAAATCCACTGAAAGTATA
651   TGCAAAATATGTGCATCAACATCTCTCTAAAAATGAATTTCTTTATTAGA
701   GAAATGCGTCATTTGTTACCAAGTCGTTTGCTTTGGCGAGTTTGCACAAA
751   CAATTTTAGCAAAATGGTTATCCAAGAATCTACACTGAGTCCAAGCTAAA
801   CAGAACAACATACGGAAAATACGGTACTCAGAGTCGCTACAAAGACAATA
851   TAGGCTAGGTGTTTTTCCATCTACTGAACAATGAATTCTTACTGAATAAC
901   ATTGGAGAAACGAAACGATTGTGCTTTGCAGGAAATGAAACTTACTGATC
951   CATACGCGCTCCTCCTAGCATTCACTTTACCGGTAAAGCAAGCCGAGCAT
1001  CATTAGTTCAGACAGTCGGGTAATCTGAAACTTTGTCGTGATAAGGATTT
1051   GTCTTTGGACATTATTAAGGATGGAGAAGTTGAGTTACACGTTCAGTC
1101   AGCAATATGAGGAG
```

Figure 3.4. Sequence of zebrafish Mx promoter region. The two ISRE motifs are boxed. The ATG is in bold and underlined. Numbers in the left margin denote the size in base pairs of the region of the zebrafish Mx promoter that was inserted into pGL3 Basic.

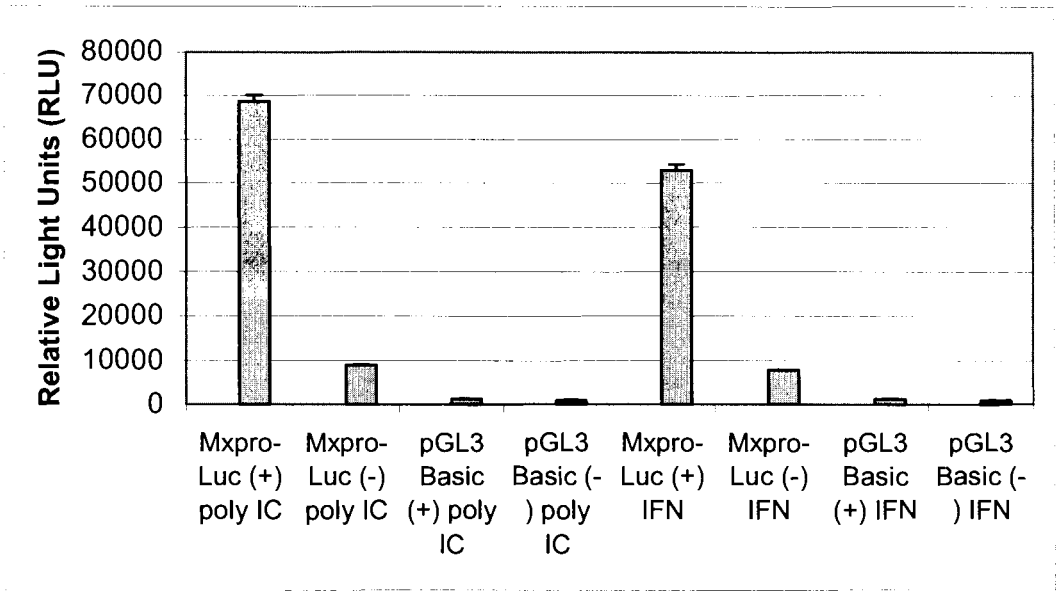

Figure 3.5. Upregulation of Mxpro-Luc in response to Poly IC. ZFL cells were transfected with either the Mxpro-Luc construct or pGL3 Basic construct and subsequently either induced with 10 μg/mL Poly IC, 10 μL zebrafish IFN supernatant, or uninduced. Mxpro-Luc(-)IFN and pGL3 Basic(-)IFN were exposed to control supernatant lacking zebrafish IFN. After 24 h, cells were assayed for luciferase activity measured by relative light units (RLU). Error bars represent the mean ± S.D. of each treatment, performed in triplicate. These results are representative of two independent experiments.

Activity of Zebrafish Mx Promoter Mutants

To further examine the role of the two ISRE motifs, as well as any putative upstream regulatory elements, a series of deletion mutants was constructed (Fig. 3.6). These constructs were then transfected into ZFL cells, induced with zebrafish IFN, and later assayed for luciferase activity. Mutant Mx187, which contains only 187 nucleotides upstream of the Mx gene start codon and includes both ISREs, demonstrated a reduction in activity to 29% of the wild type Mx promoter (Fig. 3.7). In addition, transfection of mutant constructs lacking one or both ISREs showed a further reduction in promoter activity to near basal levels. As expected, mutant Mx128, which lacks both ISREs, as well as any putative upstream regulatory elements, demonstrated virtually no promoter activity. Interestingly, the complementary mutant MxflnoISRE, which contains the entire 5' region of the Mx promoter up to but not including the two ISREs, also exhibited virtually no promoter activity (Fig. 3.7).

To determine the role of each ISRE individually, two additional sets of complementary mutants were constructed. Mutant Mx153, containing only 153 nucleotides upstream of the Mx gene start codon and including only ISRE2, and the complementary mutant MxflISRE2, containing all of the 5' end of the Mx promoter up to ISRE2 with ISRE1 removed, were examined next. The promoter activities of these two constructs were significantly diminished as compared to the wild type Mx promoter, and only slightly greater than that of the Mx128 and MxflnoISRE mutants. A final set of mutants, MxflISRE1 and truncISRE1only, similar to MxflISRE2 and Mx153, respectively, but with ISRE1 instead of ISRE2, were assayed for promoter activity. As shown in Figure 3.7, the promoter activity of these constructs was also abrogated. These data appear to suggest a synergistic effect, in which the presence of both ISRE motifs leads to much greater promoter activity than either ISRE does alone.

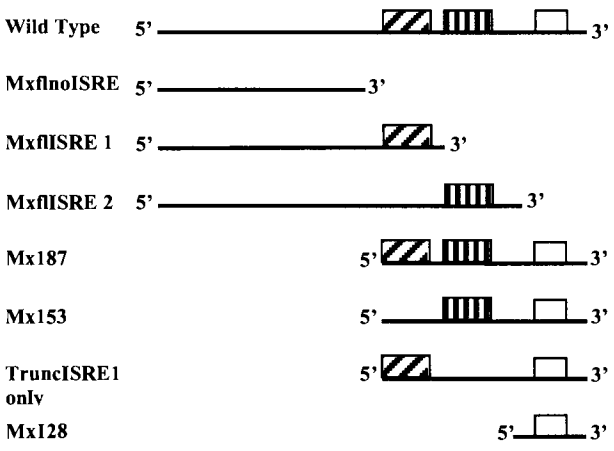

Figure 3.6. Schematic of zebrafish Mx promoter constructs. Diagonally striped box represents ISRE 1; vertically striped box represents ISRE 2, and empty box represents the start codon of zebrafish Mx.

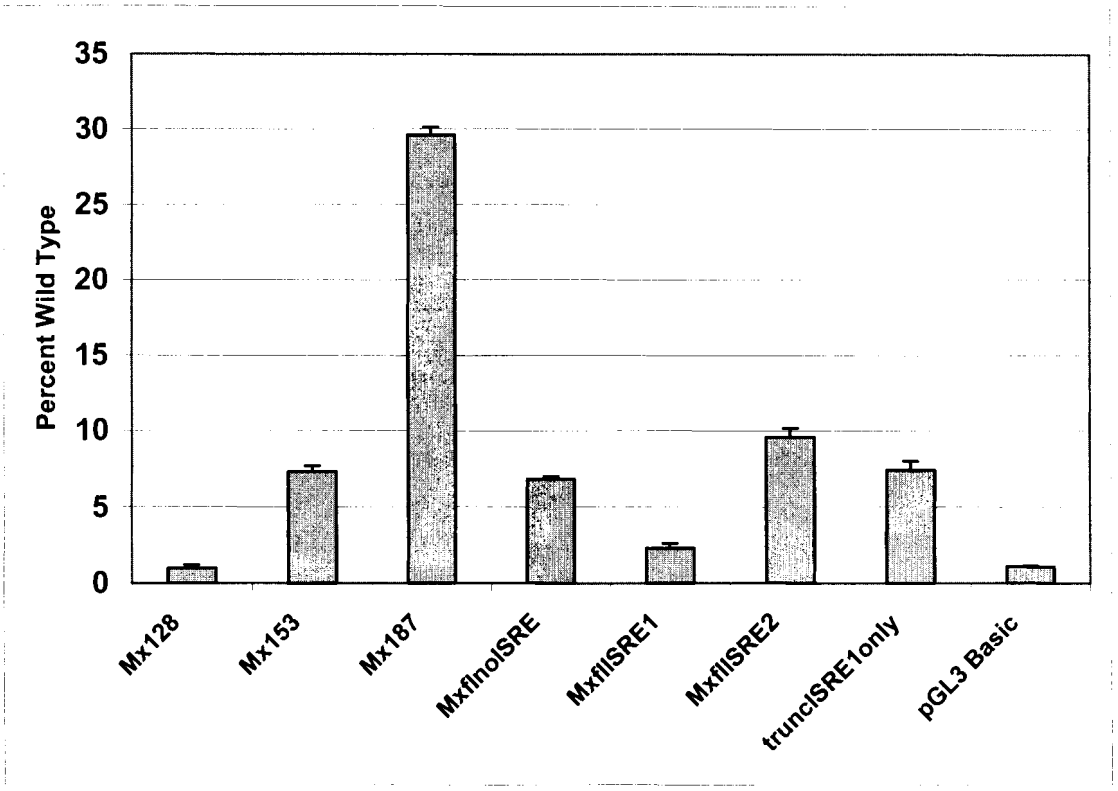

Figure 3.7. Relative activity of Mx promoter mutants compared to wild type. ZFL cells were transfected with either wild type Mx promoter, or an Mx promoter mutant and induced with 10 μL zebrafish IFN supernatant. After 24 h, cells were assayed for luciferase activity. Activity was measured in RLU, with the value for Mxpro-Luc assumed to be 100% promoter activity. Values for mutant constructs are given as a percentage of Mxpro-Luc activity. Error bars represent the mean ± SD of each treatment, performed in triplicate. These results are representative of two independent experiments.

Discussion

Mx is a crucial antiviral effector protein of the IFN signaling pathway, and, as such, has been well studied. Regulation of Mx gene expression via its IFN-inducible promoter has also been a point of interest. Use of Mx as an indicator of IFN activity has become common practice to researchers working with lower-order vertebrates such as fish [117, 138], in which a gene coding for IFN has only recently been found [136]. In this paper, we report the cloning and characterization of an Mx gene and its promoter from zebrafish. Zebrafish Mx encodes a protein of 646 amino acids in length, containing the highly conserved domains found in all known Mx proteins. ZFL cells induced with Poly IC demonstrated an increase in Mx mRNA transcription, as detected by quantitative PCR. The zebrafish Mx promoter was found to contain two ISREs that conform to the most recent ISRE consensus [49]. ZFL cells, transiently transfected with a construct containing the zebrafish Mx promoter upstream of a luciferase reporter gene, showed an increase in luciferase activity in response to induction by zebrafish IFN or Poly IC. The only other fish Mx promoters cloned to date are those of rainbow trout and fugu [40, 49]. The rainbow trout Mx promoter contains only one ISRE, whereas the zebrafish and fugu Mx promoters each contain two. ISRE 1 of the zebrafish Mx promoter conforms exactly to an ISRE of human MxA, while ISRE2 does not conform to any previously cloned Mx promoters, when comparing the nucleotides outside of the core GAAAN(1-2)GAAA element.

A search for Mx revealed several new ESTs that were unavailable at the time zebrafish Mx was first cloned. One of these ESTs, fw79f10, turned out to be a portion of our zebrafish Mx gene. However, two additional ESTs were found, both of which may represent different Mx genes. One EST, ZFT242, shows an 80% identity over residues 289 to 515 of zebrafish Mx while the other EST, fj93f01.y1, shows only 39% identity over residues 259 to 462 of zebrafish Mx. When EST fj93f01.y1 was used in a BLAST search, its highest homology was to rat Mx3, with

an identity of 44%. These results are intriguing, showing the possibility of at least three zebrafish Mx genes, two of which are similar.

Mutational analysis of the zebrafish Mx promoter has clearly demonstrated the essential role of the ISRE motif in efficient IFN-induced gene transcription (Fig. 3.7). However, an interesting phenomenon was observed in this experiment. Initially, we anticipated that mutant Mx187 would show promoter activity roughly equivalent to that of the wild type Mx promoter. In fact, a 71% decrease in promoter activity was observed. This suggested to us that additional regulatory elements might be located within the 883 nucleotides that were deleted from Mx187. Upon closer examination, this region was found to contain a total of ten elements of the type GAAANN [49, 134], which are found in the promoter region of all IFN-inducible genes. It seems reasonable to expect that these elements play a role in enhancing gene expression mediated by the ISREs, however, due to the complete lack of promoter activity from mutant MxflnoISRE, it is obvious that the ISREs are essential for this enhancement.

In addition to determining the role of both ISREs together, we also wanted to examine the role of each ISRE individually. To this end, several mutant constructs were generated containing either ISRE1 or ISRE2, both with and without the upstream promoter region (Fig. 6). The promoter activity of each ISRE individually showed only a small fraction of wild type activity. One might expect each ISRE to contribute roughly half of the total promoter activity, but the data show each ISRE mediating gene expression to no more than 10% of the wild type Mx promoter. This could be explained by a mechanism whereby the transcription factor complex must bind both ISREs simultaneously to achieve maximal promoter activity. The human MxA promoter and fugu Mx promoter also contain two ISREs in close proximity, and deletion analysis of these promoters has revealed a similar pattern, where deletion of either ISRE leads to a marked reduction in promoter activity [40, 47].

The second unexpected result was that the mutants containing the full-length promoter with either ISRE1 or ISRE2 deleted did not show increases in promoter activity over the

truncated mutants containing either ISRE1 or ISRE2. Due to the 71% reduction in promoter activity observed in mutant Mx187, we reasoned that mutants containing the putative 5' upstream regulatory region and lacking one ISRE would yield greater activity than mutants missing this upstream region. In actuality, however, no significant difference was seen. This lends further support to the idea that the two ISRE motifs act in unison, and that deletion of one will effectively abrogate all promoter activity irrespective of any additional regulatory elements.

The similarity between ISRE sequences of mammals, birds and fish is now well established. Less apparent are discrepancies in ISRE number among even closely related species, such as human (3 ISREs) and mouse (1 ISRE), and rainbow trout (1 ISRE) and zebrafish and fugu (2 ISREs each). In promoters containing two ISRE elements in close proximity, both elements appear necessary for full promoter activity. Due to the close spatial proximity of the two ISREs in the zebrafish Mx promoter, it seems unlikely that two separate transcription factor complexes would be able to bind simultaneously, hinting at the possibility of one transcription factor complex being capable of binding both ISREs at once. Additional research is needed to further elucidate the mechanism by which ISRE elements cooperate with transcription factors to regulate gene expression.

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Chapter 4

DISCUSSION AND FUTURE DIRECTION

This manuscript describes the cloning and characterization of the zebrafish innate immune effectors IFN and Mx, as well as the Mx upstream regulatory region. Zebrafish IFN was shown to behave like human IFN in terms of its expression profile, Mx-inducibility, and antiviral activity, despite its displaying only 20% identity with human IFN at the amino acid level. Analysis of the 3D structure of zebrafish IFN through the use of computer generated homology modeling has revealed a high degree of structural similarity between human and zebrafish IFN, suggesting a strong relationship between conservation of structure and function, with respect to the IFN family. An important consequence of this relationship is that it allows one to study the IFN pathway in a lower order vertebrate such as zebrafish and to extrapolate, to a certain extent, the results obtained from the zebrafish system to that of the human system.

Characterization of zebrafish IFN has important implications, not only as a tool to help broaden our understanding of the human IFN system, but also as a potential therapeutic for use in other fish systems. Nowhere is the need for effective therapeutics more pressing than in the aquaculture industry, where diseases such as ISAV are responsible for the destruction of large numbers of penned Atlantic salmon each year.

The Mx protein in one of several well characterized IFN-inducible antiviral molecules. Detection of Mx gene expression has been used in the past as an indicator of IFN activity, especially in fish systems, where a gene for IFN has only recently been uncovered. Perhaps more significant than the Mx gene, at least in terms of its use as a diagnostic, is the Mx promoter. With its well conserved ISRE sequences and strict IFN-inducible nature, the Mx promoter is a valuable tool for assessing IFN activity. In addition, the Mx promoter could potentially play a role in the generation of transgenic zebrafish. For example, a transgenic zebrafish made by linking the

zebrafish Mx promoter to a gene encoding green fluorescent protein (GFP) could serve as an excellent indicator of viral infection, in that the virally-infected Mx promoter transgenic zebrafish would produce IFN, thus inducing GFP expression in the fish. Such "sentinel fish" could conceivably be used as a frontline detection system against pathogens contaminating our water supplies.

Future experiments will be aimed at further characterization of zebrafish IFN. Protein purification of zebrafish IFN, followed by exposure of zebrafish to the purified IFN and subsequent viral challenge, would be an informative study, in that it could demonstrate whether or not zebrafish, when immersed in water containing purified IFN, could absorb enough IFN to elicit an immune response. From here, the amount of IFN and time pre/post viral infection could be manipulated to determine an optimal dosing schedule. This data could be applied to other fish species, such as salmon, as a method of prophylactically treating virally infected fish. Indeed, experiments in our lab are currently under way to determine if zebrafish IFN can be administered by immersion, as well as whether zebrafish IFN can cross-react with the type I IFN receptor on salmon cells.

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