ABSTRACT

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Mx is an interferon-stimulated gene (ISG) that has been found in every vertebrate and associated with antiviral activity. It was originally thought that chicken Mx was inactive but there are a number of conflicting reports suggesting antiviral activity exists. Moreover, these reports focus on a single nucleotide polymorphism (SNP) within the coding region of Mx. This inconsistency may be due to regulatory elements within a cassette, other than the coding region, that affect gene expression such as the 3’UTR. Furthermore, a mutation or deletion within the 3’UTR results in a reduction or even loss of gene function. Likewise, it has recently been reported that several haplotypes exist within the Mx 3’UTR of commercial chickens. Therefore, the aims of this study were to identify what variations reside within the 3’UTR haplotypes, mapping their locations, and determining what, if any, impact they may have on protein expression. Six chicken Mx 3’UTR (Mx) haplotype sequences, galgal4 Mx3’UTR, chicken GAPDH 3’UTR, and a 3’UTR negative control (NoA) were cloned into a luciferase reporter and then transfected into LMH cells. The results of these experiments demonstrated a significant decrease in LMH cell expression following transfection with pTK-Gluc-2(H2) as compared to other haplotype constructs.
The Effects of Chicken Mx 3’UTR on Gene Expression

by
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DEDICATION

To my parents H.N. and G.N.; to my grandparents N.N., M.N., P.N., G.N.; for always believing, supporting, and guiding me throughout this journey.

To J.W. and L.W. for allowing me to stay in their home while I write.

To L.W.T., you are my voice of reason.
BIOGRAPHY

Colt Wesley Nash was born on January 27, 1988 and grew up in Salisbury, North Carolina.
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CHAPTER I: LITERATURE REVIEW

Innate Immunity

Innate immunity is comprised of germline encoded components that are readily available to fight foreign pathogens, but do not directly contribute to immunological memory (Murphy 2012). This germline-encoded response acts very quickly without the need for cellular expansion. By the same token, this is important to not only eliminate foreign invasion but also to slow down pathogen replication in order to mount an adaptive response.

The innate immune system deploys a variety of multi-functional cell types that work through the production of cytokines, phagocytosis of non-self particles, and bridging the gap between innate and adaptive immunity through processing and presenting antigen (Hoebe et al. 2004; Moretta et al. 2008; Murphy 2012). These cell types include antigen presenting cells (APC) such as dendritic cells, cytotoxic cells such as natural killer cells, and phagocytic cells such as macrophages (MΦ) (Akira et al. 2006; Sansonetti 2006). These cells are activated through an assortment of pattern recognition receptors (PRRs).

Pattern Recognition Receptors

Pattern Recognition Receptors (PRRs) are non-clonal, germline-encoded receptors that recognize common structures associated with pathogens (Janeway 1989; Shearer et al. 2010). These “common structures”, known as pathogen-associated molecular patterns (PAMPs) are highly conserved across many microbial species and, more often than not,
include essential elements that contribute to virulence such as peptidoglycan or surface receptor proteins (Shearer et al. 2010). When PAMPs are recognized by PRRs, a chain of signaling events occurs, resulting in the production of type I interferons (IFNs) and/or other cytokines (FIGURE 1.1).

**Viral Infection**

The life cycle of the typical viral infection occurs in seven steps (Howley et al. 2007). A virus must (1) attach to a cell through receptors in order to gain entry. Receptor binding affects both host and tissue tropism. Once bound to its respective ligand, the virus will then (2) enter its host cell through either receptor mediated endocytosis or membrane fusion (Howley et al. 2007). Following entry, the virus must (3) uncoat and release its genome into the cell. Once the genome has uncoated, the virus (4) begins to express its viral proteins. Typically viruses first express their nonstructural proteins that are responsible for taking over the host cell machinery. At that point, the virus (5) begins genome replication. After production of structural proteins an ample amount of new daughter genomes have been made, the virus then begins to (6) assemble these genomes into new viral particles. These new viral particles are now able to (7) egress from the cell and proceed with subsequent viral infection of surrounding cells (Howley et al. 2007). Each step in this cycle involves PAMPs that are unique to viral infection, that is to say detectable by PRRs.
Toll-like Receptors

Toll-like Receptors (TLRs) are transmembrane proteins expressed on cellular membranes. Each TLR has leucine rich repeats within its ectodomain which recognize PAMPs, a transmembrane domain, and an intracellular Toll-interleukin 1 (IL-1) receptor (TIR), located within the cytoplasm, which are important for signal transduction (Azan 1998). When bound to its respective ligand, TLRs dimerize and recruit a TIR containing adapter protein (Janeway and Medzhitov 2002; Dunne and O’Neill 2003; Akira and Takeda 2004).

In general, TLR1, TLR2, and TLR6 are cell surface receptors which form heterodimers and recognize diacyl and triacyl lipopeptides (FIGURE 1.2). TLR4 and TLR5 are also expressed on the cell surface, however TLR4 recognizes lipopolysaccharide (LPS) from gram-negative bacteria and TLR5 recognizes flagella (FIGURE 1.2). TLR3, TLR7, and TLR9 are expressed within the endosome (Kim et al. 2008). TLR3 senses viral double stranded RNA (dsRNA), whereas TLR7 senses single stranded RNA (ssRNA) and TLR9 senses CpG DNA (Poltorak 1998; Alexopoulou et al. 2001; Hemmi et al. 2002; Diebold et al. 2004; Heil et al. 2004; Lund et al. 2004) (FIGURE 1.2). All TLRs with the exception of TLR3 use the adaptor protein MyD88. TLR3 instead uses the adaptor protein TIR domain-containing adaptor-inducing interferon- β (TRIF), which is primarily responsible for the production of type I interferon (Han et al. 2004) (FIGURE 1.2). TLR4 has the unique ability to signal through both MyD88 and TRIF, however TLR4 uses TRAM to activate TRIF whereas TLR3 activates TRIF directly (Yamamoto et al. 2003; Takeda and Akira 2005).
The end result of these two different adapter proteins is quite different. MyD88 signaling is primarily responsible for the activation of NF-κb, which leads to the transcription of proinflammatory cytokines (Barton and Medzhitov 2003). TRIF signaling is capable of NF-κb activation, but primarily responsible for activation of the latent transcription factors interferon regulatory factor (IRF) 3 and 7 which up regulate type I interferons (Yamamoto et al. 2002; Hoebe et al. 2003; Oshiumi et al. 2003; Yamamoto et al. 2003; Fitzgerald and Chen 2006).

_Toll-like Receptor-3 (TLR-3)_

Toll-Like Receptor-3 is expressed in the endosome of multiple cell types including; MΦ, fibroblasts, intestinal epithelial cells, dendritic cells, B-cells, T-cells, and natural killer cells (Choe et al. 2005; Murphy 2012). Crystallographic analysis shows that TLR-3 binds directly to dsRNA (Choe et al. 2005; Liu et al. 2007). The ectodomain of TLR-3 has two contact sites for dsRNA: one on the amino acid terminus and a second near the membrane proximal carboxy-terminus. The two fold symmetry of dsRNA allows it to bind simultaneously to two TLR-3 ectodomains, inducing dimerization that brings its TIR domains closer together and activates intracellular signaling (Doyle et al. 2003; Murphy 2012). TLR-3 does not signal through the conventional MyD-88 dependent pathway, but instead signals through a mechanism relying solely on TRIF (Yamamoto et al. 2003; Han et al. 2004; Takeda and Akira 2004; Guillot et al. 2005).
**Toll-like Receptor-7 (TLR-7)**

TLR-7 is found in endosomes of multiple cell types including plasmacytoid dendritic cells, monocytes, NK cells, B-cells, epithelial cells, eosinophils, and MΦ. This TLR is able to recognize guanosine and uridine rich single stranded RNA (ssRNA) from viruses such as influenza viruses and flaviviruses (Xagorari and Chlichlia 2008; Blasius and Beutler 2010; Murphy 2012).

**Toll-Like Receptor-9 (TLR-9)**

TLR-9 is found in the endosomes of multiple cell types including plasmacytoid dendritic cells, monocytes, NK cells, B-cells, epithelial cells, eosinophils, and MΦ. Furthermore, upon detecting the presence of unmethylated CpG dinucleotides, TLR-9 signals through MyD88. Because many bacteria and viruses such as herpes simplex virus, do not have DNA methyltransferase which methylate cytosine in mammalian genomic DNA, methylation is a distinguishing factor between self and non-self and therefore serves as a PAMP (Lund et al. 2003; Murphy 2012). Although chickens lack TLR-9, they are able to signal through TLR-21, which shares the same functionality. (Brownlie et al. 2009; Keestra et al. 2010).

**RIG-I-Like**

RIG-I-like helicases (RLHs), RIG-I, MDA5, and LGP2 are proteins that sense viral dsRNA within the cytoplasm. RLHs have an RNA helicase-like domain that binds to RNA,
and, with the exception of LGP2, have two amino-terminal caspase recruitment domains (CARDs) that activate signaling after interacting with adaptor proteins (Andrejeva et al. 2004; Yoneyama et al. 2004; Hiscott et al. 2006).

*Retinoic-Acid-Inducible Gene-I (RIG-I)*

Retinoic-acid-inducible gene-I (RIG-I) plays a part in viral recognition and signaling in the innate immune system. RIG-I has been identified in all mammals and in a few avian species such as Ducks and Geese (Chen et al. 2013). However, RIG-I has not been identified in chickens (Karpala et al. 2011). RIG-I is responsible for recognition of various PAMPs associated with viral infection which include cytosolic recognition of viral ssRNA, viral dsRNA, and viral genomic DNA (Nikonov et al. 2013). Specifically, RIG-I recognizes 5’ppp and polyuridine motifs that contain C nucleotides (Kato et al. 2006; Loo and Gale 2011).

*Melanoma Differentiation-Associated gene 5 (MDA5)*

Melanoma differentiation-associated gene 5 (MDA5) is a key cytosolic PRR that detects viral nucleic acids and ultimately leads to the production of interferon. Types of activating nucleic acids include dsRNA and the synthetic dsRNA analogy poly(I:C) (Chen et al. 2013). Although MDA5 and RIG-I both detect dsRNA, poly(I:C) sequences larger than 1kb are primarily recognized by MDA5, while fragments smaller than 1kb are generally recognized by RIG-I.
Laboratory of Genetics and Physiology 2 (LGP2)

LGP2 is similar to RIG-I in that its primary ligand is ds and ssRNA, however it has a greater affinity for dsRNA than that of RIG-I. LGP2 lacks a CARD and has been shown to be a negative regulator of RLR mediated signaling (Satoh et al. 2010; Chen et al. 2013).

**Interferon**

There are three types of IFNs: type I, II, and III (DeWeerd et al. 2007). IFNs are important to host immunity due to their ability to suppress or disrupt the virus life cycle (Samuel 2001). This is achieved through the up-regulation of many different antiviral genes. Nearly every antiviral response results in the production of IFNs. The IFN receptor is comprised of two subunits and effectively dimerize when bound to IFN. Once this dimerization occurs, it elicits the activation of Janus-activated kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) (Uematsu and Akira 2007; Sadler and Williams 2008). This in turn leads to the phosphorylation and activation of signal transducer of activator of 1 and 2 (STAT1 and STAT2) which then associates with IRF9 to form a trimeric complex known as the IFN-stimulated gene factor 3 (ISGF3). ISGF3 then translocates into the nucleus where the IRF9 subunit binds to the Interferon Stimulated Regulatory Element (ISRE) and starts transcription (Schindler et al. 2007). Type II signals through a (GAF) domain consisting of cGMP-specific phosphodiesterase, adenylyl cyclases, and FhiA (Haque et al. 1995; Stark et al. 1998).
Type I IFN

The Type I IFNs are comprised of IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω. However the α and β are the most commonly expressed (Siegal 1999). Most mammalian and avian species have one copy of the IFN-β gene and multiple copies of the IFN-α gene (Nanda et al. 1998; Hardy et al. 2004; Pestka et al. 2004). Type I IFNs are capable of inducing major histocompatibility complex class I (MHC-I). Therefore the type I IFN receptor and ligand are expressed by pretty much every type of cell, although, plasmacytoid dendritic cells are the primary source of IFN-α and β production (Siegal 1999). By exhibiting differential tissue expression, type I IFNs are able to fulfill different functional roles such as anti-viral and anti-proliferative activity (Jaitin et al. 2006; Kalie et al. 2008; Moraga et al. 2009).

Type II IFN

Type II IFNs are only comprised of IFN-γ and are produced by NK cells and T-Cells. The receptor for IFN-γ is expressed on all cell types. Furthermore, IFN-γ is involved in the regulation of nearly all phases of immune and inflammatory responses (Romagnani 1997; Murphy 2012). This includes the activation, growth and differentiation of T-cells, B-cells, MΦ, and NK cells. Moreover, IFN-γ enhances MHC expression on antigen-presenting cells, and is characteristic of Th1 differentiation (Romagnani 1997; Murphy 2012).
Type III IFN

Type III IFN is the newest member of the IFN family and in humans it is comprised of IFN-λ1, IFN-λ2, and IFN-λ3 (Kotenko et al. 2003; Sheppard et al. 2003). However, in chickens, only one type III IFN gene has been identified. Type III IFNs have a level of antiviral activity comparable to type I IFNs, although the chicken type III IFN activity is not as prominent (Donnelly and Kotenko 2010; Kotenko 2011). Human type III IFN receptors are expressed by epithelial cells even though pretty much all cells can produce type III IFN.

Interferon regulated antiviral mediators

The result of interferon signaling leads to the up-regulation of more than 100 different IFN stimulated genes (ISGs). The best characterized, however, are 2’-5’ oligoadenylate synthetases (OAS), serine/threonine protein kinase R (PKR), and myxovirus resistance gene (MX) (Samuel 2001; García-Sastre and Biron 2006; Stetson and Medzhitov 2006).

Each of these genes target different aspects of the virus life cycle. OAS synthesizes 2’-5’ oligoadenylates which in turn activate the latent RNase (RNase L) (Samuel 2001; Schoggins and Rice 2011). RNase L then degrades RNA in a cell in an attempt to shut off both virus replication and expression of viral genes (Samuel 2001). PKR is activated by binding double stranded RNA, leading to phosphorylation of elongation initiation factor α (EIF2α) which inhibits its ability to initiate protein translation, blocking the cell from producing viral proteins (Ko et al. 2004; Sadler and Williams 2007). Finally, Mx is a large guanosine triphosphatase (GTPase) whose specific antiviral function is still not understood,
but it appears to form oligomers around viral ribonucleoproteins complexes, blocking their ability to translocate to sites of replication (Haller and Kochs 2002; Verhelst et al. 2013).

**Myxovirus resistance (Mx) gene**

*Gene History*

The first discovery of an Mx gene (Mx1) was first reported in 1963 when mice were shown to have resistance to influenza virus infection when inoculated at various dilutions (Lindenmann et al. 1963). This event played an important role in its naming in that the “M” and “X” comes from influenza viral family *Orthomyxoviridae* (Haller et al. 2009; Verhelst et al. 2013). A second Mx gene (Mx2) was later discovered through cDNA libraries made from mRNA transcripts isolated from mouse cells treated with IFN (Staeheli and Sutcliffe 1988). Mx is an interferon inducible gene found in all vertebrate genomes and is almost exclusively responsive to RNA viruses (TABLE 1.2).

*Mx Protein Characterization*

The Mx protein is a dynamin-like large GTPase (Haller and Kochs 2002; Praefcke and McMahon 2004; Verhelst et al. 2013). This family of proteins is characterized by an N-terminal GTPase domain, a middle domain, and a C-terminal GTPase effector domain. Nevertheless, a distinguishing factor that separates Mx proteins from other dynamins is their low affinity to target cell membranes and the lack of pleckstrin homology domain (Praefcke and McMahon 2004; Verhelst et al. 2013). The most conserved domain of Mx proteins are
the GTPases which consists of a tripartite GTP-binding motif (GDXXSGKS, DLPG, and TKPD) and a dynamin signature (LPRXXGXXTR) (Song et al. 2004).

The Mx protein GTPase domain is necessary for biological activity (Pitossi et al. 1993). The turkey Mx protein excluded, all Mx protein dynamin signatures contain the penultimate threonine residue, which directs the Magnesium ions necessary to give the GTPase domain its functionality (Pitossi et al. 1993; Melén and Julkunen 1994; Song et al. 2004; Verhelst et al. 2013).

The middle domain and effector domain, although less conserved than the GTPase domain, are responsible for the structure and assembly of Mx proteins (Koch et al. 1998). Essentially, the middle domain’s primary structural role is to facilitate oligomerization, while the C-terminal GTPase effector domain’s primary functions are viral target recognition and self-assembly (Schwemmle et al. 1995; Gao et al. 2010; Mitchell et al. 2013).

Avian Mx

Avian species have one Mx gene, and so far there are no conclusive reports of any of the avian Mx genes having antiviral activity. Two groups have reported that some naturally occurring variants of Mx could demonstrate antiviral activity when expressed recombinantly in mouse cells. From these studies it was reported that Mx variants encoding an asparagaine instead of a serine at position 631 may have inhibited virus replication (Ko et al. 2002; Ko et al. 2004; Watanabe 2007). In addition to these findings, a study by Ewald et al. produced similar results when performed in vivo (Ewald et al. 2011). Conversely, studies by Schusser
et al. and Benfield et al. were unable to produce data supporting antiviral activity related to Asn631 or Ser631 in primary chicken cells or chickens (Benfield et al. 2008; Schusser et al. 2011). Together, these results imply that other factors are necessary for Mx antiviral function. Furthermore, multiple SNPs have been identified throughout coding sequence, promoter, 5’ untranslated, and 3’ untranslated regions (Li et al.; Fulton et al. 2014). Therefore, Asn631 provides only a limited amount of insight into the regulation of gene expression.

*Regulation of Mx expression*

PRR signaling leads to IFN production, which leads to polymerase binding to the Interferon Stimulated Response Element (ISRE), causing an up-regulation in Mx transcription (Schumacher et al. 1994).

After IFN has induced the activation of a promoter, (e.g., Mx gene) RNA is transcribed within the nucleus of a cell. Once transcribed, spliceosomes remove introns resulting in exons being moved closer together. Before the mRNA can localize from the nucleus into the cytoplasm, a series of adenosine nucleotides (poly-A tail) are covalently bonded to the 3’ end of the mRNA.

Once cytoplasmic localization occurs, degradation begins almost immediately as the poly-A tail is slowly shortened. The length of this poly-A tail can play a big role in the ability of the mRNA to get translated into protein. This is due to the poly-A tail having an association with the mRNA 5’ cap which together help recruit ribosomes for protein
It has been shown that not only are the length and composition of the Poly-A tail important, but also having a distinct signal sequence called the poly-A signal (PAS) (AATAA) is necessary for proper functionality. Seeing how important these structures are to functional gene expression, it is not hard to see that they are highly conserved across all eukaryotic mRNA within the 3’ untranslated region (3’-UTR) of mRNA (Conne et al. 2000; Chatterjee and Pal 2009).

3’UTR

The 3’UTR of mRNA is characterized as everything downstream of the stop codon. This region is responsible for poly-A tail formation, which is important for efficient translation and protection from cytoplasmic degradation (Haimovich et al. 2013). The PAS is a defining feature associated with a 3’UTR and is essential for proper gene function (Lagnado et al. 1994; Levitt et al. 1989). Limited information is available on chicken Mx 3’UTR, and can be summed up in one report from Li et al. that highlights the presence of a 31bp insertion-deletion (Li et al. 2007). Defects within the PAS have been associated with disease due to gene inactivation. Non-functional Poly-A tails, through mutations in the FOXP3 gene Poly-A signal, have been shown to cause a disease called immune dysfunction, poly-endocrinopathy and enteropathy, X-linked (IPEX) which results in the inability to produce $T_{reg}$-cells leading to autoimmunity (Chatterjee and Pal 2009).

Given 3’UTRs role in gene expression and the impact of a PAS on the poly-A tail, it is possible that the lack of Mx functionality was due to the 3’UTR.
CHAPTER II: 3’-UTR HAPLOTYPE EFFECT ON GENE EXPRESSION

INTRODUCTION

Myxovirus-resistance (Mx) genes are interferon-stimulated genes (ISG) found in every vertebrate (Haller and Kochs 2002). The Mx protein is characterized as a dynamin-like large GTPase, which promotes resistance to viral infections (Haller and Kochs 2002); however, not all Mx genes have demonstrated antiviral activity. Chickens, for example, have one copy of the Mx gene but most reports suggest it has no antiviral activity. There are a limited number of reports; however, that indicate a single nucleotide polymorphism (SNP) in the chicken Mx gene may confer anti-viral resistance (Ko et al. 2002; Ko et al. 2004).

Ko et al., sequenced the Mx gene from multiple lines of chickens, identifying multiple SNPs, and determined that one specific SNP, Ser to Asn at amino acid 631 was associated with increased antiviral activity when expressed in virally infected mouse 3T3 cells (Ko et al. 2002; Ko et al. 2004). In addition to these findings, a study by Ewald et al. suggested chickens expressing these different alleles had different levels of in vivo resistance when infected with highly pathogenic avian influenza (Ewald et al. 2011). Despite this, two separate studies performed by Sironi et al. and Schusser et al. reported that this SNP has no affect on the antiviral activity of Mx (Sironi et al. 2008; Schusser et al. 2011). These conflicting reports suggest that this one SNP may not be the only contributing factor to Mx antiviral activity. Furthermore, multiple SNPs have been identified throughout the coding
sequence (CDS), promoter, 5’ untranslated, and 3’ untranslated regions (Li et al.; Fulton et al. 2014).

It is known that the 3’ untranslated region has an affect on gene expression by regulating message stability and protein translation (Grzybowska et al. 2001; Hesketh 2005). Formation of a poly-A tail is dependent on elements within the 3’UTR such as the polyadenylation signal (PAS) A(A/T)TAAA, which serves as a reference point for cleavage (Levitt et al. 1989). The downstream (T- or GT-rich) elements, which bind to the PAS in order to aid in poly-A polymerase binding and cleavage (Levitt et al. 1989). The upstream elements (TGTA), which have a putative role in defining the location of the polyadenylation site (Xie et al. 2005). The cleavage site (CA) (Figure 2.2A), which is the point where poly-A polymerase cuts the 3’UTR, is the location of poly-A tail extension. A mutation or deletion in any of these structures negatively affects polyadenylation efficiency, which results in a reduction or even loss of gene function (Bennett et al. 2001; Fox and Wickens 1990).

Previous studies have suggested there the chicken Mx 3’UTR can have a 30-40 nt insertion or deletion (Li et al. 2009). We have analyzed the Mx 3’UTR from multiple lines of commercial lines and identified several haplotypes. Therefore, the aims of this study was to identify what variations reside within the 3’UTR haplotypes, mapping out there location, and determining what, if any, impact they may have on protein expression.
MATERIALS AND METHODS

Sequences analysis

Six chicken Mx 3’UTR (Mx) haplotype sequences were obtained from commercial layer-type chickens as previously described (Fulton et al. 2014), galgal4 Mx3’UTR (Ensembl_GCA_000002315.2), and chicken GAPDH 3’UTR (J00850), and alignments performed using Vector NTI (Invitrogen).

Plasmids and expression system construction

To develop assays to test for the effect of sequence differences in the chicken 3’UTR, existing reporter constructs were modified. Briefly, 2µg of the expression vector pCMV-Gluc-2 (New England Biolabs, NEB) was digested with 2 units of BglII and 2 units of HindIII (Promega). In parallel reactions, 2µg of pRL-TK (Promega) was digested with the same enzymes. Each digested plasmid was then separated by electrophoresis using a 1% agarose gel and visualized with ethidium bromide. The pGluc-2 fragment, missing the cytomegalovirus (CMV) promoter, and the herpes simplex virus-thymidine kinase (HSV-TK) promoter fragment, from pRL-TK, were extracted from the gel and purified using PCR and Gel clean up columns (Macherey-Nagel, MN). These two fragments were then combined at a 1:3 ratio of vector to insert and ligated to form a new construct pTK-Gluc-2 using the Instant Sticky-end Ligase Master Mix (NEB), and transformed into Stellar Competent cells
(Clontech). The resulting clones were then screened by enzyme digestion using EcoRI (NEB) (Figure 2.1B) and the new construct confirmed by sequencing (Genewiz).

pTK-Gluc-2 was linearized by inverse PCR (Q5 High-Fidelity DNA Polymerase, NEB) using one primer downstream of the poly-A signal site (ChMx 3’UTR FWD 2 No PolyA, 5’-GCTGGGGCTCTAGGGGATC-3’) and a second primer immediately upstream of the Gluc stop codon (ChMx 3’UTR Gluc 2 Rev (1-REV) 5’-
GTCACCACCCTCCCCCTTT-3’) (Integrated DNA Technologies, IDT). The resulting PCR product eliminated the synthetic poly-A signal encoded in the plasmid and either ligated (Blunt end ligase mastermix, NEB) without an insert to produce a construct with no 3’UTR pTK-Gluc-2(NoA); or the 3’UTR from chMx haplotype 2 (H2) (pTK-Gluc-2(H2)) or GAPDH (pTK-Gluc-2(GAPDH)) were fused together (In-Fusion Cloning System, Clontech).

The sequences for the H2 and GAPDH inserts were synthesized as double stranded DNA (gBlock, IDT) to encode 129 bases of 3’ end of the CDS fused to the end of Gluc, the entire UTR found in the mRNA, and 76 bases of downstream genomic sequence along with 18 or 21nt at the 5’ and 3’ ends, respectively, for homologous fusion to the plasmid.

The H2 construct was then modified to produce additional constructs containing the other chMx 3’UTR haplotypes. Briefly, pTK-Gluc-2(H2) was linearized by inverse PCR (var-reg H2 fwd, 5-GGGAGCCTTCAGCTG-3’ and var-reg H2 rev 5’-
ACTAATTCTGCTGGTCAG-3’, IDT) and inserts synthesized (IDT) to produce pTK-Gluc-2(H1), pTK-Gluc-2(H3), pTK-Gluc-2(H4), pTK-Gluc-2(H5), pTK-Gluc-2(H6), and pTK-Gluc-2(Gal) using In-Fusion (Clontech). All constructs were confirmed by sequence analysis.
Effect of chMx 3’UTR on Gene Expression

LMH cells were propagated in Dulbecco’s Modified Eagle Media (DMEM, Corning) supplemented with 10% FBS (Atlanta Biologicals) and 1mM L-glutamine (Sigma) and incubated at 37°C with 6% CO₂. For expression analysis, 1.25x10⁵ LMH cells were seeded per well, in quadruplicate wells of a 24-well cell culture plate (Cellstar), and cultured overnight to a density of 70% confluency. Cells were then transfected with reporter construct (pTK-Gluc-2) and/or control plasmid (pSV40-Cluc, NEB) using 25µl X-fect (Clontech) following the manufacturer’s instructions. Cells were incubated with DNA and transection reagent at 37°C and 6% CO₂ for 4hrs. The medium was then removed, replaced with fresh medium, and the cells incubated for 48hrs at 37°C and 6% CO₂. At the end of the 48hr incubation, the supernatants were collected from each well, centrifuged at 16,000 x g for 30 seconds, and the clarified supernatants transferred to new tubes and stored at -80°C.

Gluc and Cluc assay reagents (NEB) were then prepared following the manufacturer’s instructions. Briefly, replicate 20µl aliquots of supernatant were added to an opaque flat bottom 96-well plate (Corning) and 50µl of either Gluc or Cluc assay reagent (NEB) was added and the relative light units (RLUs) measured using a luminometer (Berthold).

Plasmid Linearization

To eliminate the possibility of downstream elements within the vector affecting luciferase expression pTK-Gluc-2 and pTK-Gluc(NoA) were linearized prior to transfection.
pTK-Gluc-2 was linearized by digesting 5µg of plasmid with 5 units of XbaI (Promega), cleaving the plasmid 5nt downstream of the synthetic poly-A signal sequence, and 63nt downstream of the Gluc stop site. The removal of the synthetic poly-A site to generate the NoA construct also removed the XbaI site; therefore, the plasmid was mutated to introduce a BmtI site using inverse PCR (Mx Trans linear BmtI fwd: 5’-
/5phos/AGCGATTAGGGTGATGGTTCACGT-3’ and Mx Trans linear BmtI Rev: 5’-
/5Phos/AGCTAATGCGCCGCTACAGG-3’), and Q5 High Fidelity Polymerase (NEB). The resulting PCR product was blunt end ligated by Blunt/TA Ligase Master Mix (NEB), and mutated clones identified by restriction digestion using BmtI and confirmed by sequence analysis. 5µg of the resulting construct was then linearized using 5 units of BmtI, which cleaves pTK-Gluc-2(NoA) 97nt downstream of the Gluc stop site. In addition 5µg of pSV40-Cluc was digested with 5 units of SalI (NEB) to serve as matched linearized transfection control plasmid. 5µg of pTK-Gluc-2, pTK-Gluc-2(NoA), and pSV40-Cluc were each mock digested in control reactions, all six digestion reactions (cut and uncut) where then purified using PCR Clean-Up kit (Clontech), transfected into LMH cells, and luciferase activity measured as described above.

**Detection of polyadenylated Gluc in transfected LMH cells**

Total RNA was isolated from transfected LMH cells using the RNA Mini Prep kits (MN). 250ng of DNA-free total RNA was reverse transcribed using Oligo-dT primers following the standard protocol for ThermoScript II (Invitrogen). Gluc cDNA was detected
by PCR (Forward 1-Colt, 5’-ATGGGAGTCAAAGTTCTGTTTGCCCTG-3’, Middle-Fwd-Colt, 5’-TGCACCAGGGGCTGTCTGATC-3’, and Reverse 1-Colt, 5’-AGGTCAGAACAATCGACGTTGGC-3’) and the GoTaq Master Mix (Promega). Amplicons were visualized by agarose gel electrophoresis and ethidium bromide staining.

**Statistical Analysis**

Data was analyzed by multivariable one-way ANOVA using Prism 6 (GraphPad Software).

**RESULTS**

**Variability in chMx 3’UTR sequence**

Previous reports have suggested that the 3’UTR of the chicken Mx mRNA can vary by as much as 31nt in length (Li et al. 2007). Examination of this region of the Mx suggests that the polymorphic region corresponds to the location where the poly-A signal sequence is located (FIGURE 2.2B). To better understand how variation in this region may affect Mx gene expression, genomic sequence from the last 129nt of the Mx coding sequence through to 76nt downstream of the end of 3’ UTR was aligned (FIGURE 2.2B). This analysis demonstrates this region of the Mx gene is nearly identical, with the exception of a region between nt 221 and 264 of the 3’UTR (FIGURE 2.2B). Variation in this region appears to result in at least seven distinct haplotypes, with at least three that appear to be missing the standard A(A/T)TAAA poly-A signal sequence motif (FIGURE 2.2A). By comparison, the
3’UTR of the GAPDH gene (FIGURE 2.2A and C) contains the prototypical signal sequences thought to be required for efficient polymerization of the poly-A tail and subsequent protein translation. The only clear representation of a polyadenylation factor existing within Mx 3’UTR are the DSE (Edmonds 2004; Hollerer et al. 2014; Jurado et al. 2014).

**Validation of pTK-Gluc-2 plasmid and expression system optimization**

Following the construction of the pTK-Gluc-2 plasmid (FIGURE 2.1), LMH cells were transfected with this construct or the pSV40-Cluc control plasmid to determine the optimal concentrations of the reporter and control plasmid needed for consistent luciferase detection (FIGURE 2.3). Initial experiments investigated the amount of luciferase activity with increasing plasmid for each construct (FIGURE 2.3A). From the results of these experiments we determined 0.5µg of plasmid DNA produced the greatest amount of luciferase activity for both plasmids.

A similar dose response was observed in subsequent experiments when equal amounts of both plasmids were co-transfected into LMH cells (FIGURE 2.3B). The results of these experiments demonstrated the greatest level of Gluc and Cluc activity was detected in cells co-transfected with 0.5µg of plasmid both pTK-Gluc-2 and pSV40-Cluc. Finally, the amount of Gluc and Cluc activity was measured following transfection with different amounts of plasmid DNA (FIGURE 2.3C). These results demonstrated the highest activity when 0.5µg of pTK-Gluc-2 and 1µg of pSV40-Cluc were used. From these co-transfection
experiments, the ratio of Gluc to Cluc activity was calculated (FIGURE 2.3D) to identify the plasmid combination that provided the greatest activity of each luciferase as well as the greatest Gluc to Cluc ratio.

**Mx 3’UTR variants affect expression**

A panel of pTK-Gluc-2 plasmids was produced encoding each of the 6 3’UTR haplotypes found in commercial layer-type chickens and the galgal4 reference sequence (FIGURE 2.2B). These constructs, pTK-Gluc-2(H1), pTK-Gluc-2(H2), pTK-Gluc-2(H3), pTK-Gluc-2(H4), pTK-Gluc-2(H5), pTK-Gluc-2(H6), pTK-Gluc-2(NoA), and pTK-Gluc-2(Gal), were transfected into LMH cells with pSV40-Cluc control and assayed for differences in the relative expression of Gluc (FIGURE 2.4). The results of these experiments demonstrated a significant decrease in LMH cells expression following transfection with pTK-Gluc-2(H2) as compared to other haplotype constructs. Interestingly, there was no significant change in expression between pTK-Gluc-2 and the negative control, pTK-Gluc-2(NoA).

**Linearized vs. Circular plasmid transfection**

Because LMH cells transfected with the NoA negative control construct were found to have luciferase expression (FIGURE 2.5), despite having no plasmid encoded poly-A signal, it was thought that the NoA RNA transcripts maybe polyadenylated via signal elements in the downstream neomycin resistance cassette. To address this, the pTK-Gluc-2
and pTK-Gluc-2(NoA) constructs were linearized downstream of the Gluc open reading frame (FIGURE 2.5A) to ensure termination of the RNA transcript. Transfections were then performed using both linear and circular plasmids. When the LHM cells were assayed for relative luciferase activity, both pTK-Gluc-2 and pTK-Gluc-2(NoA) demonstrated Gluc activity (FIGURE 2.5B). pTK-Gluc-2(NoA) was found to have lower relative Gluc activity than pTK-Gluc-2, and interestingly cells transfected with linearized plasmid were found to have more relative Gluc activity than their circularized matched control (FIGURE 2.5B).

**Confirming Gluc(NoA) is Polyadenylated**

To confirm the RLU detected in the pTK-Gluc-2(NoA) transfected cells were from polyadenylated Gluc, mRNA extracted from LMH cells was reverse transcribed using oligo-dT to produce a cDNA library; and PCR was performed with cDNA templates from linear TK-Gluc-2 (TK-L), linear TK-Gluc-2(NoA) (NoA-L), circular TK-Gluc-2 TK-C), and circular TK-Gluc-2(NoA) (NoA-C), using two different primer sets (FIGURE 2.5C). F1C-R1C produces a 467bp product whereas MFC-R1C produces a 263bp product (FIGURES 2.5A and C). Each of the oligo-dT primed libraries produced a PCR product of the expected size, while no product was observed in the no RT control (FIGURE 2.5C).

**DISCUSSION**

Mx has been associated with enhanced resistance to viral infection in mice as well as many other species (Dittmann et al. 2008; Horisberger and Hochkeppel 1985). Historically,
the chicken Mx was commonly thought to be non functional. This idea was challenged by Ko et al., who examined a variety of different chicken breeds, and identified multiple Mx alleles. Though systematic in vitro testing of the various SNPs they reported that some alleles have antiviral activity. Furthermore they describe that a single polymorphism (Ser631 to Asn631) could confer antiviral activity (Ko et al. 2002; Ko et al. 2004). In the decade since this initial discovery, Mx has become the focus of several research groups; moreover the idea of “functionality” is questionable due to very disparate results. Another group has suggested that chickens with single nucleotide polymorphisms, identified by Ko et al. in 2002, are more resistant to viral infections, specifically influenza, than other chickens (Ewald et al. 2011); indeed, the majority of reports suggest that the chicken Mx lacks antiviral activity (Benfield et al. 2008; Sironi et al. 2008; Benfield et al. 2010; Schusser et al. 2011; Wang et al. 2012).

An explanation for these conflicting findings remains ambiguous. One possibility for this discrepancy may be due to a limited understanding of the entire Mx gene. Notably, the sequence analysis described here, has shown a potential for regions outside the CDS, especially within the 3’UTR. On a percentage bases, the 3’UTR has as many changes/differences as any other region of the Mx gene. Furthermore, when one examines where in the in 3’UTR these changes are concentrated, it does not seem unreasonable to expect one or more of the different 3’UTR haplotypes to affect gene expression.

The 3’UTR provides many of the primary elements required for efficient translation. These elements include PAS, that is generally 10-30nt upstream of the CA site, the point at which the 3’UTR is severed and polyadenylation polymerase (PAP) begins extending the
poly-A tail. A downstream element, characterized as a T- or GT- rich region that aide in PAP binding and cleavage by annealing to the PAS, exits 10-50nt downstream of the cleavage site. Upstream elements, (TGTA) range between 40-50nt upstream of the cleavage site (CA) (Darmon 2012; Xie et al. 2005), have a putative role in defining the PAS location. These elements are without a doubt the most common, however, on a completely different level, there exists variations among the PAS, which is referred to as alternative cleavage and polyadenylation sites (APA). These primarily consist of multiple PAS that provide “options” for polyadenylation signaling (Giammartino et al. 2011), and the presence of cytoplasmic polyadenylation element (CPE) which produce a longer poly-A tail (FIGURE 2.2A) (Hesketh 2005).

Comparison of the relative luciferase activity detected in cells transfected with the different Mx 3’UTR constructs demonstrated that H2 resulted in significantly less activity than the other constructs. Additionally, the expression values for SV40-Cluc between wells and plates were non-significant. This suggests that the H2 construct is lacking important elements, such as a PAS, required for efficient translation. Based on examination of the Mx 3UTR sequences, H3, H5, and H6 each appear to have the prototypical poly-A signal sequence; while Gal4, H1, H2, and H4 do not. These 4 would be expected to show a reduction in gene expression. So it was interesting, that only H2 was significantly lower than the others. It is possible that Gal4, H4, and H1 have sequences very similar to a PAS. Moreover, this similarity is characterized by a one bp deference in not only Gal4 and H4, both having sequence TTAATT (nt374-379), but also H1, having the sequence TTTAAA
(nt375-380) (FIGURE 2.2B). To illustrate this, Gal4 and H4 are one bp from TTATTT (Proudfoot 2011) and H1 is one bp away from TATAAA (Edmonds 2004; Elkon et al. 2013; Jurado et al. 2015).

The pTK-gGluc-2(NoA), was expected to serve as the negative control, and produce essentially no RLU. The fact that cells transfected with this construct produced as much or more than the other constructs remains puzzling. Since this construct had the poly-A signal removed, we were concerned that the RNA polymerase was producing transcripts that included the downstream antibiotic resistance gene and its functional 3’UTR was allowing for expression of the Gluc ORF. To try and account for this, the plasmid was linearized downstream of Gluc; however, this construct also produced Gluc luciferase activity (FIGURE 2.5B ). Moreover, to ensure our constructs were polyadenylated, we performed Olio-dT primed RT-PCR (FIGURE 2.5C)

Regardless of the issues with the NoA negative control, it is still interesting that the H2 construct consistently produced less luciferase activity than any of the other 3’UTR tested. This suggests that the differences in the Mx 3’UTR may affect the level of Mx gene expression and therefore its potential antiviral function. It is still unclear as to what it is about H2, specifically that is affecting function when the NoA control is still functional. More studies are needed to fully understand how the differences in this 3’UTR affect expression, as well as the frequency by which this UTR is found in poultry.

Data presented here is the first examination of variation within the 3’UTR of the Mx gene (chicken or otherwise) and its functional significance. The results of these studies
suggest that genetic variation in Mx; with consequences for Mx expression and activity are not just restricted to non-synonymous changes in the coding region.

CHAPTER III: CONCLUSION

This study was conducted in order to explore other possible factors contributing to chicken Mx gene expression. Given the antiviral potential of chicken Mx in poultry production, research focused on identifying factors related to a lack of gene function is important. In the decade since Ko et al. reported a SNP within the chicken Mx CDS was responsible for antiviral activity, there has been a lot of interest focused on this topic. In contrast, a report by Sironi et al. refuted the data published by Ko et al., concluding that a SNP does not affect Mx antiviral function. Together, this suggests that regulation of antiviral activity involves more components than just a SNP found within the Mx CDS. This study sought to answer what impact, if any, these variations have on chicken Mx expression.

Research synopsis

Our first challenge was developing an assay to study the effects of 3’UTR polymorphisms. To my knowledge, no such expression model exists. Consequently, we developed a unique reporter assay to model 3’UTR activity. The foundation for this assay is the Gluc luciferase reporter under the control of the CMV promoter. The CMV promoter produced RLU values above the linear range for our luminometer. For this reason, we decided to substitute the CMV promoter for the less active HSV-TK promoter.
Before this project could continue further, it was necessary to show that the pTK-Gluc-2 could produce a functional luciferase enzyme activity. LMH cell were transfected with pTK-Gluc; which resulted in luciferase activity, and confirmed the functionality of our recombinant reporter system. The assay was then optimized based on cell number, transfection reagent and amounts of pTK-Gluc-2 and control plasmid SV40-Cluc. Once the validated we began preparing the 3’UTR haplotype constructs to be analyzed.

We then compared gene expression between each of the six ChMx 3’UTR haplotypes. Chicken 3’UTR positive control (pTK-Gluc-2(GAPDH)), positive control for the expression vector (pTK-Gluc2), and a negative vector control (pTK-Gluc-2(NoA)), lacking the 3’UTR, were also included. Results from this experiment showed that expression levels from H2 were lower that all other constructs. However, because expression in NoA had not been eliminated, we were unable to fully understand the cause for results seen in H2.

**pTK-Gluc-2(NoA)**

Protein expression without a 3’UTR, observed in the linear and circular (NoA) constructs, is one of the most noteworthy problems we encountered. Based on RT-PCR analysis using oligo-dT primed cDNA from the (NoA) transfected cells, Gluc mRNA was poly-adenylated, but where the signal for this is coming from is unknown. Analysis of the plasmid sequence between the end of the Gluc ORF and the BmtI site shows no classic PAS. In order to solve this problem I would first need to locate the putative poly-A signal within (NoA). To do this would require identification of the mRNA cleave site using 3’RACE.
Next, I would use inverse PCR to produce linearized constructs that terminate closer and closer to the end of the CDS (upstream of the cleavage site) until luciferase activity disappears. Lastly, I would use site directed mutagenesis to mutate individual nucleotides in the region presumed to encode the mystery PAS until luciferase activity is lost to confirm I have located the poly-A signal.

**H2 Expression**

It is interesting to note that expression levels from H2 were significantly lower than all other constructs, including (NoA). This suggests that the H2 3’UTR is capable of affecting gene expression. Furthermore, it is possible that this decrease maybe due to H2 affecting enzyme activity and not quantity. To address this, I would compare the Mx constructs using a western blot, and then confirm these results using qPCR.

**Future Direction**

For this assay to be of any use in the future, luciferase expression must be entirely eliminated. That is to say, a change in gene expression, or any other observation, is a result of the 3’UTR and not pTK-Gluc-2(NoA), is only possible if the response from (NoA) is zero. That being said, a commercial plasmid, designed specifically to evaluate 3’UTR functionality does not exist. With this in mind, after continually observing expression after removal of the Gluc vector 3’UTR, it is clear that not enough is known about the plasmid.
Therefore, it may be necessary to design an expression system having only the essentials for prokaryotic amplification and eukaryotic expression. Based on what we know about how 3’UTR in translation work, they will complex with 5’UTR. So moving forward, this expression system would include the necessary machinery for prokaryotic amplification such as origin of replication, prokaryotic promoter, and an antibiotic resistance gene. This would also have to include the necessary eukaryotic expression components that include a TK promoter, Mx 5’UTR, Gluc reporter lacking access to a PAS, and a multi-cloning site (MCS) for inserting Mx 3’UTR, to look at how they interact.
### TABLE 1.1: HUMAN AND BIRD PRR COMPARISON

<table>
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<tr>
<th>Pattern Recognition Receptor</th>
<th>Human Origin of Ligand</th>
<th>Chicken Origin of Ligand</th>
<th>Duck/Goose Origin of Ligand</th>
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## Table 1.2: Antiviral Spectrum of MX Proteins MX

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<tr>
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FIGURE 1.1: MODEL OF INNATE IMMUNE RECOGNITION OF VIRUS IN BIRDS

The plasma membrane receptor of TLR15 recognizes CpG-ODN from viruses and bacteria. Viral recognition relies on intracellular PRRs, whose ligands are dsRNA derived from viruses or virus-infected cells (TLR3), ssRNA derived from RNA viruses (TLR7), CpG-ODN (TLR21), short 5’ppp dsRNA (RIG-I), and long dsRNA (MDA5). TLR3, TLR7 and TLR21 localize mainly in the ER in the steady state and traffic to the endosome, where they engage with their ligands. The recognition triggers the downstream signal transduction to activate NF-κb or IRF3/7, which induces inflammatory cytokine production.
FIGURE 1.2: THE INTERFERON (IFN)-SIGNALING CASCADE

Three IFN receptors located on the cell surface include: type I IFNs which act through IFN-α receptor 1 (IFNAR1) and 2 (IFNAR2) heterodimers; type III IFN which act through interleukin-10 receptor 2 (IL-10R2) and IFN-λ receptor 1 (IFNLR1) heterodimers; and type II IFN which acts through a IFN-γ receptor 1 (IFNGR1) and 2 (IFNGR2) heterodimer. Binding of both type I and III IFNs to their IFNAR1/2 or IL-10R2/IFNLR1 complexes, causes phosphorylation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which in turn phosphorylate the receptors at specific intracellular tyrosine residues. This leads to the recruitment and phosphorylation of signal transducers and activators of transcription 1 and 2 (STAT1 and 2). STAT1 and 2 associate to form a heterodimer, which in turn recruits the IFN-regulatory factor 9 (IRF9) to form the IFN-stimulated gene factor 3 (ISGF3). Binding of type II IFN dimers to the IFNGR1/2 complex leads to phosphorylation of preassociated JAK1 and JAK2 tyrosine kinases, and transphosphorylation of the receptor chains leads to recruitment and phosphorylation of STAT1. Phosphorylated STAT1 homodimers form the IFN-γ activation factor (GAF). Both ISGF3 and GAF translocate to the nucleus to induce genes regulated by IFN-stimulated response elements (ISRE) and gamma-activated sequence (GAS) promoter elements, respectively, resulting in expression of antiviral genes.
FIGURE 2.1: DEVELOPMENT OF EXPRESSION CONSTRUCT TO ASSAY FOR FUNCTIONAL EFFECTS OF CHMX 3’UTR VARIATION

(A) The reporter constructs pCMV-Gluc-2 and pRL-TK were digested with BglII and HindIII to remove the CMV and TK promoters respectively. The TK promoter was then ligated into the promoterless pGluc-2 backbone, generating a new construct pTK-Gluc-2. (B) Clones containing this new plasmid were identified by EcoRI enzyme digestion and electrophoresis. Lanes 1, 3, and 5 show undigested pCMV-Gluc-2, pRL-TK, and pTK-Gluc-2. Lanes 2, 4, and 6 show pCMC-Gluc-2, pRL-TK, and pTK-Gluc-2 following EcoRI enzyme digestion. Lane M = molecular weight marker.
**3’UTR Functional Elements**

**Step #1**
- **CDS** → **AATAAA** → **GGTTGTTGTT**
  - Poly A Signal (Cleavage Factor)
  - G/T rich region (Cleavage Factor)

**Step #2**
- **CDS** → **AATAAA** → **TTGGTTGTTGG** → **CA**
  - Poly A Polymerase
  - CLEAVAGE SITE

**Step #3**
- **CDS** → **AATAAA** → **AAAAAA**
  - Poly A Polymerase extends Poly A Tail

**FIGURE 2.2A: FUNCTIONAL ELEMENTS IN 3’UTR POLYADENYLATION**

The beginning of mRNA Polyadenylation occurs once cleavage factors, (Poly-A signal and G/T rich region), dimerize. PAP then binds to the homodimer and moves 5’ to 3’ until a cleavage site (CA) is located, then all nucleotides downstream of the cleavage site are removed and PAP begins extending the Poly-A tail.
**FIGURE 2.2B: COMPARISON OF CHMX 3’UTR SEQUENCES**

Region of chMx gene including the last 129 nt of the Mx coding sequence (brown), the mRNA 3’UTR (blue) and 76 nt of downstream genomic sequence (orange) from galGal4, accession #Z23168, and 6 haplotypes identified in commercial layer-type chicken lines were aligned using Clustal W. Sequence in yellow show the adapter sequences on the ends of the H2 sequence used for fusion to pTK-Gluc-2. Sequence capitalized in bold denote the putative poly-(A) signal. “*” denote the sequence, similar to a PAS, located in Gal4/H4, nt374-379. “x” denote the sequence, similar to a PAS, located in H1 nt375-380. Underlined sequences denote locations where DNA was inserted into the pTK-Gluc-2(H2) plasmid to generate the H1, H3, H4, H5, H6, and Gal constructs. “~” denote nt identical to galGal4. “—” denote gap or missing nt. Note numbers to the right of the brown sequence reflect nt number in the coding sequence, and numbering restarts (v) with the start of the 3’UTR (blue).
FIGURE 2.2C: COMPARISON OF CHMX 3’UTR SEQUENCES

Sequence of the corresponding region of the chicken GAPDH 3’UTR (accession # J00850). In addition to the putative poly-A signal (capitalized in bold), the other prototypical signaling elements, upstream signal elements (USE, capitalized and underlined) and downstream signal elements (DSE, capitalized and italics).
FIGURE 2.3: OPTIMIZATION OF PTK-GLUC-2 EXPRESSION ASSAY

Duplicate wells of LMH cells were transfected with (A) increasing concentration of pTK-Gluc-2 or pSV40-Cluc in separate reactions, (B) co-transfection with pTK-Gluc-2 and pSV40-Cluc in increasing concentrations, and (C) co-transfected with inverse concentrations of the two plasmids. 48hrs post-transfection supernatants were collected and assayed for Gluc and Cluc activity. (D) The average ratio (Gluc/Cluc) was calculated for duplicate wells of co-transfected cells (black bars). Results representative of 3 independent experiments.
FIGURE 2.4: VARIATION IN CHMX 3’UTR HAS VARIABLE EFFECT ON LUCIFERASE EXPRESSION

Replicate wells of LMH cells were transfected with 1ug of reporter pTK-Gluc-2 (TK), pTK-Gluc-2(Gal4), pTK-Gluc-2(H1), pTK-Gluc-2(H2), pTK-Gluc-2(H3), pTK-Gluc-2(H4), pTK-Gluc-2(H5), pTK-Gluc-2(H6), pTK-Gluc-2(GAPDH) or pTK-Gluc(NoA), and 0.5µg of pSV40-Cluc control plasmid. 48hrs post-transfection supernatants were assayed for Gluc activity and normalized for transfection efficiency based on Cluc activity (Gluc/Cluc). Average luciferase ratios (solid bars) and standard deviations (error bars) for each reporter construct was determined and analyzed by ANOVA. “a” denotes significance difference from pTK-Gluc-2(H2) (p<0.05). “b” denotes significant differences from pTK-Gluc-2(GAPDH) (p<0.05). Results are representative of 4 independent experiments.
FIGURE 2.5: LMH EXPRESSION OF GLUC FOLLOWING TRANSFECTION WITH INTACT OR CIRCULAR PTK-GLUC-2 WITH OR WITHOUT POLY-A SIGNAL

(A) Diagram of pTK-Gluc-2 and pTK-Gluc-2(NoA) following digestion with Xbal or BmtI respectively. Arrows depict relative location of primers Forward 1-Colt (F1C), Middle-Fwd-Colt (MFC), and Reverse 1-Colt (R1C) used to detect Gluc mRNA; black bars indicate expected amplicon size. (B) Replicate wells of LMH cells were transfected with 1ug of linear or circular versions of pTK-Gluc-2 or pTK-Gluc-2(NoA) and the respective linear or circular pSV40-Cluc. 48hrs post-transfection supernatants were assayed for Gluc activity and normalized for transfection efficiency based on Cluc activity (Gluc/Cluc). Average luciferase ratios (solid bars) and standard deviations (error bars) for each reporter motif were determined ANOVA. * Denotes a significance from circular pTK-Gluc-2 (p<0.05) and ** denotes a significant from circular pTK-Gluc-2(NoA) (p<0.05). (C) Presence of Gluc mRNA in LMH cells from panel B was detected using primers seen in panel A, and following the isolation of DNA-free total RNA followed synthesis of oligo-dT primed cDNA. Black lines under the gel indicate the primer pair used for those lanes above. Controls were non-reverse transcribed (noRT) RNA from both TK and NoA transfected wells.
REFERENCES


APPENDICES
OTHER RESEARCH PROJECTS AND LESSONS LEARNED

Effects of Fluoxetine on growth and feed efficiency

Following undergrad I had no research experience but was interested in graduate school. To learn if a career in discovery research was for me, and to gain experience, I worked as a PBS under the direction of Dr. Jim Croom. Our project’s primary focus was determining what affect Fluoxetine might have on weight gain. Dr. Croom, having been prescribed Fluoxetine for depression, noticed an increase in body weight. Consequently, Dr. Croom wanted to find out if Fluoxetine could alter resting metabolic rates in young broilers.

Although the experiment had already been conducted, the data still needed to be analyzed. Before data could be evaluated, it had to first be assembled and then organized. Therefore, I transferred all data to a central spreadsheet and organized the values into groups based on experimental topic. Next, analysis of variance (ANOVA) was performed to evaluate our data. During this process, I became familiar with the analytical aspects of research and, consequently, why ANOVA was chosen. Furthermore, I learned that the relationship between statistical models and data analysis is invaluable for interpreting results. Based on my analysis we concluded that Fluoxetine significantly increases feed efficiency and growth in young broilers.

The results from this study were presented at the 2012 annual Poultry Science Association, in Athens Georgia. This was the first time I presented an abstract at a scientific meeting. Although it was an exciting area of research, funding for this project was
discontinued. It would have been interesting to investigate if the findings were repeatable on a larger scale and to evaluate the potential for Fluoxetine use in the broiler industry. Because the use of Fluoxetine as a feedstuff may not be economically feasible. Therefore, it would have been of interest to investigate if an herbal substitute exists and, if so, how it would compare to Fluoxetine.

**Effect of astrovirus nonstructural proteins on host cell function**

In addition to my interest in nutritional physiology, infectious diseases, specifically viruses, also fascinate me. I was fortunate to have the opportunity to continue my graduate program working under the direction of Dr. Matthew Koci. Initially, I selected a project involving the turkey astrovirus. Astrovirus are characterized as positive sensed, single strand RNA, and are non-enveloped. Diseases from astrovirus are primarily associated with gastroenteritis but have been known to cause hepatitis and nephritis. Moreover, diseases associated with avian astrovirus include runting stuntin syndrome (RSS), poult enteritis syndrome (PEC), and poult enteritis mortality syndrome (PEMS).

While we have known about astrovirus and their impact on animal health for decades, we still know very little about how they cause disease. With the turkey astrovirus specifically, further complications arise from the fact that they do not replicate in cell culture. To better understand the host/viral interactions associated with TAstV2, I wanted to identify how the open reading frame-1a (ORF-1a) nonstructural proteins expressed by astrovirus affect gastro enteritis.
Because TAstV2 will not propagate in cell culture, we decided to clone ORF-1a into an adenoviral expression vector. We chose this vector because, unlike lentiviral vectors, an adenoviral vector will not integrate its DNA into host chromosomes during viral gene replication. To create our adenovirus vector, we used AdEasy XL Adenoviral Vector System (Stratagene). The first step in the developing the adenovirus vector was cloning ORF-1a into a shuttle vector (pShuttle-CMV). Due to difficulties with cloning ORF-1a into pShuttle-CMV, I was unable to complete this project. Furthermore, structural characteristic of ORF-1a, being AT-rich (58% AT) and having tandem “T” repeats, may influence gene confirmation. Therefore, a conformational motif of ORF-1a, caused by tandem “T” repeats, could inhibit cloning by disrupting covalent bonds between ORF-1a and pShuttle-CMV. Nevertheless, an alternative method for observing how ORF-1a non structural proteins affect host cells would be direct transfection of ORF-1a mRNA.

**Lessons from Mx 3’UTR not in Chapter II**

In addition to these other projects, which did not become the focus of my thesis, I learned several additional lessons from working with the chMx 3’UTRs and expression system. One of the pitfalls I experienced specifically with my project, dealt with variations in the number of LMH cells added to each well of a 24-well culture plate. To ensure all wells have the same number of cells, calculate the total number of cells you need and suspend them in the total volume of medium needed, prior to seeding them in the well.
I encountered another project-specific problem while trying to locate mRNA cleavage sites using 3’RACE on TK-Gluc-2 (TK-L), TK-Gluc-2(NoA) (NoA-L), TK-Gluc-2 TK-C), and TK-Gluc-2(NoA) (NoA-C). Specifically, multiple bands were observed in all samples after PCR amplification. Moreover, adding a temperature gradient to the PCR reaction did not improve band specificity. This suggests that the RACE Oligo-dT was non-specifically annealing to a RNA poly-(A) tail. Therefore, to limit non-specific annealing during reverse transcriptase, it will be necessary to use a RACE Oligo-dT with the terminal base “A”.

My growth, development, and understanding about science and research

Throughout the course of my thesis project, I have experienced many things I feel may be useful to help future students avoid pitfalls. There are two general pitfalls I had that apply to not just my project but to research in general. First, the most important tool to any researcher is their laboratory notebook. Therefore, to ensure accurate documentation of all materials, methods, procedures, and data, never leave lab without writing down everything you did that day. Second, one of the biggest pitfalls I have experienced with time management was one of my most significant struggles as a young scientist. Poor time management can not only negatively affect daily tasks, but also keep a student from finishing on time by delaying the overall project. Therefore, before beginning an experiment, I have found it is best to first write down what procedures will be done in chronological order. Next, figure out what materials and how much of each are needed to finish the experiment. By
doing this, I ensured that my time was being used efficiently and that no unnecessary delays resulted from having to order more supplies.
FIGURE 3.A: LMH EXPRESSION OF GLUC FOLLOWING TRANSFECTION WITH INTACT OR CIRCULAR PTK-GLUC-2 WITH OR WITHOUT POLY-A SIGNAL

(A) Diagram of pTK-Gluc-2 and pTK-Gluc-2(NoA) following digestion with XbaI or BmtI respectively. Arrows depict relative location of primers Middle-Fwd-Colt (MFC) and Reverse 1-Colt (R1C) used to detect Gluc mRNA, and the black bars indicate expected amplicon size. (B) Presence of Gluc mRNA in LMH cells from panel B was detected using primers seen in panel A, and following the isolation of DNA-free total RNA followed synthesis of oligo-dT primed cDNA. Black lines under the gel indicate the primer pair used for those lanes above. Non-reverse transcribed (noRT) RNA from both TK and NoA transfected wells was used as a control.