ABSTRACT

BROOKS, ELAINE BARBARA. The Chicken Mx Gene and its Influence on Host Response to Influenza Infection. (Under the direction of Dr. Matthew D. Koci and Dr. Christopher M. Ashwell.)

Avian influenza has long been a concern for the poultry industry, with previous outbreaks resulting in major economic losses. In recent years, the growing number of bird-to-human transmission of avian strains, in conjunction with the discovery that the devastating influenza outbreak of 1918 arose from a strain consisting solely of avian genes, has raised concern that the next influenza pandemic may be caused by a highly pathogenic avian influenza (HPAI) strain. Since such viruses can arise from low pathogenic (LPAI) strains, control of infection at the farm level becomes much more important.

Understanding how to enhance the ability of poultry to overcome an LPAI infection may provide insight into other prevention methods, by controlling the spread of infection of an LPAI strain before it mutates into an HPAI strain, able to decimate entire flocks. As the host innate immune system is believed to play a key role in the pathology of influenza, augmenting the poultry innate resistance to influenza may be a possible method of disease control. The Mx protein, in particular, has been shown to possess innate antiviral activity to several RNA viruses, including influenza. The current studies examined the chicken genome for additional Mx-like genes and the influence of the Mx sequence on response to influenza infection.

The chicken genome was analyzed for genes with homology to Mx to determine if poultry, like mammals, possess multiple Mx genes. Three potential genes with limited

similarities in predicted structural motifs to Mx were identified. RT-PCR analysis of these candidate genes demonstrated that they are expressed in normal chicken embryo fibroblast cells. This is in contrast to the known chicken Mx, whose expression is regulated by interferon (IFN), which is released in response to viral infection. Thus, it is likely that these candidate genes more closely resemble another family member of large GTPases called dynamin rather than the antiviral Mx.

These results lead us to conclude that chickens have only one Mx gene. To further characterize the role that Mx plays in the anti-influenza response, I examined the Mx gene of multiple genetic lines of poultry for sequence variation. Several polymorphisms were identified, some of which encoded amino acid changes. In a recent study by Ko et al., a specific amino acid substitution near the carboxy-terminal was described as conferring antiviral activity of the chicken Mx to a variety of RNA viruses. This mutation, of serine to asparagine, was observed in two of the sampled lines in this experiment. However, there did not appear to be a correlation between a lower viral output after infection and the presence of the antiviral asparagine at amino acid 639.

In conclusion, chickens appear to have only one Mx gene with the characteristic protein domains and interferon regulation. Variations within this gene have been shown to influence the antiviral capabilities of Mx, though the reported single amino acid substitution near the C-terminal may not be the only factor in conferring this antiviral phenotype.

THE CHICKEN MX GENE AND ITS INFLUENCE ON HOST RESPONSE TO INFLUENZA INFECTION

by

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DEDICATION

To

My family, for all of your love and support.

BIOGRAPHY

Elaine B. Brooks, daughter of Janet P. Brooks and Charles P. Brooks, was born on December 21, 1979 in Syracuse, NY. Growing up in Baldwinsville, NY, she attended C.W. Baker High School, graduating in 1998, and went on to pursue a Bachelor of Science degree at Cornell University in Ithaca, NY. Here, her interest in biological systems was nurtured in the physiology program in the College of Agriculture and Life Sciences. Upon completion of her B.S. degree in 2002, Elaine moved to Manhattan to work in an immunology research laboratory at Weill Medical College of Cornell University on human visceral leishmaniasis. After two years of practical experience, she decided to continue her education at North Carolina State University in Raleigh, NC to pursue a Master of Science degree in physiology under the guidance of Dr. Matthew D. Koci and Dr. Christopher M. Ashwell.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER III

LIST OF FIGURES

CHAPTER I

CHAPTER II

1.1. CHAPTER I

LITERATURE REVIEW

ELAINE B. BROOKS

1.1 AVIAN INFLUENZA

Viruses are submicroscopic, non-living particles composed mostly of nucleic acids with a protein coat which infect the cells of biological organisms. Since they lack reproductive machinery, viruses are similar to obligate intracellular parasites in that they are incapable of self-reproduction outside of the host cell.

1.1.1 Classification of influenza viruses

Influenza viruses are members of *Orthomyxoviridae* family, and are enveloped, segmented, negative-sense (complementary to mRNA), single-stranded RNA viruses which are divided into three types: A, B, and C, based on the antigenic differences between their nucleocapsid (NP) and matrix (M) proteins (Lamb et al., 1996). Type A viruses infect a number of species, including a wide variety of wild and domesticated birds, while types B and C only affect humans. Subtype classification of influenza A is based on the serotype of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). There are 16 HA and 9NA recognized subtypes, all of which are found in avian species. Like several other enveloped viruses, influenza requires cleavage of a surface glycoprotein (HA) for activation (Taubenberger, 1998). The amino acid sequence of the HA1 region, which results from cleavage of the hemagglutinin by endogenous proteases and is responsible for HA antigenicity, differs from one subtype to the next by 30% or more (Rohm et al., 1996).

1.1.2 Influenza Life Cycle

Each of the eight gene segments within the virion (Figure 1-1) has a specific role in the viral life cycle (Figure 1-2).

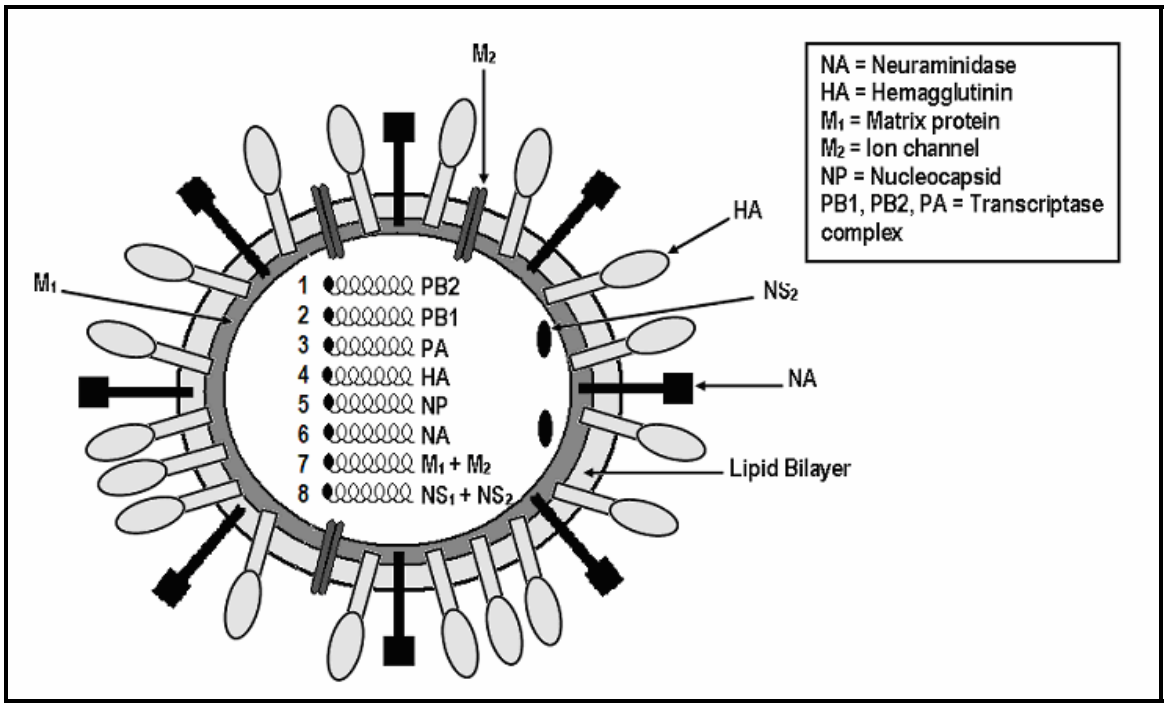

FIGURE 1-1: Structure of the influenza virion. The three integral membrane proteins embedded in the surface of the virion are hemagglutinin (HA), neuraminidase (NA), and the matrix 2 (M2) protein. Each of these is capable of eliciting an antibody response by the host. The virion encodes for six internal proteins: three viral polymerase genes (PB1 and PB2, which are basic, and PA, which is acidic), nucleoprotein (NP), nonstructural genes (NS1 and NS2), and the matrix 1 (M1) gene. Figure adapted from Fields Virology (Lamb et al., 1996) by Elaine B. Brooks.

HA binds specifically to sialic acid residues on the host cell membrane, which are either in α(2,3) linkages to galactose, as is the case in birds, or in α(2,6) linkages, found in the humans. The HA subtypes are specific for one of these linkages. Receptor-mediated endocytosis through this interaction is the mechanism by which the virus enters the cell. A conformational change in HA is induced by the low pH in the endosome, which initiates membrane fusion (Bullough et al., 1994). Once endocytosed, the matrix 2 (M2) protein, an integral membrane protein, functions as a proton channel to acidify the inside of the endosome which facilitates virion uncoating (Pinto et al., 1992). This allows the release of the RNA-dependent RNA polymerase complex, or ribonucleoprotein (RNP)

complex, which is comprised of the four proteins, three viral polymerases (PB1, PB2, and PA) and NP, and the RNA segments, into the cytosol for transport to the nucleus and viral replication (Nath et al., 1990; Mukaigawa et al., 1991; Neumann et al., 1997).

Viral RNA (vRNA) transcription employs the 5' cap of cellular mRNAs, cleaved preferentially at a purine residue by a viral endonuclease, as primers for transcription (Lamb et al., 1996). Most of the gene segments (HA, NA, NP, PB1, PB2, and PA) encode one protein, while the remaining two segments (matrix, M, and nonstructural, NS) each encode two proteins which are translated through mRNA splicing events, resulting in M1, M2, NS1, and NS2 (Lamb et al., 1996). These newly synthesized viral RNAs are encapsidated with nucleoprotein within the nucleus.

From here, the HA, NA, and M2 vRNAs are translated first, forming the three integral membrane proteins of the virion. Both HA and NA are transported to the rough endoplasmic reticulum to be glycosylated and are subsequently integrated into the cell membrane. Late in infection, the M1 protein must be transported back into the nucleus to permit the newly assembled RNPs to migrate out of the nucleus (Lamb et al., 1996). Progeny viral particles are assembled in the cytoplasm and interact with the matrix proteins associated with the plasma membrane to form a bud. NA, the other surface glycoprotein on the influenza virion, acts as a sialidase, cleaving sialic acid and releasing the progeny virion bud from the cell surface, thus facilitating virus spread.

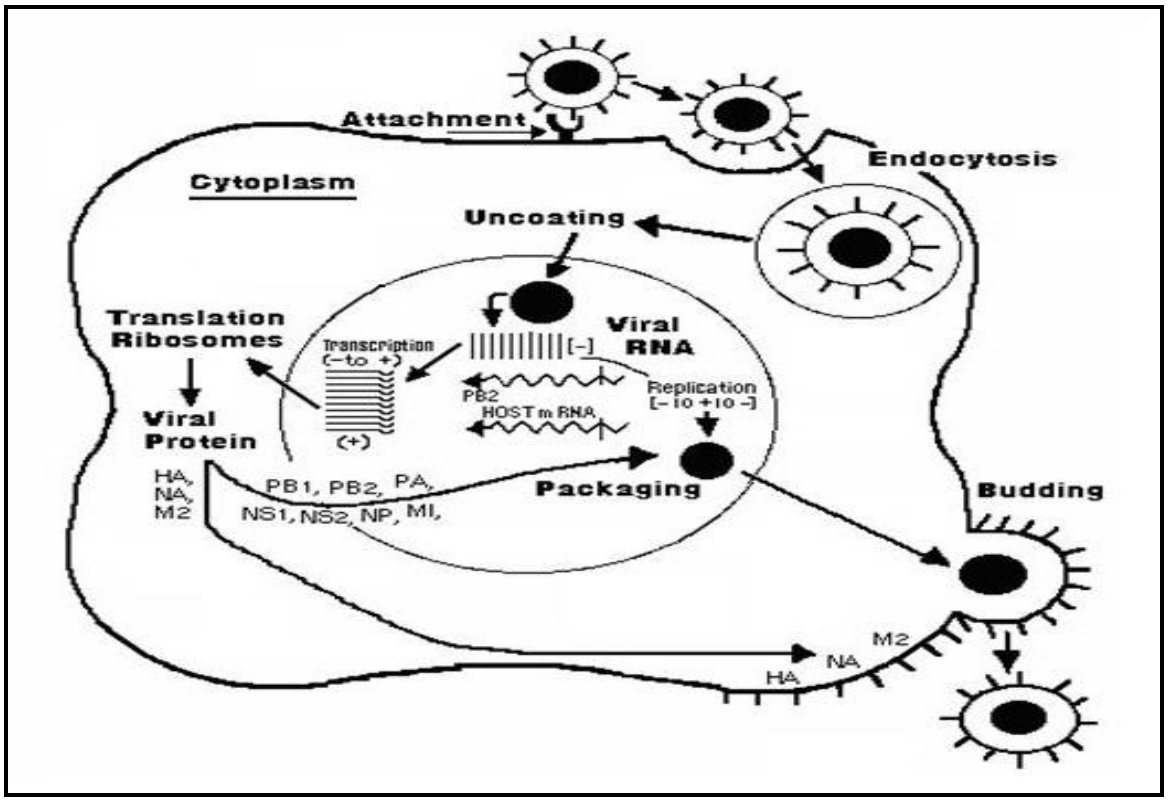

FIGURE 1-2: Diagram of influenza life cycle. The virion enters the host cell via receptor-mediated endocytosis upon binding of viral hemagglutinin to sialic acid residues on the host cell membrane surface. The matrix 2 proton channel acidifies the endosome, initiating membrane fusion and virion uncoating. Each of the 8 viral gene segments are transcribed in the nucleus, employing the 5' cap of cellular mRNAs as primers, and are then translated in the cytoplasm. The HA and NA proteins are subsequently glycosylated at the endoplasmic reticulum and, along with the M2, migrate to the cell membrane to await budding. The remaining viral proteins and newly synthesized gene segments are packaged in the nucleus. The newly packaged material migrates to the cell membrane where budding occurs and releases the progeny virion. Adapted from Viruses (Lawrence et al., 2001) by Elaine B. Brooks.

1.1.3 Viral Evolution

Antigenic drift vs. antigenic shift

Antigenic variation occurs continually in influenza viruses due in part to their segmented genome. This results from one of two processes: antigenic drift or antigenic shift. Viral changes resulting from point mutations are thought to be driven by immunological pressures on the HA and NA. This antigenic drift is known to occur in

human viruses, accounting for the need to develop new vaccine strains every few years, but has also been detected amongst avian strains (Kida et al., 1987).

Antigenic shift results either by direct transmission of nonhuman strains to humans or when more than one type of influenza virus infect the same cell, as there is a reassortment of genes within the infected cell (Webster et al., 1982). The latter is the classic model for antigenic shift as such mixed infections occur relatively frequently in nature and can lead to this genetic reassortment (Hinshaw et al., 1980).

"Mixing vessel"

Prior to the Hong Kong outbreak of the avian H5N1 in 1997, the transmission of avian influenza viruses from birds directly to humans was believed to be impossible, since avian and human cells preferentially bind to different sialic acid residues (Bush, 2004). While this difference was thought to be the primary barrier for direct transmission, receptors for both types of linkages are found on swine cells, allowing pigs to be co-infected by both avian and human strains. This led to the idea that swine can serve as an intermediate host or "mixing vessel" where avian strains could adapt to be transmissible to humans (Ito et al., 1998; Bush, 2004).

Role of waterfowl

Waterfowl play a particularly important role in the spread of influenza. All HA and NA subtypes are capable of being maintained in aquatic birds, including ducks, gulls, and shorebirds (Sharp et al., 1997; Suarez et al., 2000). Influenza viruses are secreted from the intestinal tract of infected birds and are transmitted via ingestion of fecally-contaminated water, though infected waterfowl rarely show signs of the disease. Additionally, influenza viruses appear to have reached an optimal state of adaptation,

where little to no selective advantage is obtained from amino acid changes (Gorman et al., 1991). The combination of this adaptation with the lack of symptomatic disease makes waterfowl an ideal natural influenza reservoir.

1.1.4 Pathogenicity of influenza in poultry

The appearance of disease resulting from avian influenza viruses ranges from asymptomatic to mild to severe respiratory infection to rapid fatal systemic disease. One of the differences between highly pathogenic avian influenza (HPAI) strains and low pathogenic (LPAI) strains is systemic versus local viral replication. Since HA cleavage is necessary for the virus to be infectious, this distinction is related to differences in how the HA is cleaved. In HPAI, this is done by endogenous proteases present in most cells in the body, whereas only trypsin-like enzymes found primarily in the respiratory tract and the gut are capable of cleaving LPAI (Stieneke-Grober et al., 1992; Steinhauer, 1999).

1.1.5 Influenza outbreaks

Human outbreaks

Previous human influenza pandemics have been responsible for millions of deaths worldwide. The most devastating pandemic, the "Spanish influenza" of 1918, is estimated to have killed 20-50 million people (Taubenberger et al., 2005; Tumpey et al., 2005). Subsequent influenza pandemics in 1957 and 1968 were far less catastrophic but did have one thing in common; they were caused by hybrid viruses possessing a combination of avian and human viral genes, confirming that avian influenza viruses are important contributors to human pandemics.

More recently, the impact of highly pathogenic avian influenza strains on human disease has become more evident. In 1997, the avian H5N1 was isolated from a 3-year-

old in Hong Kong (Yuen et al., 1998). While HPAI viruses had been identified prior to this outbreak, their effects had been confined to poultry. Of greater concern was the realization of the virulence potential of HPAI to humans, as the Hong Kong isolates were not reassortants as in the 1957 and 1968 pandemics but rather were made up entirely of avian viral genes (Subbarao et al., 1998). This impact was compounded by the discoveries made from the reconstruction of the 1918 Spanish influenza virus, specifically that viral gene sequences resemble avian H1N1 more so than any mammalian H1N1 strain (Taubenberger et al., 2005; Tumpey et al., 2005). Such findings have made it evident that avian influenza strains have the capacity to not only infect humans directly but also to cause severe disease.

Poultry outbreaks

Originally known as the fowl plague, HPAI viruses have been affecting poultry for well over a century. First described in 1878 in Italy (Perroncito, 1878), the causative agent of disease (H7N7) was eventually isolated in 1902, marking the first isolation of influenza virus documented. However, it was not until 1955 that the fowl plague virus was demonstrated as a member of the influenza A virus group (Schafer, 1955). Since then, outbreaks have been recognized across the globe, greatly impacting the poultry industry and, more recently, posing a threat to human health.

In 1983, an avirulent H5N2 strain emerged in Pennsylvania (Eckroade et al., 1986). Later that year, after many flocks had been decimated, virulent influenza viruses were isolated from chickens. Sequencing studies of these viruses revealed that their genes were derived from an avirulent virus, rather than the virulent H5 strain which was

responsible for previous outbreaks. In order to eradicate this virus, more than 17 million birds had to be destroyed, costing over 61 million dollars (Eckroade et al., 1986).

Studies of these Pennsylvania avian viruses showed that point mutations in the HA cleavage site were responsible for the acquisition of virulence by the avirulent strain (Bean et al., 1985). Since the HA gene was previously identified as a critical determinant of virulence (Webster et al., 1986), comparisons of HA amino acid sequences of virulent and avirulent viruses showed a single substitution suggested to be responsible for acquiring virulence. The threonine to lysine change at residue 13 coincided with the loss of an oligosaccharide side chain, thought to block the cleavage site in avirulent strains (Kawaoka et al., 1984). The H5N2 that caused the 1983 Pennsylvania outbreak possessed a carbohydrate side chain which blocked the basic residues of the cleavage site. Once that side chain was lost from the avirulent strain, the HA became highly cleavable, and the virus became virulent (Kawaoka et al., 1984).

Ten years later, avirulent H5N2 viruses were once again isolated in Pennsylvania. However, through phylogenetic analysis of the HA genes, these were determined to share an ancestry with viruses isolated from shorebirds in 1991 and did not originate from the 1983 strain, suggesting introduction of this new virus by waterfowl (Saito et al., 1994). The acquisition of virulence by the 1983 viruses combined with the finding that the 1993 avirulent viruses also required only a single mutation to become virulent (Horimoto, 1995) show the ability of avirulent strains to mutate into potentially hazardous influenza viruses in nature.

1.2 HOST RESPONSE TO INFLUENZA

Type I interferons (IFN) produced by virally-infected cells play a crucial role in the host's early defenses against viruses, inducing expression of a number of host genes, including Mx (Muller et al., 1994; Horisberger, 1995). The murine Mx1 protein was originally discovered in a genetic line of laboratory mice (A2G) that were influenza resistant (Lindenmann, 1964). Mx proteins were found subsequently in other vertebrates, including chickens (Pavlovic et al., 1993; Bernasconi et al., 1995), and have since been recognized to be involved in the response to other RNA viruses.

1.2.1 Regulation of Mx Expression

The kinetics of Mx activation are quite rapid, meaning that cells are capable of responding to influenza infection quickly by producing Mx proteins which remain within the infected cell and interferons which are released into the extracellular environment to stimulate Mx expression in neighboring cells (Horisberger, 1995). In this way, the interferon-system participates in limiting viral spread and initiating recovery, giving the immune system an opportunity to mount its own response through T-cells and B-cells. There is a clear correlation between influenza infection and the induction of Mx expression (Arnheiter et al., 1990; Horisberger, 1995).

The Mx gene expression is stimulated via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, which begins when IFN-1 binds to its receptor and follows several steps including protein phosphorylation and activation of the STAT1 and STAT2 transcription factors which migrate to the nucleus to initiate gene transcription (Levy, 1995). An important component of Mx, which is responsible for its response to the presence of interferon, resides within the promoter.

IFN-responsive genes in mammals contain a sequence element within the promoter, designated the IFN-stimulated response element (ISRE), which is present in all Mx genes. *In vitro* studies suggest that endogenous IFN is induced and in turn activates the chicken Mx promoter, which seems to respond with a high selectivity to IFN (Schumacher et al., 1994).

1.2.2 Structural Motifs

Three structural domains have been described in known Mx proteins which are implicated in the antiviral activities of Mx against RNA viruses. Since both dynamin and Mx proteins are members of the protein family of large GTPases, they possess some common structural motifs, including a GTPase domain near the amino terminus. However, there are some differences that distinguish Mx proteins from other GTPases.

Dynamin-specific domains

A microtubule-associated mechanochemical enzyme (Kochs et al., 2005), dynamin proteins are characterized by 5 distinct domains: an N-terminal GTPase domain, a GTPase effector domain with a tendency to form coiled coils, a middle domain of unknown function, a pleckstrin-homology (PH) domain, and a C-terminal proline-rich domain (van der Bliek, 1999). The first two of these are also found in Mx proteins and are described further in the following sections. However, one of the main structural differences between dynamin and Mx lies in the absence of two of these domains in defined Mx proteins: the PH domain, which has been implicated in membrane binding, and the proline-rich domain, which contains several Src-homology type 3 (SH3) binding sites (van der Bliek, 1999; Haller et al., 2002).

GTP-binding domain

Mx proteins have been characterized by motifs demonstrated to be necessary for their antiviral activity to certain RNA viruses. One of these domains is a tripartite consensus motif that is characteristic of proteins with GTP-binding and GTP-hydrolyzing abilities and resides near the amino terminus, which is the region of highest sequence conservation among Mx proteins (Dever et al., 1987). Such GTPase activity is also found in dynamin, suggesting that it may be involved in intracellular protein translocation and motility (Kochs et al., 2005). This motif, which constitutes about one-third of the amino terminal (Melén et al., 1992) has been demonstrated to be essential for antiviral activity by Pitossi et al. (1993), who found a direct correlation between the GTP-binding and hydrolysis capabilities and their antiviral activity.

Perhaps one of the reasons for the importance of this motif lies in the presence of a self-assembly sequence (SAS) located between the first and second conserved GTP-binding sequences (Nakayama et al., 1993). This sequence, along with a central interactive domain and a leucine zipper, are known to be involved in self assembly and association with other molecules, including viral target structures. The binding and hydrolysis of GTP induce conformational changes in Mx and are suggested to be essential for viral target recognition and antiviral activity (Kochs et al., 2002).

Central interactive domain

Located in the middle of the molecule, the central interactive domain has also been implicated in the antiviral capabilities of Mx proteins (Kochs et al., 1998). Interactions between this domain and the α-helix that results from the leucine zipper region is necessary for the self-assembly characteristic of Mx proteins and their ability to

associate with other molecules (Di Paolo et al., 1999). The importance of this region was confirmed through the use of monoclonal antibodies to the central interactive domain and other regions. Binding of a monoclonal antibody to an epitope in this domain resulted in the prevention of human MxA from associating with the viral target structures and abolished its antiviral activity. Antibodies to other regions had no such effect, demonstrating that the central interactive domain is required for function (Flohr et al., 1999).

Leucine zipper motif

A notable feature of Mx proteins is their ability to form high molecular weight oligomers *in vivo* and *in vitro* (Melén et al., 1992; Kochs et al., 2002). Through cross-linking experiments, Melén et al. revealed that human, rat, and murine Mx proteins exist predominantly in trimers. At the carboxy-terminus of all known Mx proteins, there is a leucine zipper motif which plays a role in oligomerization (Melén et al., 1992; Yap et al., 2003), is involved in GTPase activation, and corresponds functionally to the GTPase effector domain present in dynamin (Schwemmle et al., 1995). It is the backfolding of the leucine zipper on the central interactive domain, thus increasing the GTPase activity, that defines the C-terminal as the effector domain.

In mice, this motif is also responsible for intracellular localization of the protein. The stretch of basic and charged amino acids at the C-terminal of murine Mx serves as a nuclear localization signal, directing the protein to the nucleus where it interferes in influenza RNA transcription (Zurcher et al., 1992). Additionally, point mutations within human and rat Mx exposed that this effector domain is a key determinant for recognition of the viral target structures (Zurcher et al., 1992; Johannes et al., 1997).

Given the importance of this motif to the antiviral function of Mx proteins made evident in other species, it can be concluded that it has a role in chicken Mx antiviral activity as well. Although the primarily cytoplasmic chicken Mx had not previously shown much viral inhibition (Bernasconi et al., 1995), Ko et al. found that a single amino acid substitution immediately upstream of this motif, from serine to asparagine at position 639, confers antiviral activity in chickens, suggesting that the sequence near the carboxy-terminus is indeed important in determining antiviral activity towards influenza and other RNA viruses.

1.2.3 Protein localization and antiviral activity

Some mammalian species are shown to have more than one Mx gene and protein isoform, whose intracellular localization and antiviral activity vary (Lee et al., 2002). Mice and humans both possess two Mx genes. Murine Mx1 is a nuclear Mx protein which selectively inhibits the primary transcription of orthomyxoviruses (Pavlovic et al., 1993; Nagata et al., 1997). Such nuclear protein localization is not the only factor in determining antiviral activity (Garber et al., 1993), but it is necessary for specifically inhibiting influenza mRNA synthesis (Zurcher et al., 1992). Cytoplasmic Mx proteins, like human MxA, also possess antiviral activity. However, in contrast to the nuclear murine Mx, they interfere with a poorly defined step along the influenza multiplication pathway between primary transcription and genome replication (Pitossi et al., 1993). Currently, there is only one known Mx gene in chickens, which produces a cytoplasmic protein. While transfected cells expressing the chicken Mx protein initially showed no enhanced resistance to influenza A virus (Bernasconi et al., 1995), more recent

experiments demonstrate the chicken Mx's ability to participate in the innate antiviral response to influenza (Ko et al., 2002).

1.3 CURRENT STUDY

From the preceding literature review, the potential of Mx proteins to effect an innate antiviral response to influenza challenge is clear, though such activity has not been consistently demonstrated in chickens. Given that other species are known to possess more than one Mx, the first part of this research examines the chicken genome for the possibility of Mx homologs with similar antiviral activity. The second part of the study focuses on the known chicken Mx gene and the effects of sequence variation within that gene between sampled genetic lines when challenged with a low pathogenic H9N2 strain of avian influenza.

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2.1. CHAPTER II

EXAMINATION OF THE CHICKEN GENOME FOR CANDIDATE MX GENES

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2.1 ABSTRACT

Chicken Mx is an interferon-inducible gene that has been shown to possess innate antiviral activity. Previous reports have demonstrated potential sequence variations which may lead to differences in response to a number of RNA viruses, including influenza. Since other species, specifically mammals, possess multiple Mx genes which contribute to the antiviral response, the chicken genome was examined for the presence of genes with homology to Mx. Three genes were identified from this search and were subsequently compared with the antiviral chicken Mx. Defined Mx proteins have a leucine zipper motif that is thought to play a role in oligomerization at the carboxy-terminus and a tripartite GTP-binding motif near the amino terminus, both of which have been suggested to participate in the antiviral response. Each candidate gene was analyzed for the presence of structural motifs found in Mx and was assayed for expression. The results suggest that these candidate genes are more similar to another large GTPase, dynamin, than Mx, as they possess dynamin-specific motifs not found in Mx proteins and do not appear to be interferon-regulated.

(*Key words*: Mx family, antiviral, chicken)

2.2 INTRODUCTION

Acute viral infections are a major cause of morbidity and mortality in humans and animals worldwide. Understanding the host mechanisms involved in the antiviral response is essential to developing more effective therapies. The type-1 interferon pathway plays a central role in the innate response to viral infection, inducing expression of multiple genes known to participate in the antiviral response. One of the best characterized interferon-regulated antiviral genes is the Mx gene. The Mx proteins are members of the large GTPase superfamily and have been reported to participate in the host response to viral infections in a variety of vertebrates, including mice, fish, humans, and chickens (Pavlovic, 1991). Humans and mice, as well as others, express multiple Mx genes (Lee, 2002). Mammals, in particular, are known to possess multiple Mx genes which function in the antiviral response to different viruses. Previous studies of the chicken response to viral infection led to the identification of one Mx gene, which was originally reported to possess no antiviral activity. Recently, studies by Ko et al. demonstrated variability within the Mx gene of chickens and suggest certain mutations conferring an antiviral phenotype (Ko et al., 2002).

Based on these observations, and in order to understand more about the role of Mx in the poultry innate response to viruses, the chicken genome was examined to identify other as yet unappreciated Mx genes.

2.3 MATERIALS AND METHODS

Search for Candidate Mx Genes and Primer Design

The Ensembl Genome Browser was used to search for genetic sequences similar

to Mx, generating a protein family using the Markov cluster algorithm, which groups proteins based on sequence similarity (Enright et al., 2002). Using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), primers (10μM) were designed to flank the transcript of Mx gene on chicken chromosome 1 (Mx), as well as the identified candidate genes on chromosomes 1 (GTPase1), 8 (GTPase8), and 17 (GTPase17). However, as the transcript is not known for these, alignments were done of all proposed transcripts and primers designed for the homologous region so that the product, if present, would amplify regardless of which transcript was made. The forward (F) and reverse (R) primer sequences for each gene were as follows: 1) MxF 5'-GCCAGAAGAACAGCAGAACA-3', MxR 5'-GGTTGCTGCTAATGGAGGAT-3', 2) GTPase1F 5'-ATGGAGGCGCTGATCCCGGT-3', GTPase1R 5'-TCACCAAAGATGTGTCTCCCGA-3', 3) GTPase8F 5'-TCCGTGCTGGAGAACTGCGT-3', GTPase8R 5'-GCTGGCAGGGTATCCCGAAT-3', 4) GTPase17F 5'-GGATCTGATCCCGCTGGTCA-3', GTPase17R 5'-CAATGGAGAGAAGCTGGCTCTG-3'.

Mx Expression Analysis

Chicken embryo fibroblast cells (CEFs) were isolated (Bernasconi et al., 1995) from fertile eggs of selected genetic lines and cultured in Mediatech's high-glucose Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS (Mediatech), 1% of 100X L-glutamine (Sigma), and Sigma's antibiotic/antimycotic (100units/mL penicillin, 100μg/mL streptomycin, 0.25μg/mL amphotericin B).

CEFs were cultured in T25 flasks at 37°C and 5% CO_2 and, once at 70% confluency, were inoculated with 30μL of an H9N2 strain of avian influenza (A/TK/WI/66) at 50% tissue culture infectious dose ($TCID_{50}$) of $3.2x10^6$/mL, and

incubated for 48 hours at 37ºC in DMEM supplemented with 0.75% FBS and 2µg/mL TPCK-trypsin (Pierce). Total RNA was isolated using Trizol (Invitrogen) and was used to generate cDNA through oligodT. Genes were amplified through polymerase chain reaction (PCR) under the following conditions: initial denaturation at 95ºC for 2 minutes, followed by 30 cycles of PCR, with 30 seconds denaturing at 95ºC, annealing temperatures of 55ºC for Mx, 54ºC for GTPase1, and 57ºC for GTPase8 and GTPase17, and extension time of 1 minute per kilobase of product. The expected PCR product size for each gene was approximately 2.1kb for Mx, 2kb for GTPase1, 770 base pairs for GTPase8, and 1.1kb for GTPase17. PCR products were electrophoresed on a 1% agarose gel to observe the presence of PCR amplicons of the Mx transcript. Mock-infected CEFs cultured in influenza culture media underwent the same procedure and were used to compare basal versus induced expression of Mx genes. Primers for the glycolysis enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were used as a control, to normalize RNA levels across treatments.

2.4 RESULTS

The chicken genome was screened using the Ensembl Genome Browser to identify additional candidate Mx genes. Three putative genes (Figure 2-1) were then compared with the antiviral chicken Mx. The most notable similarities between all four proteins were the presence of a GTP-binding domain and a dynamin structural motif (Figure 2-2). However, the candidate Mx genes did not appear to have the C-terminal leucine zipper, which is known to have a role in oligomerization of Mx proteins (Melén et al., 1992). Additionally, the putative genes on chromosomes 8 and 17 possess other

dynamin-like motifs that specifically not found in Mx proteins, including the plekstrin homology (PH) domain and a proline-rich region.

Although several polymorphisms were observed in the putative sequences, the regulation of expression of these genes differed from the reported chicken Mx. Data resulting from the RT-PCR analysis of uninfected versus infected cells indicated that each of the candidate genes (GTPase1, GTPase8, and GTPase17) are each constitutively expressed. In contrast to Mx, whose expression is induced upon viral infection and initiation of the interferon system (Figure 2-3), these putative family members were shown to have no change in expression when challenged with H9N2 (Figure 2-4).

2.5 DISCUSSION

The Mx family is part of the interferon-regulated antiviral response to infection with RNA viruses in mammals and chickens. Mammals appear to have multiple antiviral Mx genes, although only one Mx gene has been described in chickens. Mutations in Mx have been described to be involved in differences in susceptibility to influenza infection among strains of mice (Horisberger et al., 1983; Garber et al., 1993).

Since other species are known to possess multiple Mx genes, the possibility of other potential Mx family members in the chicken genome was explored. Although the three previously unidentified genes revealed from this analysis had similar features to the established chicken Mx, they did not appear to have all of the same structural motifs associated with the antiviral activity of Mx, particularly the C-terminal leucine zipper. These genes appear to resemble more closely the structure of dynamin, which employs GTPase activity to regulate receptor-mediated endocytosis within the cell (Sever et al.,

1999), though the functions of these genes are still unknown. Of particular importance is the presence of certain protein motifs that are found in dynamin but are not in Mx GTPases (Haller, 2002). The first of these is the pleckstrin homology (PH) domain, which is necessary for dynamin membrane localization and receptor-mediated endocytosis (Salim et al., 1996). Figure 2-2 shows that two of the candidate Mx genes (GTPase8 and GTPase17) contain this PH domain whereas antiviral chicken Mx does not. The second of these domains is a proline-rich domain that is near the carboxy-terminal of dynamin proteins, which is important for protein-protein interactions for a number of cellular processing, including clathrin-mediated endocytosis (Hinshaw, 2000). While this domain also is present in the candidate genes on chromosomes 8 and 17, Mx proteins lack this region (Haller, 2002).

While the candidate gene on chromosome 1 (GTPase1) does not possess these two dynamin domains, its expression was regulated differently than Mx. RT-PCR analysis of the expression of each of these putative Mx genes demonstrated that they are constitutively expressed (Figure 2-4), suggesting that they are not regulated by interferon like the antiviral chicken Mx (Figure 2-3). Given this observation, along with the dynamin-specific domains present in GTPase8 and GTPase17, it seems that these candidate Mx family members function more like dynamin than the antiviral Mx on chromosome 1. Further studies are necessary to fully appreciate the role of these genes in the cell and their role, if any, in the antiviral response.

2.6 TABLES AND FIGURES

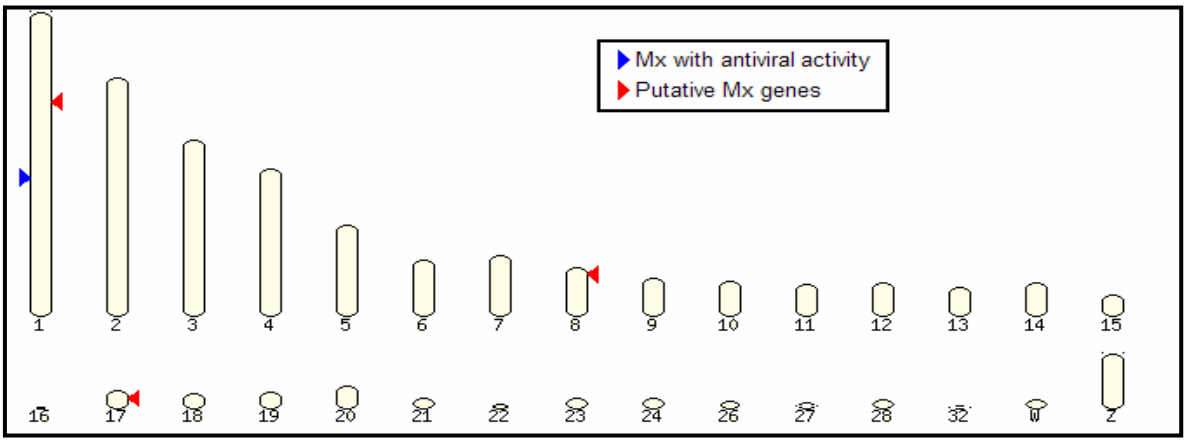

FIGURE 2-1: Schematic of the chicken genome, with markers identifying putative Mx family members. The previously described (blue arrow) chicken Mx gene was entered into the Ensembl Genome Browser (http://www.ensembl.org) in order to search for other putative Mx gene family members (red arrows).

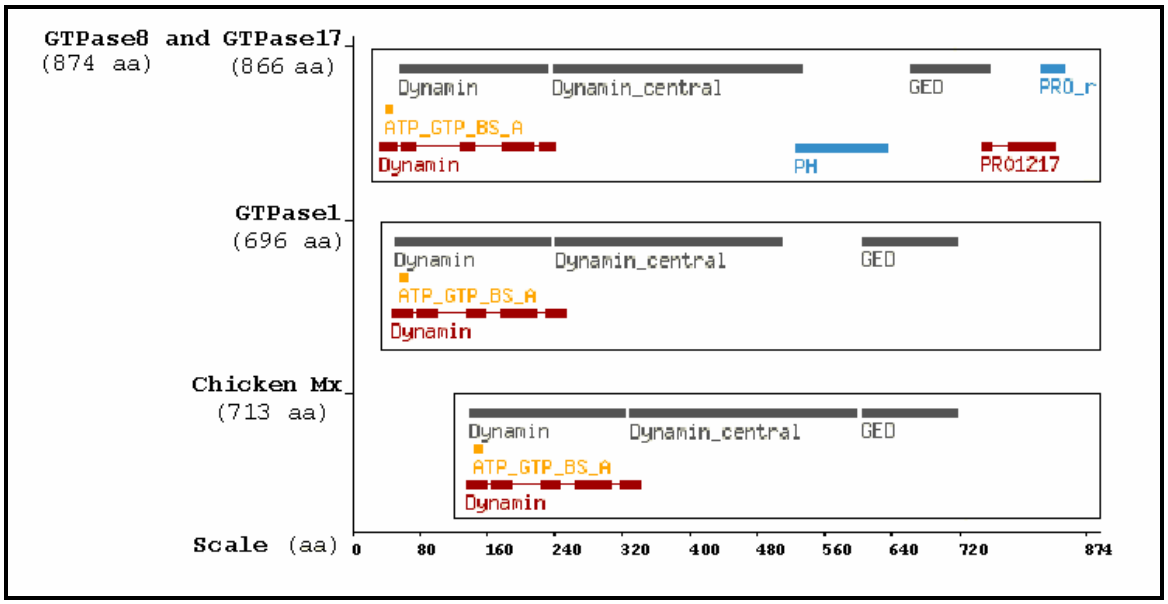

FIGURE 2-2: Structural domains of the chicken Mx and the three candidate genes, GTPase1, GTPase8, and GTPase17. While GTPase1 has similar domains to Mx, both GTPase8 and GTPase17 contain the plekstrin-homology (PH) domain and a proline-rich region, characteristic of dynamin proteins and not found in known Mx proteins. Adapted from the Ensembl Genome Browser website by Elaine B. Brooks.

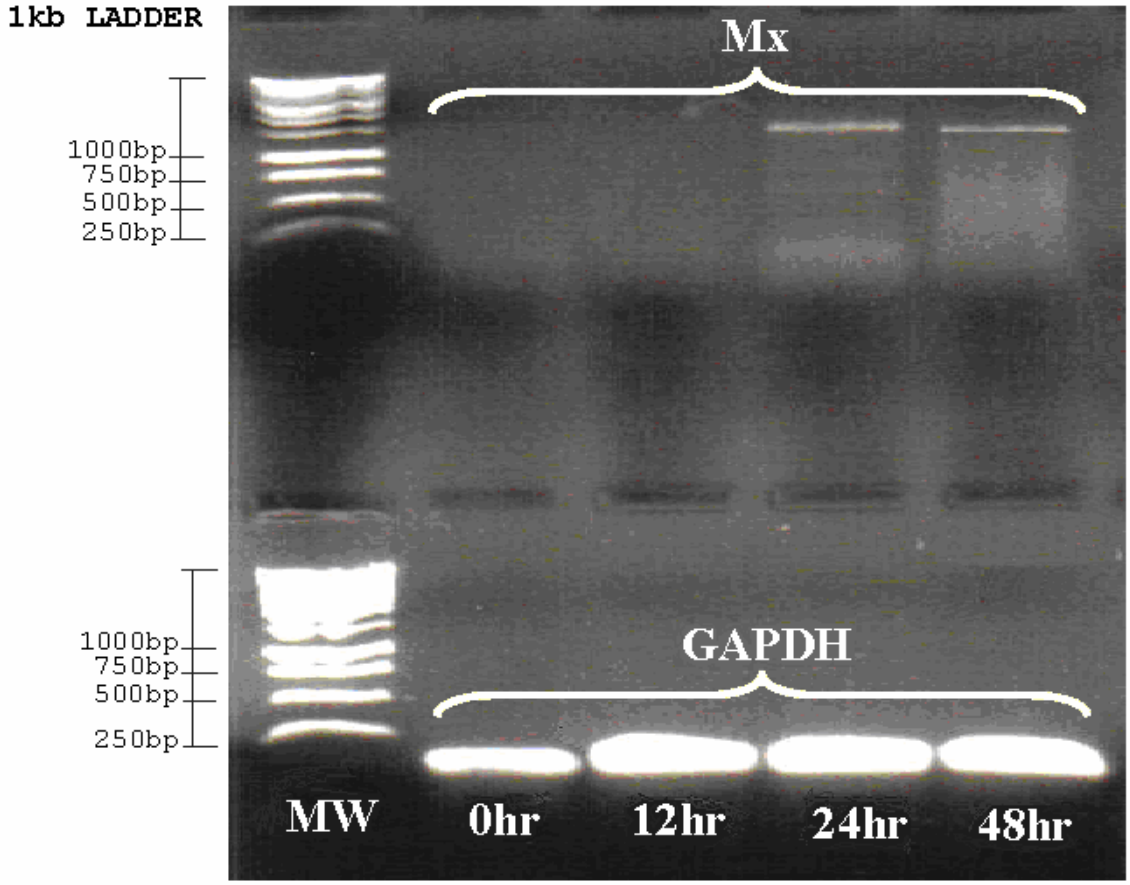

FIGURE 2-3: RT-PCR products of Mx at 0hr, 12hr, 24hr, and 48hr post-infection with avian influenza show that Mx expression is induced by interferon, which is produced in response to viral challenge. RT-PCR products of GAPDH at each time point were used as the control.

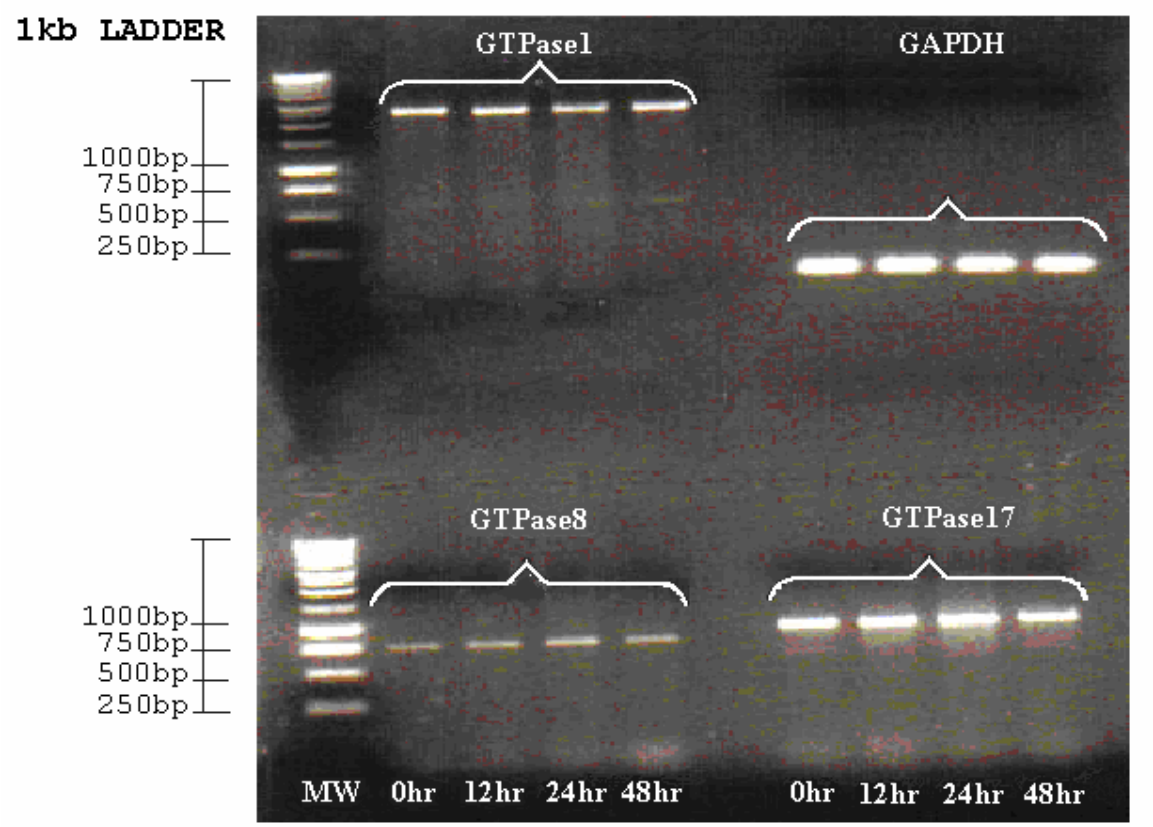

FIGURE 2-4: RT-PCR products of candidate Mx genes at 0hr, 12hr, 24hr, and 48hr post-infection time points. Primers to the glycolysis enzyme GAPDH were used as a control. Each of these genes is expressed at all four time points, indicating that their expression is not interferon-induced like Mx.

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3.1. CHAPTER III

PREDICTED RESPONSE TO AVIAN INFLUENZA A CHALLENGE AMONG DOMESTIC CHICKEN LINES BASED UPON VARIABILITY WITHIN THE CHICKEN MX GENE

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3.1. ABSTRACT

Avian influenza outbreaks are a continuing concern for the poultry industry, having caused significant economic losses in the past. Recent outbreaks across the globe of new strains of highly pathogenic avian influenza (HPAI) have had devastating effects on infected flocks and resulted in global awareness of the need to develop better techniques for disease prevention (Kwon et al., 2005; Nakatani et al., 2005). Since HPAI viruses may arise from mutations in low pathogenic avian influenza (LPAI) viruses, it is important to understand the host factors important in the cellular response to avian influenza infection. One way to do this is to augment the poultry innate resistance to influenza. The Mx protein has been demonstrated to be an important part of the innate antiviral response. Genetic variability of the Mx gene among chicken lines may reveal a sequence which confers greater influenza resistance. Genomic DNA from several lines of chickens was sequenced to determine the variability within the Mx gene, with several synonymous and nonsynomymous changes observed, suggesting possible functional differences among lines. Since a specific amino acid substitution immediately upstream of the leucine zipper motif has been demonstrated to confer antiviral activity in prior studies (Ko et al., 2002), mutations found near the C-terminus may shed light on protein functionality. The goal was to gain a greater understanding of Mx sequence variability and how that may shed light on its involvement in genetic resistance to certain viruses.

(*Key words*: Mx, influenza, antiviral, chicken)

3.2. INTRODUCTION

The innate immune response of the host is believed to be a key factor in the clinical course and pathology of influenza. An explanation for variations in the degree of illness that exist amongst avian genetic lines may lie in differences within their innate immune system. The Mx gene, in particular, is known to participate in the cellular response that inhibits influenza multiplication (Pavlovic et al., 1990; Ko et al., 2004). Chickens appear to have only one antivirally active Mx gene, located on the first chromosome[1].

Chicken interferon type 1 is induced in response to viral infection and activates the chicken Mx gene. Members of a family of large GTPases, Mx proteins are characterized by a high molecular weight, a tendency to self-assemble, and antiviral activity against various RNA viruses (Haller, 2002). All Mx proteins contain a tripartite consensus GTP-binding domain near the amino terminus, providing it with GTPase activity, which has been demonstrated to be essential for antiviral activity (Pitossi et al., 1993; Kochs et al., 2005). At the carboxy-terminus of Mx proteins, there is a leucine zipper motif which plays a role in oligomerization (Melén et al., 1992; Yap et al., 2003). Both of these domains have been implicated as necessary for the antiviral activity of Mx proteins.

The purpose of this study was to evaluate the degree of variability between sampled genetic lines of chickens and its association with changes in antiviral activity to RNA viral challenge.

[1] Results previously found by the author.

3.3. MATERIALS AND METHODS

Genomic Sequencing

Genomic DNA from several domestic genetic lines of chickens was obtained from a commercial hatchery (Hy-Line International). Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design primers (10μM), which flank each of the 13 exons of chicken Mx gene (Table 3-1). The exon sequences were amplified through polymerase chain reaction (PCR) under the following conditions: initial denaturation at 95ºC for 2 minutes, followed by 30 cycles of PCR, with 30 seconds denaturing at 95ºC, an annealing temperature of 55ºC for 30 seconds, and extension time of 1 minute per kilobase of product (product size = 2.1 kb). PCR amplicons for each Mx exon were produced and used as a template for DNA sequencing reactions following the manufacturer's instructions (ABI). Sequencing reactions were analyzed using an ABI 3100, and the resulting sequence data was analyzed using Vector NTI v. 10 (Invitrogen). Polymorphisms were identified and compared with the Mx transcript.

Mx Gene Expression

To test for gene expression regulation, chicken embryo fibroblast cells were isolated from fertile eggs of available lines of chickens (Bernasconi et al., 1995) and cultured in Mediatech's high-glucose Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS (Mediatech), 1% of 100X L-glutamine (Sigma), and Sigma's antibiotic/antimycotic (100units/mL penicillin, 100μg/mL streptomycin, 0.25μg/mL amphotericin B). CEFs were cultured in T25 flasks at 37°C and 5% CO_2. Once at 70% confluency, cells were inoculated with 30μL of an H9N2 strain of avian

influenza (A/TK/WI/66) at 50% tissue culture infectious dose ($TCID_{50}$) of $3.2x10^6$/mL, and incubated for 48 hours at 37ºC in DMEM supplemented with 0.75% FBS and 2µg/mL TPCK-trypsin (Pierce). Total RNA was isolated using Trizol (Invitrogen) and was used to generate cDNA through oligodT. Genes were amplified through polymerase chain reaction (PCR) under the above conditions and were subsequently electrophoresed on a 1% agarose gel to observe the presence of PCR amplicons of the Mx transcript. Primers for the glycolysis enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were used as a control, to normalize RNA levels across treatments.

Viral Titration

The H9N2 avian influenza virus, A/TK/WI/66, was obtained from the National Veterinary Services Laboratory. Virus was propagated using MDCK cells in DMEM supplemented with 2% Bovine Serum Albumin and 1µg/mL TPCK trypsin (Pierce). All viral titers were calculated using the Reed-Muench calculation for $TCID_{50}$.

3.4. RESULTS

Sequence data demonstrate that variations between sampled genetic lines do exist, some encoding for amino acid substitutions (Table 3-2). Recent experiments have demonstrated that polymorphisms within the chicken Mx gene may, in fact, alter the antiviral response (Ko et al., 2004). As depicted in the schematic below Table 3-2, most of the observed variants occurred within encoding regions for protein motifs. Based upon these mutation loci, certain amino acid substitutions were predicted to affect the protein's antiviral capabilities as these motifs are implicated in the Mx antiviral response to influenza.

Using the trends observed from sequencing data, representative genetic lines were then tested *in vitro* for differences in response to avian influenza challenge using a one-step growth curve. Figure 3-1 depicts Mx expression through RT-PCR with correlating viral titer for each of the growth curve time points. GAPDH was used as the control for each sampled time point. No Mx products were observed prior to the 48-hour time point. At 48 hours post-inoculation, only lines 7 and 8 showed RT-PCR product.

3.5. DISCUSSION

The objective of this study was to explore the variability of the chicken Mx gene and discern if observed polymorphisms altered the response to influenza challenge. Although the chicken Mx protein was thought to be devoid of antiviral activity (Bernasconi et al., 1995), recent reports have suggested that polymorphisms do exist in this chicken Mx gene and that these polymorphisms could result in differences in the antiviral response (Ko et al,. 2002; Ko et al., 2004). Analysis of the sequenced lines in this study revealed several mutations, some of which encoded amino acid substitutions relative to the published sequence. Several of these changes were found in conserved motifs within Mx that have been described to be associated with its antiviral activity. The serine to asparagine substitution at amino acid 639, which was shown to confer an antiviral response to influenza challenge by Ko et al., 2002, was observed in two of the commercial genetic lines, suggesting that these two may be better equipped to respond to influenza infection.

Based on the sequencing results in Table 3-2, lines 3 and 7 would be expected to have a greater antiviral response to influenza infection, as each has the reportedly

antiviral amino acid at position 639, asparagine, whereas lines 6 and 8 have a serine at this locus. However, the viral titers and corresponding RT-PCR results do not echo this expectation. According to Figure 3-1, lines 3 and 6 produced the highest viral titers during the sampled time points of the one-step growth curve. The matching expression profiles for these lines show no Mx transcript for any of the time points. In contrast, at 48 hours after infection, lines 7 and 8 do appear to have transcribed Mx and also showed less viral output.

A possible explanation for these unexpected results lies in the observed sequence variation from the genomic DNA of line 6. The expression profile determined through RT-PCR shows that the full Mx transcript of line 6 is not expressed during the sampled time points. The single base change observed from the genomic sequence data (C to T) would result in the insertion of a stop codon in place of the glutamate normally at that position (amino acid 440). Even if chicken Mx is being transcribed in this genetic line, the resulting mRNA would be truncated, possibly nonfunctional, and would not amplify under the PCR conditions used. Although lines 6 and 8 are genetically related with similar genomic sequences, this truncated Mx may be responsible for the difference in response to infection of line 6 when compared with line 8. While no such truncation variation was observed in lines 3 and 7, the difference in their response may be due to regulation of Mx rather than the presence of a functional versus truncated transcript.

Although a specific amino acid substitution was shown to be the deciding factor in conferring the Mx antiviral activity against influenza (Ko et al., 2004), our results do not demonstrate the consistency of this change. With two of the tested lines possessing the variation for the reportedly antiviral residue, the expectation was that these would

respond similarly to the viral challenge. Yet, the results observed showed a closer relationship between the presence of an Mx transcript compared with response to infection rather than the sequence of that transcript at position 639.

Further studies are needed to determine the cause of this variation in Mx expression as well as to understand the biological significance of the observed amino acid changes to the cells' ability to respond to avian influenza infection both *in vitro* and *in vivo*.

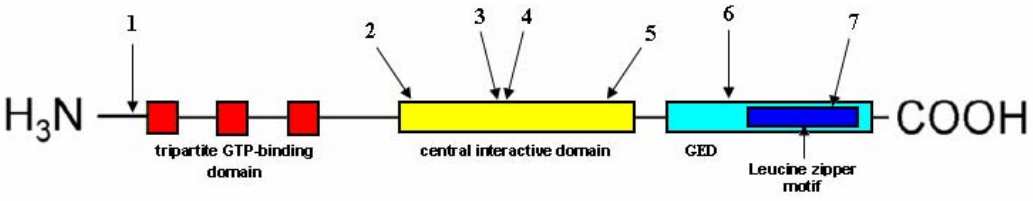
3.6. TABLES AND FIGURES

TABLE 3-1: Forward and reverse primers to amplify each of the 13 Mx exons.

Exon	Primer Sequence (5'→3')
1-Forward	GAGCAAGCCAGAAGAACAGC
1-Reverse	TGGAGAGTATCTGTGCCTTTCC
2-Forward	TTTGTTTGTTTCTGGAATCAGC
2-Reverse	TCAGGTGAAAACCTGAGAAGG
3-Forward	CCCAGTCCACTCACACAATG
3-Reverse	GAGCAAGGGCAATACGCTAC
4-Forward	TGCTTTCCTCTTTCCACCTC
4-Reverse	CCTTTGCATAAATTGGCAGA
5/6-Forward	GGAGTGGTCGCATCCTACAT
5/6-Reverse	CAATTCGTTGCAGAAGTCCA
7-Forward	GGCATACTTCCCACAAGCAG
7-Reverse	TGAAAGGAAGAAGGGTTGGA
8/9-Forward	CCAGCTGTGTTCAGCCTACC
8/9-Reverse	TCAGCTGCAAGTGATGGTTT
10-Forward	TTCTTTCTTAACCAGAAATTTATGAAG
10-Reverse	TGTGTGGCCTGTGAGACGTA
11-Forward	AGATGCCCAGCTATTTCAGC
11-Reverse	AGGTTATGGCTTGTCCCTCA
12-Forward	CAGAACTTGTTCTCTTCTTTTCCA
12-Reverse	CTGGTGTACTGTGTTGTAGTCTGC
13-Forward	AGCAACTCCATACCGTGTTTT
13-Reverse	TGCTAGAAAGCAAAAGCAGAAA

TABLE 3-2: Outline of observed Mx polymorphisms between sampled genetic lines. The variant numbers in the table correspond to the numbered arrows in the schematic drawing, indicating the relative locus of each polymorphism within the Mx protein. Variants 6 and 7 are both contained within the GTPase Effector Domain (GED), though variant 6 occurs just upstream of the leucine zipper motif within this domain.

VARIANT NUMBER	BASE VARIATION	POSITION (NUC/AA)+	AMINO ACID CHANGE	LINES WITH REPORTED BASE	LINES WITH VARIANT
1	A to G	338/113	Lys to Arg	3,7,8	6
2	A to G	982/327	Tyr to Cys	3,7	6,8
3	A to G	1307/436	Glu to Gly	7,8	6
4	C to T	1318/440	Glu to Stop	7,8	6
5	C to T	1667/556	Ala to Val	6,8	3,7
6	G to A	1916/639	Ser to Asn	6,8	3,7
7	G to A	2043/681	NO CHANGE	6,8	3,7



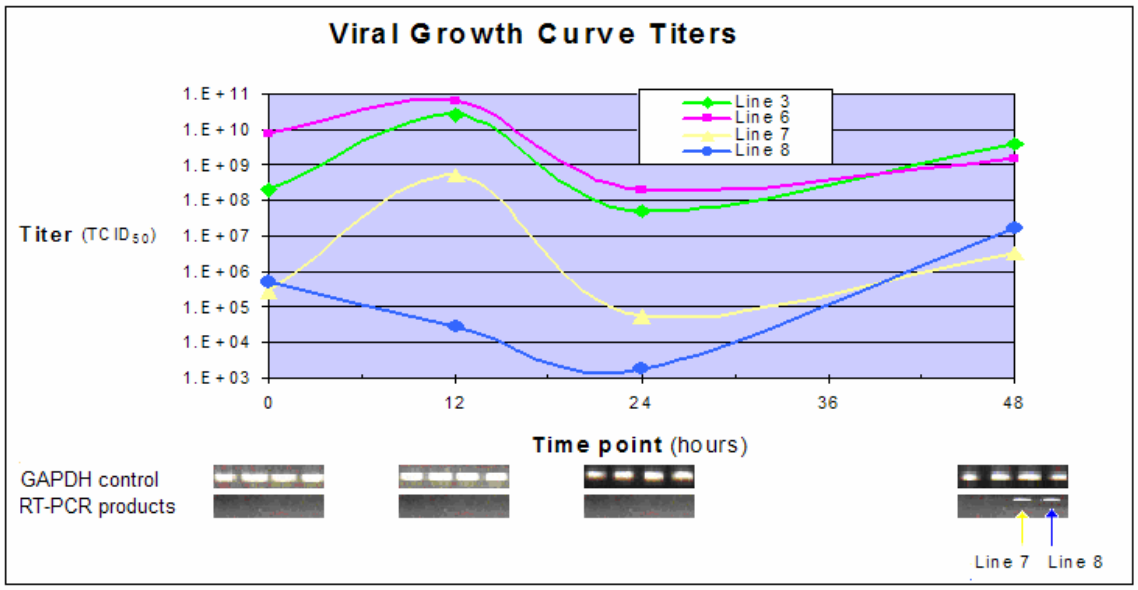


FIGURE 3-1: Influenza titers and corresponding RT-PCR products for each commercial genetic line (3, 6, 7, and 8). Primers to GAPDH were used as the control.

3.7. REFERENCES

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4.1. SUMMARY

Avian influenza outbreaks have resulted in major economic losses to the poultry industry in the past and continue to be a concern, particularly in light of the growing number of direct bird-to-human transmissions of avian strains, like the highly pathogenic H5N1 strain in Asia (Subbarao et al., 1998; Yuen et al., 1998). This, in conjunction with the discovery that the 1918 influenza outbreaks arose from a strain consisting solely of avian genes (Taubenberger et al., 2005; Tumpey et al., 2005), has raised concern that the next influenza pandemic may be caused by a highly pathogenic avian influenza (HPAI) strain. Since such strains can arise from low pathogenic (LPAI) strains, as suggested by sequencing studies of the HPAI viruses that decimated Pennsylvanian poultry flocks in 1983 (Bean et al., 1985; Eckroade, 1986), control of infection at the farm level becomes much more important.

As the host innate immune system is believed to play a key role in the pathology of influenza, augmenting the poultry innate resistance to influenza may be a possible method of disease control. The Mx protein, in particular, has been shown to possess innate antiviral activity to influenza. Having been described in multiple species including chickens (Pavlovic et al., 1993; Bernasconi et al., 1995), it is believed to be an important component to the antiviral response. Previous studies in mice have demonstrated the presence of multiple Mx genes (Lee, 2002) and that specific Mx mutations confer different susceptibility to influenza A (Horisberger et al., 1983; Garber et al., 1993).

This study began with a search for potential Mx family members within the chicken genome, which revealed three genes, on chromosomes 1, 8, and 17. Each of these possesses the three main domains present in all known Mx proteins: a GTP-binding

domain (Melén et al., 1992; Pitossi et al., 1993), central interactive domain (Kochs et al., 1998), and a GTPase effector domain (Melén et al., 1992; Schwemmle et al., 1995). However, further examination revealed some distinct differences that lead to the conclusion that these candidate genes are likely more similar to another GTPase, dynamin, than to Mx. Two dynamin-specific motifs, a plekstrin-homology (PH) domain and a proline-rich region, are present in the candidate genes on chromosomes 8 and 17. Since these motifs are not found in defined Mx proteins (van der Bliek, 1999; Haller, 2002), GTPase8 and GTPase17 were discarded as potential Mx family members. GTPase1, in contrast, does not appear to possess these dynamin-specific motifs. Therefore, RT-PCR was used to determine how expression of these genes is regulated. Since each of these is constitutively expressed and their expression does not appear to be influenced by influenza infection, we concluded that they are not interferon-regulated and hence, not Mx-like.

In light of these findings, the focus of the second part of the study was on sequence variations within chicken Mx and their effect on response to influenza challenge. The Mx GTPase motif near the amino terminus and the leucine zipper motif at the carboxy-terminus, which functions as the GTPase effector domain, have been implicated as important to the antiviral activity of Mx (Melén et al. 1992; Pitossi et al., 1993). With this in mind, polymorphisms within regions encoding these motifs were hypothesized to have a greater influence on the antiviral response to influenza.

The sequencing data revealed that two of the sampled lines possessed an amino acid substitution immediately upstream of the leucine zipper motif that was previously reported to confer antiviral activity against influenza by Ko et al. However, there did not

appear to be a correlation between presence of this polymorphism in the sequence and a lower viral output. Rather, the results observed showed a closer relationship between the presence of an Mx transcript and response to infection as opposed to the sequence of that transcript at position 639. Lines 3 and 7 both possess the reportedly antiviral asparagine while 6 and 8 have the serine, yet it was lines 7 and 8 that showed lower viral outputs. Although there does not appear to be a close relationship between the sequences of lines 7 and 8, these are also the only two that showed expression of the Mx transcript following viral challenge. While Ko et al. (2002) showed a specific amino acid substitution to be the deciding factor in conferring the Mx antiviral activity against influenza, our results do not demonstrate the consistency of this change. Therefore, it may be that a difference in regulation of the gene has a greater impact on its antiviral activity than an isolated amino acid change. Further studies are needed to appreciate the role of interferon in the activity of Mx. Given the results observed, the regulation of Mx expression should be examined for differences in the inducibility of the interferon-stimulated response element within the promoter (Schumacher et al., 1994).

In summary, chickens do appear to have only one Mx gene with the characteristic protein domains and interferon regulation. Variations within this gene have been shown to influence the antiviral capabilities of Mx, though the reported single amino acid substitution near the C-terminal (Ko et al., 2002; Ko et al., 2004) may not be the only factor in conferring this antiviral phenotype. Further studies of the regulation of Mx expression may explain these observed differences in Mx response to influenza challenge.

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