DISSERTATION

THE MX SYSTEM IN *BOS TAURUS*:

CLONING AND CHARACTERIZATION OF cDNAS AND LINKAGE MAPPING OF THE GENE TO *BOS TAURUS* CHROMOSOME 1

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY NORMAN MATTHEW ELLINWOOD ENTITLED THE MX SYSTEM IN *BOS TAURUS*: CLONING AND CHARACTERIZATION OF CDNAS AND LINKAGE MAPPING OF THE GENE TO *BOS TAURUS* CHROMOSOME 1 BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE MX SYSTEM IN *BOS TAURUS*: CLONING AND CHARACTERIZATION OF CDNAS AND LINKAGE MAPPING OF THE GENE TO *BOS TAURUS* CHROMOSOME 1

The Mx protein, an interferon-induced intracellular GTPase, is among the best characterized antiviral proteins, having *in vivo* and *in vitro* activity against negative sense RNA viruses. Mouse, rat, and human Mx systems are well characterized, with each species having multiple genes, at least one of which, in each species, produces an antiviral protein. The Mx system has not been critically evaluated in any agricultural species. Complete understanding of the cattle Mx system might aid the selection, or transgenic engineering, of cattle for disease resistance, as well as aid research in the pathogenesis of viral disease in cattle. To investigate cattle Mx, cDNAs from *Bos taurus* Mx mRNAs were characterized. Alleles identified allowed linkage mapping of the bovine Mx locus. Induction of Mx mRNA by interferon was confirmed by Northern analysis. Mx proteins were characterized *in vitro* by immunoblot analysis.

Allelic cDNAs encoding bovine Mx proteins were isolated from an endometrial phage library. The open reading frames of two clones predicted proteins of 654 (Mxl, GenBank accession AF047692) and 648 (Mxl-a, GenBank accession U88329) residues. Both alleles had the tripartite GTPase domains, dynamin signatures, and leucine zipper

motifs common to Mx proteins. Bovine protein sequences showed highest identity to ovine Mx (93%) and were similar to human MxA (73%) and mouse Mx1 (63%).

A 13 bp deletion in the 3'-untranslated region of Mx1 was found in some cattle. Polymorphism segregation was consistent with codominant inheritance. The MX1 locus was mapped between microsatellite markers BM1824 (LOD = 10.24) and BMS4043 (LOD = 3.96), between 114.7 and 118.6 cM from the centromere on the USDA map of cattle (*Bos taurus*) chromosome 1 (BTA1).

Rapid induction of Mx mRNA in Madin-Darby bovine kidney (MDBK) cells was seen in response to interferon. The monoclonal antibody 2C12 cross-reacted with a cytoplasmic moiety found in MDBK cells exposed to interferon. Immunoblots of SDS/PAGE separated total cell lysates of interferon-treated MDBK cells identified an immunoreactive, chemiluminescent signal migrating at approximately 75 kDa. The signal increased in a dose response to type I interferon. Detectable signal could be distinguished in cells treated with as little as 0.1 U interferon/ml.

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DEDICATION

To my parents John and Joan Ellinwood, who provided me the tools and opportunities to exact from life the best of fortunes.

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REVIEW OF THE LITERATURE

Introduction

Mx proteins are large intracellular GTPases that are stringently induced in cells by exposure to type I interferons. Some, but clearly not all, Mx proteins possess potent antiviral activity which has been demonstrated both in cultured cells and whole animals. This antiviral activity has stimulated great interest in Mx proteins, which has led to cloning of Mx cDNAs from a large group of vertebrates, ranging from humans to fish. Despite the fact that Mx genes appear to have been highly conserved during vertebrate evolution, a normal cellular function for these proteins, particularly those without known antiviral activity, has not been identified.

A bulk of our understanding of the Mx system derives from study of Mx genes and proteins from mice and humans, and thorough investigation of the Mx system in an economically important agricultural species has not been accomplished. Potential benefits of a more complete knowledge of the Mx system in cattle could include selection for greater disease resistance, potential transgenic utilization, and use in diagnostic and research applications.

Interferon

Interferon was discovered by Isaacs and Lindenmann (1957) as a substance produced by the chorioallantoic membrane of chick embryos inoculated with influenza

virus, that, upon exposure to other cells, induced resistance to virus replication. Since that initial report, a whole sub-discipline has arisen around the study of interferons, with important implications for a wide range of fields, from viral disease pathogenesis to clinical medicine to very basic molecular biology.

The interferon system as we now understand it is comprised of two distinctly different kinds of proteins, designated type I and type II interferon, which differ in their gene structure, biochemical properties and immunologic function. Type I interferons are acid-stable proteins secreted in response to viral infection. They contain between 140 and 150 amino acids and exert their biological effects as monomers. The primary *in vitro* effects of type I interferons are the ability to inhibit cell proliferation and induce an antiviral state in responsive cells. In a given species of vertebrates there are at least two and in some cases up to four classes of type I interferon, designated α, ω, β, δ, and τ, which are distinguished by the cell types of origin and the circumstances of production. Leukocytes are the primary source of interferon-α and -ω, which have immunomodulatory and antiviral effects. Interferon-β, which is principally antiviral in its activity, has the potential to be produced by most cells in a body. All species whose type I interferon systems have been studied in depth have interferon-α, -ω, and -β. Interferon-δ and -τ are produced by embryonic tissues during implantation in swine and ruminants, respectively, have less antiviral potency, and play a role in the maternal recognition of pregnancy. These last two forms of interferon have been given separate designations based on sequence variation, though the possibility exists that they serve analogous functions, and may derive from an individual ancestral gene. All type I interferon genes lack introns and reside on the same chromosome in a given species, with the majority of type I interferon

groups having multiple genes. Type I interferons are able to compete for binding on the same receptor, only one of which has been identified, although accessory proteins may exist which modify the affinity for and signal transduction of, different type I interferon ligands.

Type II interferon, designated γ, is comprised of a single intron-containing gene. Although there is but one interferon-γ gene in a given species, the gene product dimerizes to bind to and activate the type II interferon receptor. Interferon-γ is an acid-labile, secreted protein of length similar to type I interferon (140-150 amino acids). It is produced by activated lymphocytes and has a multifaceted role in the modulation of immune responses. Relative to type I interferon, interferon-γ has weaker antiviral activity, and has a specific role in the immune responses to mycobacteria and protozoa.

Type I Interferon: Response to Virus

Type I interferon is produced naturally by cells in response to virus exposure. Infection is not a prerequisite for induction as inactivated virus may induce interferon (Winship and Marcus, 1980). Components of the viral particles that have been implicated as inducers are viral glycoproteins (Ito, 1994) and nucleic acids (Tytell and Field 1972). Double stranded RNA is an excellent artificial inducer of interferon, a fact that has implicated viral nucleic acids as key factors in viral induction of interferon. The ability of virus to induce interferon can be type and strain specific. Not all viruses are equally good inducers of interferon, nor within a strain of virus are all isolates equal in their capacity to induce (Marcus *et al.*, 1992). There is also variability in the susceptibility of virus to interferon's antiviral activity (Samuel, 1991). There is doubtless an element of host responsiveness to viral induction as well. Following an appropriate stimulus, cells secrete

measurable levels of interferon within 12-24 hours. Circulating interferon is then able to bind to specific cell membrane receptors on as yet uninfected cells, thereby inducing the target cell to produce a host of antiviral proteins.

Type I Interferon: In vivo Inhibition of Viral Replication

When a vertebrate host is infected with a virus, type I interferons are produced and secreted by cells at the initial site of infection. By binding it's cognate receptors, which are ubiquitously expressed, circulating interferons can activate a variety of antiviral responses in cells directly peripheral to the initial site of infection as well as throughout the body, rendering the host resistant to what might otherwise be an overwhelming infection. In this way interferons act as an early alarm to viral infection, limiting spread by inducing an antiviral state in as yet uninfected cells. An early and striking illustration of the importance of the type I interferon system's response to viral infection was provided by studies in which interferon was immunoneutralized (Gresser *et al.*, 1976; Gresser *et al.*, 1976a). Mice inoculated with encephalomyocarditis virus and treated with anti-interferon antisera were more severely affected compared to controls. Relative to untreated animals, mice treated at the time of viral inoculation with 1.2×10^6 neutralizing units showed clinical signs earlier (24-36 hours post infection versus 72-96 hours in untreated mice), succumbed earlier and with higher frequency (100% dead at 48 hours versus 90% dead at five days), and had greater viremia, higher viral titers in spleen, liver, kidney and heart, but lower titers in brain. Similar results were found using herpes simplex, Moloney sarcoma, vesicular stomatitis and Newcastle disease viruses. More recent studies of the *in vivo* role of type I interferon during viral infections have utilized two lines of mice engineered to carry null mutations in a common subunit of the type I interferon receptor ($INFAR1^{-/-}$,

Müller *et al.*, 1994; Hwang *et al.*, 1995). The hallmark of these mice was a complete non-responsiveness to type I interferon, making them exquisitely sensitive to viral infections. Homozygous null mutant animals were 10^6 fold more sensitive to vesicular stomatitis virus (VSV) than controls, succumbing to as few as 30 plaque forming units (PFU) of VSV. Semliki forest virus (SFV) replication in the liver of receptor knockout mice 24 hours post infection was elevated roughly 3 x 10^9 fold over controls. Mice were normal with respect to litter size, growth, and development. Both lines had slight functional, numerical, or biochemical abnormalities in myelogenous cell lineages, but otherwise appeared to have normal cellular and humoral immunity. From these studies a crucial role for type I interferon emerges as part of an acute response system which forestalls the lethal effects of viral infection until such time as the immune system can mount an effective response.

Type I Interferon: Receptors and Signal Transduction

The type I interferon receptor is a multi-subunit receptor member of the interferon subfamily of the cytokine receptor superfamily (reviewed, Domanski and Colamonici, 1996; Pestka, 1997). When the interferon-α receptor 2 (INFAR2), which is the ligand binding subunit of the receptor, binds type I interferon, oligomerization of the receptor subunits occur, thus activating a signaling transduction cascade involving Janus-activated kinases (Jaks) and signal transducers and activators of transcription (Stats, reviewed, Haque and Williams, 1998). This signaling cascade leads to the binding of a class of *cis*-acting sequences known as interferon stimulated response elements (ISRE) by the *trans*-acting interferon stimulated gene factor-3 (ISGF-3), a trimeric complex of Stat1, Stat2, and the nuclear protein p48, thus initiating transcription of type I interferon stimulated genes.

The Mx System

Exposure of cells to type I interferon can activate transcription of over 50 different genes, over thirty of which have been characterized (De Maeyer and De Maeyer-Guignard, 1998). The antiviral activities of certain interferon-stimulated proteins are exerted in concert with each other. An example of such an effector protein collaboration is provided by RNase L and 2', 5'-oligoA synthetase. These enzymes are produced by cells exposed to interferon where they remain inactive until, in the presence of virus, 2', 5'-oligoA synthetase catalyzes the formation of 2', 5'-oligoA from ATP, which in turn activates RNase L to degrade viral RNA genomes and transcripts (reviewed, Castelli *et al.*, 1998). One or more Mx proteins are among those proteins induced in cells by binding of type I interferon to its receptors. In contrast to the cooperative interactions among other interferon-induced proteins, Mx proteins appear to exert their antiviral activity independent of other interferon-induced proteins. Additionally, Mx proteins are the only interferon-induced gene products for which naturally-occurring alleles have been identified that impact host susceptibility to disease.

The Mx System in Mice: The Mx$^+$ Phenotype

The Mx system was discovered by Lindenmann (1962) as a trait in the inbred mouse strain A2G that conferred resistance in adult mice to lethal infection with influenza A virus. From the initial observations made by Lindenmann, efforts to elucidate the phenotype and pattern of inheritance of Mx-dependent viral resistance followed different lines of inquiry (reviewed by Haller, 1981). Lindenmann (1964) found that the trait was inherited in a simple autosomal dominant manner in adult mice and the name Mx, for myxovirus resistance, was proposed. Mice are either Mx$^+$, *i.e.* resistant, or Mx$^-$, *i.e.*

susceptible to influenza virus, although earlier nomenclature used the form Mx/Mx for resistance and +/+ for susceptible. The Mx^+ A2G mice were found resistant to neurotropic, pnuemotropic (Lindenmann *et al.*, 1963), and hepatotropic (Haller *et al.*, 1976) mouse-adapted strains of influenza A virus. In order to distinguish whether the Mx^+ phenotype was dependent on other immunological functions, genetic crosses were produced, which demonstrated that the Mx^+ phenotype was wholly independent of B and T cell function (Haller and Lindenmann, 1974; Haller *et al.*, 1976). Congenic mice (Balb.A2G) developed from the initial cross of the Mx^+ A2G strain with the Mx^- BALB/c mice (Staeheli *et al.*, 1984), have an Mx^+ phenotype, supporting the conclusion that the Mx^+ phenotype is independent of background genetics. Different forms of immunosuppression were found to have no effect on the Mx^+ phenotype (Haller *et al.*, 1976; Fiske and Klein, 1975). A series of studies implicated with ever stronger evidence that Mx was linked to type I interferon activity (Haller *et al.*, 1979; Haller *et al.*, 1980; Haller, 1980; Arnheiter *et al.*, 1980; Arnheiter and Haller, 1983). With the availability of antisera to type I interferon, investigators were able to show that *in vivo* immunoneutralization of interferon fully abrogated the resistant phenotype (Haller *et al.*, 1979a). In the same study, resistant mice, not given anti-interferon serum, had lower serum levels of interferon during viral infection compared to susceptible mice, echoing earlier findings of low interferon levels in organs of infected, resistant mice (Fiske and Klein, 1975). However, these immunoneutralization experiments as well as the findings of lower interferon responses in resistant mice provided only indirect evidence of the role for interferon in the Mx^+ phenotype. Conclusive evidence for a primary and obligate role required further *in vivo* and *in vitro* studies. Interferon treatment of newborn A2G mice,

which are, under normal circumstances susceptible to influenza virus until weaned, caused the premature expression of the adult, resistant, phenotype (Haller *et al.*, 1981). Macrophages, initially thought to express the resistant phenotype *in vitro* (Lindenmann *et al.*, 1978), were later found to express resistance in an interferon dose-dependent manner (Haller *et al.*, 1979b). Using macrophages held in culture, Haller and co-workers (1980) demonstrated that the *in vitro* phenotype of A2G mice macrophages, which had been shown to co-segregate with the *in vivo* phenotype (Lindenmann *et al.*, 1978), involved an increased sensitivity to the antiviral effects of interferon. Haller and colleagues provided evidence from bone marrow transplant studies that macrophages alone were unable to confer resistance (1979b). This finding was extended by Arnheiter *et al.*, (1980), who demonstrated that hepatocytes in culture were capable of expressing the Mx^+ phenotype, thereby providing further evidence that Mx^+ phenotype was not restricted to cells of the immune system. Studies using heterokaryons of mouse embryo fibroblasts from Mx^+ and Mx^- mice further illustrated that the Mx^+ phenotype was both dependent on type I interferon and was not restricted to components of the immune system (Ruff, 1983). The finding that the Mx^+ phenotype was type I interferon-dependent, was expanded by Staeheli and colleagues (1984), when they showed that type I, but not type II interferon, was able to induce resistance in cells from Mx^+ mice.

The Mx1 Protein: From Phenotype to Macromolecule

Comparative 2-D gel analysis of interferon-induced cultures derived from A/J (Mx^-) and A2G (Mx^+) mice allowed researchers to show that only A2G cells produced a 72,500 Da protein (Horisberger *et al.*, 1983) which was eventually designated Mx1 (Hug *et al.*, 1988). Staeheli and coworkers (1983) elaborated on this finding by demonstrating

that the mRNA associated with this 72,500 Da protein was induced in cells treated with type I interferon, but absent in untreated cells. Identification of the Mx1 protein up until this point required the arduous process of 2-D gel analysis, which made isolation of significant amounts of protein a troublesome task. The Mx1 protein had no known substrate affinity or enzymatic activity, which might have allowed for purification. Employing a clever strategy, researchers were able to produce specific antibodies to Mx1 before they had isolated a pure protein. Inoculation of Balb/cJ mice with splenocytes from Newcastle disease virus (NDV)-infected congenic mice (Balb.A2G), allowed for the production of polyclonal antibodies that were shown to recognize the then putative mouse 72 kDa Mx protein (Staeheli *et al.*, 1985). An outgrowth of these efforts was the finding that Mx functioned as a minor histocompatibility antigen (Speiser *et al.*, 1990). The polyclonal antiserum precipitated an interferon-induced protein with identical 2-D gel mobility profiles as seen in earlier studies. In addition to producing a polyclonal antiserum, the authors used a similar strategy to produce monoclonal antibodies. One of the resulting hybridoma cell lines, 2C12, produces an IgG1 antibody that also precipitated an approximately 72 kDa protein, exclusively in type I interferon-induced mouse cells bearing the Mx^- phenotype. Anti-Mx antibodies allowed for the demonstration that the mouse Mx1 protein segregated to the nucleus (Dreiding *et al.*, 1985), and helped to confirm the antiviral nature of Mx by showing, through intracellular microinjection studies, that immunoneutralization of the 72 kDa Mx1 protein obliterated the antiviral phenotype of Mx^- cells (Arnheiter and Haller, 1988).

Molecular Genetics of the Murine Mx System

Employing congenic strains of mice and the polyclonal antiserum described above, Staeheli and co-workers (1986b) cloned the cDNA of mouse Mx1 mRNA. The 3200-bp cDNA encoded a hydrophilic protein of 631 amino acids, possessing a string of basic amino acids at the carboxy terminus, and was not homologous to any protein known at that time. The Mx1 protein segregated to the nucleus, and was demonstrated to have anti-influenza virus activity. In these respects the cDNA product conformed to the expected characteristics of a protein responsible for the Mx^- phenotype. A second mouse Mx transcript was identified using the Mx1 cDNA as a molecular probe of Northern blots of RNA derived from cells of the Mx^- BALB/c mice. This second gene, designated Mx2, showed close genetic linkage to that of Mx1, and was transcribed in an interferon-inducible manner in all Mx^- laboratory mouse strains studied (Staeheli and Sutcliffe, 1988). A comparison of the cDNA sequence to Mx1 identified a potential supernumerary cytosine base at position 1366 of the Mx2 cDNA that created an early stop codon, thought to lead to metabolic instability of the transcript. Removal of this C residue lead to a stable transcript and production of a full-length protein that segregated to the cytoplasm and had antiviral activity against VSV (Zürcher *et al.*, 1992a). All laboratory mouse strains have null mutations in the Mx2 gene (Staeheli and Sutcliffe, 1988), while the Mx^- strains have one of two different null mutations in the Mx1 gene in addition to the null mutations in the Mx2 gene (Staeheli *et al.*, 1988). Recently a wild type Mx2 protein has been identified, which identifies the supernumerary nucleotide to be an adenine at 1367, not the cytosine at 1366, and confirms the anti-VSV and cytoplasmic character of the protein (Jin *et al.*, 1999).

Initial work using Southern blot analysis revealed that the Mx1 gene contained introns and was at least 10 kbp in length (Staeheli *et al.*, 1986b). Hug *et al.* (1988) examined the organization of the BALB/A2G mouse Mx1 gene, finding that it spanned 55 kbp and possessed 14 exons and 13 introns. The first intron is at least 30 kbp, separating the non-coding exon 1 from exon 2. Also contained in this large intron is an alternate exon, designated 1a, which is seen in approximately one in ten transcripts. The gene locus for mouse Mx1 was mapped to chromosome 16 (Staeheli *et al.*, 1986c) using somatic cell hybrids and was linkage mapped to 71.20 cM from the centromere on chromosome 16 (Reeve *et al.*, 1988; Mouse Genome Database, 1999). The orientation of the gene is unknown. Although Mx1 and Mx2 genes, being non-recombinant, show tight linkage (Staeheli and Sutcliffe, 1988), nothing is yet known of the genomic structure of Mx2.

Mx Gene Transcription: Induction and Kinetics

The promoters of the mouse Mx1 (Hug *et al.*, 1988), human MxA (Chang *et al.*, 1991; Horisberger *et al.*, 1990a; Ronni *et al.*, 1998), and chicken Mx (Schumacher *et al.*, 1994) genes have been cloned and evaluated (Table 1.3). The conclusions that can be drawn from these different studies support the following two observations: interferon response elements (ISREs) located between −120 and −50 relative to the transcription start site mediate type I interferon-induced gene transcription, and Mx genes show heterogeneity in the proximal elements, with different genes having a variable complement of common elements such as Sp1 sites and TATA box sequences. The promoter region of the human MxA gene lacks both TATA and CCAAT boxes, but has a functional Sp1 binding site 31 to 28-bp upstream from the transcription initiation site. The proximal

sequence of the mouse promoter has an Sp1 binding site and a TATA box, and, although the latter does not conform to the consensus, it is identical to the TATA box sequence of diverse interferon-α genes (Hug *et al.*, 1988; Weissmann and Weber, 1986). The chicken promoter has a TATA-like sequence that does not conform to the consensus, and is located atypically close to the initiation site for a TATA element.

The human MxA promoter has three ISREs, the most distal of which (–505 to -490) appears to have no positive effect on induced transcription from the Mx promoter. The second ISRE (-103 to -89) has a very strong positive effect on interferon-induced transcription, while the most proximal ISRE (-45 to -57) is obligatory for interferon-mediated transcription. The proximal pair of ISREs have been shown to mediate their activity by binding the *trans*-acting interferon-stimulated gene factor 3 (ISGF3), a complex of Stat1, Stat2 and p48, which is a type I-specific interferon-activated initiation factor. The less exhaustive analyses of the mouse and chicken promoters have identified ISREs in regions of the promoters that upregulate transcription in response to interferon. Enhancer elements may be contained within the first intron of the human MxA promoter as inclusion of this intron increased interferon-mediated induction with respect to controls (Ronni *et al.*, 1998).

Despite the presence of response elements in the human MxA promoter for interleukin-6 and NF-κB, neither interleukin-6 nor TNF-α were found to induce transcription from the MxA promoter. Induction by interferon-γ was documented, even at concentrations as low as 1 U/ml, though conditions of induction did not include immunoneutralization of type I interferon (Ronni *et al.*, 1998). Even at maximal levels of induction, interferon-γ was still 1000 fold less active an inducer of transcription than

interferon-α. Interferon-γ induction was not mediated by ISREs being bound by Stat1/Stat1/p48 complex, the *trans* factor that is the hallmark of interferon-γ induced transcription.

TABLE 1.1. SUMMARY OF ANALYSES OF MX PROMOTERS

SPECIES GENE	*TRANS*-ACTING FACTORS	ACTIVE	*CIS*-ACTING ELEMENTS	LOCATION	ACTIVE	ENHANCER	REFERENCES
Human huMxA	ISGF3	Y	Sp1	-36 - -28	y	Intron 1	Chang *et al.*, (1991)
	Stat1/Stat1/p48	N	ISRE1	-45 - -57	y		Horisberger *et al.*, (1990a)
			ISRE2	-89 - -103	y		Ronni *et al.*, (1998)
			NFκ-B RE	-234 - -244	n		
			IL-6 RE	-350 - -400	n		
			ISRE3	-490 - -505	n		
Mouse muMx1	na		TATA box	-27 - -20	na	na	Hug *et al.*, (1988)
			Sp1	-43 - -34	na		
			ISRE	-131 - -118	y		
Chicken gdMx	na		TATA box	-18 - -11	na	na	Schumacher *et al.*, (1994)
			ISRE	-50 - -61	y		

Less rigorous evaluation of Mx inducers have been conducted using a variety of different viruses and cytokines. It has been speculated that Mx may have been induced by virus to a greater degree than other interferon genes and hence was able to exercise its antiviral effect at a critical point in the process of viral infection of a cell, so that Mx's antiviral properties may have been as much a matter of timing as any innate antiviral properties. Viruses tested as inducers of Mx include VSV (Goetschy, *et al.*, 1989), NDV (Hug *et al.*, 1988; Aebi *et al.*, 1989; Goetschy, *et al.*, 1989; Bazzigher *et al.*, 1992; Schumacher *et al.*, 1994) and influenza virus (Goetschy, *et al.*, 1989; Bazzigher *et al.*, 1992; Ronni *et al.*, 1995; Ronni *et al.*, 1997). Experimental design flaws plague many of these earlier studies and a clear consensus is difficult to extract from a review of these reports. Only influenza A virus and NDV have been implicated as having direct Mx-inducing capacity. Bazzigher and colleagues showed convincingly that primary induction

of Mx by NDV in primary and permanent cell lines is not significant, and even when evident at low levels, it was cell type-dependent and much lower than the virus induction of other interferon-inducible genes (1992). Ronni *et al.*, (1995) found that fresh human peripheral blood mononuclear cells did produce Mx in response to influenza virus, and that production of Mx mRNA was not dependent on synthesis of new protein. Unfortunately immunoneutralization of type I interferon was not conducted. Assessment of Mx production in response to virus infection of mice that are unable to respond to type I or type II interferon should be possible but has regrettably not been reported.

It is a hallmark of the receptor family to which the type I interferon receptor belongs, that there can be overlap of intracellular signaling pathways used by the various cytokines receptors (Williams and Haque, 1997), and exactly which cytokines were able to induce Mx production was not initially clear. Staeheli had shown that interferon-γ did not induce the Mx^+ phenotype in Mx^- cells (1984). Since this initial study, numerous cytokines have been evaluated as primary inducers of Mx including FGF, G-CSF, G/M-CSF, IGF, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, PDGF, TGFβ2, and TNFα/β (Goetshy *et al.*, 1989; von Wussow *et al.*, 1990b; Simon *et al.*, 1991; Files *et al.*, 1998; Ronni *et al* 1998). None of these cytokines have been found to be primary inducers of Mx. By employing an immunoneutralization strategy, secondary induction of Mx, through production of type I interferon, has been shown for IL-1α, IL-2, TNFα and interferon-γ (Simon *et al.*, 1991; von Wussow *et al.*, 1990b). Conditioned media from fresh human peripheral blood mononuclear cells exposed to bacterial lipopolysaccharide, phytohemagglutinin, or pokeweed mitogen failed to act as primary inducers of Mx (von Wussow, *et al.*, 1990b). Although probably not a direct inducer of Mx, interferon-γ may

prime Mx induction, rendering the Mx gene more sensitive to transcriptional stimulus (Geotschy *et al.*, 1989). To summarize, the Mx promoter is refractory to the effects of cytokines other than type I interferon as primary inducers of transcription. The transcription of Mx also appears to be very poorly induced by virus alone.

The kinetics of Mx1 transcription in response to type I interferon have been evaluated in mouse cells (Staeheli, *et al.*, 1986a). Studying the kinetics of transcription initiation and transcript accumulation in mouse fibroblast cell culture, Staeheli and co-workers found Mx1 gene transcription was initiated in a dose-dependent fashion after exposure to interferon-α and/or -β. Transcription, which was not dependent on new protein synthesis, was first detected at 1.5 hours after exposure to interferon, and peaked between 1.5 and 4.5 hours. No initiation of transcription was found after 8 hours in spite of what was considered to have been continual exposure to interferon. The accumulation of Mx mRNA peaked between 4.5 and 8 hours, with a decline to 40% of maximum levels at 16 hours. The 5' and/or 3' UTRs of human MxA and MxB (Horisberger *et al.*, 1990b), and pig Mx1 (Müller *et al.*, 1992b) contain ATTTA sequences, and such sequences are thought to play a role in increased catabolism of mRNA (Brawerman, 1989). In fully-induced cell monolayers Mx transcripts are estimated to account for 0.1% of total mRNA, as estimated from percentages from phage clones and by comparison to mRNA hybridization signal of a housekeeping gene. It was concluded that control of transcript levels is mainly, if not exclusively, at the step of initiation of transcription. No evidence was found for an increase in Mx mRNA stability as a contributing factor to accumulation of mRNA levels. Further reports on the induction of Mx transcription have neither refuted these earlier findings nor added any significant detail to our understanding of Mx

transcription (Geotschy *et al.*, 1990; Ronni *et al.*, 1993; Ronni *et al.*, 1998). In all the salient features mentioned above, the kinetics of Mx transcription are similar to many other interferon-induced genes. Given the observed parameters of Mx transcription, it would appear that Mx is similar to other interferon-induced proteins in that the signal that initiated transcription is the same signal that activates repressors of transcription (Larner *et al.*, 1986; Harada *et al.*, 1989). After initial stimulation, Mx transcription becomes responsive again to interferon in approximately 18 hours (Ronni *et al.*, 1993).

Conservation of Mx in the Vertebrate Subphylum

Regarding the Mx system in mice as a whole, a pattern of findings is seen to which most other species conform. The Mx system is characterized by the existence of multiple type I interferon-induced genes in a given species, with some variability in cellular

TABLE 1.2. MX PROTEINS FOR WHICH CDNAS EXIST

Species	Name	Protein Length	Cellular Localization	Reference
Human	huMxA	662	cytoplasmic	Aebi *et al.* (1989)
	huMxB	715/633	cytoplasmic/nuclear	Aebi *et al.* (1989)
Mouse	muMx1	631	nuclear	Staeheli *et al.* (1986b)
	muMx2	655	cytoplasmic	Jin *et al.* (1999)
Rat	rnMx1	652	nuclear	Meier *et al.* (1988)
	rnMx2	659	cytoplasmic	Meier *et al.* (1988 & 1990)
	rnMx3	659	cytoplasmic	Meier *et al.* (1988 & 1990)
Porcine	ssMx1	663	cytoplasmic	Müller *et al.* (1992b)
	ssMx2	Unknown	unknown	Müller *et al.* (1992b)
Ovine	oaMx	653	unknown	Charleston and Stewart (1993)
Equine	ecMxA	660	unknown	Chesters *et al.* (1997)
Duck	apMx	721/702	cytoplasmic/nuclear	Bazzigher *et al.* (1993)
Chicken	gdMx	705	cytoplasmic	Bernasconi *et al.* (1995)
Trout	omMx1	621	cytoplasmic	Trobridge and Leong (1995)
	omMx2	636	cytoplasmic/nuclear	Trobridge *et al.* (1997)
	omMx3	623	cytoplasmic	Trobridge *et al.* (1997)
Flounder	poMx1	620	unknown	Lee *et al.* (2000)
Salmon	asMx1	623	unknown	Robertsen *et al.* (1997)
	asMx2	623	unknown	Robertsen *et al.* (1997)
	asMx3	623	unknown	Robertsen *et al.* (1997)

localization, as well as in the virus types which are sensitive to the various forms of Mx. For example, mouse Mx1, a nuclear protein, is active against influenza virus, while mouse Mx2 is a cytoplasmic protein with activity against VSV. The rat Mx1 and Mx2 proteins follow this pattern exactly, but the cytoplasmic rat Mx3 protein shows no antiviral activity. In contrast is the human Mx system consisting of MxA (for acidic) and MxB (for basic), of which MxA is cytoplasmic while MxB is both cytoplasmic and nuclear. Human MxA protein has antiviral activity against *both* influenza virus and VSV, plus several other viruses, while human MxB is apparently devoid of antiviral activity. Multiple Mx proteins have now been identified in a number of vertebrates, using various polyclonal sera as well as the 2C12 monoclonal antibody. The cDNAs of a variety of vertebrate Mx transcripts have been cloned and characterized. It appears that the Mx gene, often found as part of a multiple gene family in a given species, has been universally conserved in the vertebrate

TABLE 1.3. MX PROTEINS IN SPECIES WITH INCOMPLETE GENETIC CHARACTERIZATION OF MX

SPECIES	NUMBER	LOCATION	REFERERENCE
Monkey	1	Cytoplasmic	Horisberger and Gunst (1991)
Marmoset	1	Cytoplasmic	Horisberger and Gunst (1991)
Hamster	2	cytoplasmic/nuclear	Horisberger and Gunst (1991)
Guinea pig	2	cytoplasmic/nuclear	Horisberger and Gunst (1991)
Rabbit	1	Cytoplasmic	Mortier and Haller (1987) Horisberger and Gunst (1991)
Bovine	2	Cytoplasmic	Horisberger (1988) Horisberger and Gunst (1991)
Caprine	1	Cytoplasmic	Mortier and Haller (1987)
Equine	2	Cytoplasmic	Horisberger and Gunst (1991) Heinz *et al.* (1994)
Pig	2	cytoplasmic	Horisberger and Gunst (1991) Horisberger (1992b)
Dog	1	cytoplasmic	Horisberger and Gunst (1991)
Cat	1	cytoplasmic	Horisberger *et al.* (1990b)

subphylum. Table 1.2 summarizes findings of Mx proteins for which cDNAs exist. Table 1.3 summarizes the findings of Mx proteins from species where molecular genetic data is either incomplete or does not exist.

Mx Alleles

Multiple alleles at both the mouse Mx1 and Mx2 loci have been identified. In the creation of the congenic BALB/A2G mouse strain, Mx1 and Mx2 genes demonstrated linkage, with no recombination seen in the generation of the congenic strain. Wild mice have been identified which have functional Mx1 alleles similar to A2G mice as evaluated by restriction fragment length polymorphism (RFLP) analysis, resistance to influenza virus, inheritance, and nuclear staining (Haller *et al.*, 1986). Over 66 strains of laboratory mice have been evaluated for the Mx^{-} phenotype and all but two are susceptible to influenza virus. The two resistant strains, A2G mice, the strain in which the original Mx^{-} phenotype was identified, and SL/NiA, a Japanese sub-line of SL mice (Abujiang *et al.*, 1996), share the same Mx1 allele seen in wild Mx^{-} mice as evaluated by the criteria listed above. The A2G mouse strain was derived at Glaxo Laboratories between 1942 and 1950 from an illegitimate mating between an A strain and unknown mouse (Staats, 1980). The Mx^{-} and M^{-} alleles in wild mice are estimated to occur with equal frequency and resistant and susceptible individuals have been identified among wild mice from both Europe and North America (Haller *et al.*, 1987). In evaluations of these animals it was found that one Mx^{-} wild mouse strain exhibited much lighter nuclear staining of interferon treated macrophages using the monoclonal antibody 2C12, but was indistinguishable from other Mx^{-} lines when using a polyclonal antibody, suggestive of a qualitative rather than quantitative difference in the Mx1 protein. Using RFLP analysis, Staeheli *et al.*, (1988),

identified three Mx1 alleles, designated types 1 through 3, in laboratory mouse strains which were also seen in inbred strains founded from wild mice. Type 1 includes A2G and SL/NiA. Type 2 is represented by BALB/c and includes nearly all inbred laboratory mouse strains. This allele of Mx1 is characterized by a genomic deletion, encompassing exons 9 to 11. The deletion leads to truncated mRNA and an out of frame mutation, generating a premature stop codon. Type 3 mice include CBA/J and two other lines. These lines have an A to T point mutation at bp 1378 of the Mx1 cDNA which generates a premature stop codon. The Mx1 mRNAs of RFLP types 2 and 3 are unstable in cells, and cycloheximide treatment is required to increase mRNA levels to the point where hybridization signal is visible by Northern analysis. There is no evidence of translation of these mRNAs. CBA/J mice (type 3) were found to use the alternate exon 1a in all cDNAs analyzed while the BALB/A2G transcripts contained exon 1a only one tenth of the time. Whether this was a chance finding, the result of cycloheximide treatment, or indicative of a genuine phenomenon was not rigorously assessed. If genuine, it may represent allelism at the MX1 loci or at loci involved in splicing. The RFLP type 3 allele is likely ancestral to the type 2 allele as they both share 6 silent mutations relative to the type 1 allele. The type 2 allele did not arise as part of the development of inbred strains of mice as all three types were identified in lines recently founded from wild mice (Staeheli *et al.*, 1988). In contrast to this last finding is a recent study of eight inbred mouse strains founded from wild stock form Asia, Europe, and North America (Jin *et al.*, 1998). All stains were found to be M^- as assessed by immunohistochemistry, and RT PCR. Within these Mx^+ strains two new alleles additional to the one represented by A2G, were identified by sequence analysis of exon 10 of Mx1, one of which had two point mutations over a span of six bp which led to

two successive amino acid substitutions relative to the Mx1 of A2G mice. The Mx2 gene is linked to Mx1 (Staeheli and Sutcliffe, 1988), however linkage mapping has not addressed their proximity. In all Mx^- laboratory strains studied, Mx2 has an inactivating frameshift mutation (Staeheli and Sutcliffe, 1988)

Excluding studies of mice, only a limited number of reports of Mx alleles exist in the published literature. Swine are reported to have an unstable Mx2 mRNA which impeded acquisition of a full cDNA (Müller *et al.*, 1992b), though two proteins have been identified in other studies (Horisberger and Gunst, 1991; Horisberger 1992b). However it remains an unanswered question whether the cloning difficulty was the result of innate instability of the transcript or instability conferred by a significant mutation. Assessment of equine Mx proteins identified species of Mx which could only be induced in peripheral blood mononuclear cells (PBMC) from 50% of horses tested (Heinz *et al.*, 1994). A larger and more basic equine Mx was identified, though only in cell culture, and was absent in PBMC of the population assessed. While these finding are intriguing, and suggest the possibility of equine MxA or MxB alleles, the existence of such has not been rigorously proven.

No natural polymorphisms in human Mx genes are known which influence MxA antiviral activity. Petersen and colleagues (1989) have reported two RFLPs linked to the human MX1 gene. A single nucleotide polymorphism (SNP) in the MxA cDNA has been reported (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?type=rs&rs=13920)(Locus ID = 4599, NCBI SNP ID = rs13920, Assay ID = ss16280) . The polymorphism would be predicted to lead to an amino acid substitution (H595R). This SNP has not been authenticated in the published literature. Recently, a human MxA polymorphism has been

used to establish a link between MxA genotype and increased risk for alopecia areata, an inflammatory condition of the skin and nails (Tazi-Ahnini *et al.*, 2000).

In conclusion, alleles of Mx genes have been studied to a significant extent only in the mouse, where it was found that the inactivating mutations, which are found in domestic strains, are also found in populations of wild mice. Two substantially differing alleles exist for duck Mx, neither of which is associated with antiviral activity (Bazzigher *et al.*, 1993). More recently RFLPs have been identified for sheep and trout Mx genes and at least in the case of the trout, linkage has been shown to a rhabdovirus resistant phenotype (Phua and Wood, 1997; Trobridge *et al.*, 2000). With regard to mice it is notable that a large number of wild mice possess mutations in both Mx1 and Mx2 genes. It is lamentable that no controlled epidemiological assessment of wild caught mice has been conducted. Such a study could help to identify the role of Mx in a natural environment

Mx Genomics

Using the Mx1 cDNA as a probe in Southern blot analysis of mouse-hamster somatic cell hybrids, the Mx locus was mapped to mouse chromosome 16 (Staeheli *et al.*, 1986c). Linkage analysis, reported in the same study, excluded a centromeric location on mouse chromosome 16, a finding confirmed by Reeves *et al.*, (1988). The loci of Mx1 and Mx2 are now mapped at the extreme end of chromosome 16, at 71.2 cM from the centromere (Mouse Genome Databases, 1999). The comparatively early mapping of mouse Mx1 paved the way for the Mx gene being listed for use as a type I anchor locus for comparative mapping (O'Brien *et al.*, 1993). In this capacity the Mx1 gene has played a role in syntenic mapping studies of a number of domestic animals. The confirmed and

predicted chromosomal location for numerous vertebrates for which Mx mapping data exits or can be predicted by synteny with other mapped genes is presented in Table 1.12.

The complete genomic structure of the mouse Mx1 gene is known (Hug *et al.*, 1988). The mouse Mx1 gene contains 14 exons, spanning 55 kbp. There is a very large intron, ~30 kbp, between exon 1a, which is non-coding, and exon 2. This large intron contains an optional exon, denoted exon 1b. It was assumed that exon 1b is included in transcripts as the result of splicing variation. Human MxA is also known to have an optional noncoding exon in the 5'-untranslated region (UTR) of 50% of transcripts (Horisberger *et al.*, 1990a). A large 5' genomic fragment of a perch Mx gene has been characterized (Staeheli *et al.*, 1989). The portion of the perch gene that was characterized was demonstrated to be homologous to the mouse Mx1 gene from exon 3 to exon 8. Intron-exon boundaries were completely conserved between these two vertebrates.

TABLE 1.4. CHROMOSOME LOCATION: MX GENES OF SELECTED SPECIES

SPECIES	GENE	CHROMOSOME (PREDICTED)	METHOD	REFERENCE
Human	MX1(MxA)	21	somatic cell hybrid analysis	Horisberger *et al.*, (1988)
		21q22.3	ISH	Huber *et al.*, (1988)
	MXB	21	somatic cell hybrid analysis	
Mouse	Mx1 & Mx2	16, 71.2 cM	somatic cell hybrid analysis	Staeheli *et al.*, (1986c)
			linkage mapped	Reeves *et al.*, (1988)
				Mouse Genome Database (1999)
Rat	Mx1	RNO 10	somatic cell hybrid analysis	Levan *et al.*, (1991)
Swine	Mx1	SSC 13, 154 cM	somatic cell hybrid analysis	Rettenberger *et al.*, (1996)
			linkage mapped	Archibald *et al.*, (1995)
Horse	MX1(MxA)	ECA 26q16-q17	direct mapping	Lear *et al.*, (1998)
Sheep	MX1	OAR 1q	somatic cell hybrid analysis	Broad *et al.*, (1995)
Bovine		(BTA1)	predicted by synteny with:	McAvin *et al.*, (1988)
			SOD1	Skow *et al.* (1988)
			PAIS and PRGS	Barendse *et al.* (1993)
			CRYA-1	Schmutz *et al.*, (1996)
			COL6A1	Barendse *et al.* (1997)
Cat		(CD 2)	predicted by synteny with:	O'Brien and Nash (1982)
			SOD1	Watson *et al.*, (1986)
			ETS2	

Mx: Member of a Family of Large Intracellular GTPases

Mx is a member of a family of large (~70-100 kDa) intracellular GTPases, all members of which possess low level, innate GTPase activity. These GTPases possess conserved structural elements, which include, in the amino-terminal portion, the tripartite GTPase domains (Dever *et al.*, 1987), and a characteristic sequence referred to as the dynamin family signature which acts as a self-assembly domain (Vallee and Okamoto, 1995). The carboxy-termini have characteristic domains which have helped in the distinguishing of subfamilies. A classification scheme has been proposed for this family of proteins (Liu and Robinson, 1995) which divides it into subfamilies based on structural similarity. One group consists of mammalian dynamins I, II, and III, as well as the product of the *Drosophila* gene *shibire* (Utrrutia *et al.*, 1997). This subfamily is characterized by pleckstrin homology and proline-rich domains in the carboxy terminus, which are involved in interactions with phospholipids and SH3 domain containing proteins. Rat dynamin (Obar, *et al.*, 1990) is involved in receptor-mediated endocytosis via clathrin-coated pits (Vallee *et al.*, 1993), and in signal transduction of ligand-induced activation of mitogen-activated protein kinase (Whistler *et al.*, 1999). The *Drosophila* protein *shibire* (van der Bliek and Meyerowitz, 1991; Chen *et al.*, 1991) is needed in the recycling of synaptic vesicles and in the scission of coated and uncoated vesicles from the plasma membrane. A second subfamily, characterized by a lack of pleckstrin homology and proline-rich domains in the carboxy terminus, is composed of the yeast proteins Vps1p and a recently isolated mammalian protein called *dymple*. The yeast protein Vps1/Spo15 (Rothman *et al.*, 1990; Yeh *et al.*, 1991) is involved in the retention of proteins in the *trans* Golgi. The human protein *dymple* (Kamimoto *et al.*, 1998) has an as yet

undetermined function. A third subfamily is composed of two plant proteins, soybean phragmoplastin (Gu and Verma, 1996), which is involved in cell plate formation, and the *Arabidopsis* protein aG68 (Dombrowski and Raikhel, 1995). The interferon-induced Mx protein and the yeast protein Mgm1 (Jones and Fangman, 1992) constitute the most divergent subfamily. The yeast protein Mgm1 is necessary for the maintenance of the mitochondrial genome in yeast. The Mx proteins are distinguished within this subfamily by the leucine zipper motifs in the carboxy terminus.

Enzymatic Properties, Structure and Cellular Biochemistry

All Mx proteins studied to date have three characteristic primary protein sequences. These include, in the amino portion, a tripartite sequence which confers GTPase activity, the dynamin family signature, which acts as a self-assembly domain (Nakayama *et al.*, 1993), and in the carboxy terminus, a leucine zipper motif, which is responsible for oligomer formation (Melén *et al.*, 1992). Post-translational modification of Mx proteins involving phosphorylation has been excluded (Weitz *et al.*, 1989; Melén *et al.*, 1992). In mouse Mx1 there are two *N*-linked glycosylation target sequences (Staeheli *et al.*, 1986b), but no documentation of glycosylation at these sites has been reported. The possibility of *O*-linked glycosylation has been addressed and no evidence was found for such (Melén *et al.*, 1992). Immuno-precipitation of mouse Mx1 produced by *in vitro* translation yielded a protein with similar mobility to Mx1 produced in cell culture, as assessed by SDS/PAGE (Staeheli *et al.*, 1985), suggesting that no significant post-translational modifications of Mx proteins occur. Enzymatic GTPase activity has been characterized in mouse Mx1 (Nakayama, *et al.*, 1991; Nakayama, *et al.*, 1992; Nakayama *et al.*, 1993; Pavlovic *et al.*, 1993; Melén *et al.*, 1994), human MxA (Horisberger, 1992a;

Pavlovic, *et al.*, 1993; Melén *et al.*, 1994; Richter *et al.*, 1995) and MxB (Melén *et al.*, 1992). The intact GTPase function of MxA and Mx1 is a requirement for antiviral activity (Melén and Julkunen, 1994; Pitossi *et al.*, 1993). Later studies have indicated, that at least in some situations, only GTP binding but not hydrolysis is important for virus-Mx interactions (Kochs and Haller, 1999a). The GTPase activity of Mx is wholly dependent on Mg^{++} and is both heat and cold sensitive. The Mx proteins tested showed no significant ATPase activity. There are discrepancies in the values reported in earlier studies, probably due to the purity of the proteins, since it has been shown that structural cellular proteins will co-purify with Mx (Horisberger, 1992a). However the more recently reported measures of GTPase activity are within a similar range. Hydrolysis values of Mx are ~2-20 mol GTP/minute/mol protein, which puts the GTPase activity at roughly the same level as seen with G-protein linked receptor-induced GTPase activity of the alpha subunit of Gs (Graziano and Gilman, 1989). This level of GTPase is still much lower than that seen for microtubule activated dynamin GTPase activity (200 mol GTP/minute/ mol protein, Shpetner and Vallee, 1992), thus placing the level of Mx GTPase more in line with a signaling rather than a mechanoenzymatic activity. The innate GTPase activity of Mx is quite unlike that of better known GTPases such as $p21^{ras}$, in that Mx innate GTPase activity is several orders of magnitude higher. Additionally, Mx does not appear to require guanine nucleotide release proteins and GTP activating proteins for continued cycling. The high K_d for GTP (~5-35 μM), versus the K_d for GDP (~100 nM), would make it appear there is likely little effect on the GTPase activity of Mx which results from normal guanidine nucleotide concentrations. The much lower affinity for GTP is illustrated by cross-linking studies that demonstrated that only a small fraction of Mx is in

its GTP bound form (1.1 nM for 1 μM protein). In addition to the requirement of the amino terminal sequences for GTPase activity, an intact carboxy terminus is also required. Mutant forms of MxA, with an internal deletion of amino acids 301-576 maintained GTPase activity, where as a mutant lacking the 7-carboxy amino acids had lost all GTPase activity (Schwemmle *et al.*, 1995a).

In addition to the GTPase sequences of Mx proteins, there exists a self-assembly sequence, also known as the dynamin family signature, which lies between the first two of the tripartite GTPase domains. Nakayama and colleagues (1993) have demonstrated through mutational studies that this domain maps to amino acids 51-99 of mouse Mx1. This domain leads to the self-assembly of Mx proteins into polymers. The native form of the Mx polymers appears as horseshoe like aggregates, which, when exposed to GTP, form globular or helical structures, as visualized by negative staining electron microscopy. Chemical cross-linking studies have shown most Mx in cells exist primarily as trimers (Melén *et al.*, 1992). In this same study, it was shown that the carboxy terminus of Mx1 contains both a leucine zipper motif and a nuclear transport sequence, which, when part of a chimeric molecule, could act to aggregate monomers as trimers and target them to the nucleus. Leucine zippers, which differ slightly between different Mx proteins within the same species, as is seen with human MxA and MxB, do not lead to the formation of heteromeric oligomers (Richter *et al.*, 1995; Melén and Julkunen, 1997). Ponten *et al.*, (1997) have shown that aggregation can also be conferred by the sequence of human MxA from amino acids 359-572. This region of the protein is neither part of the leucine zipper nor the self assembly domain. Taking the findings together gives a picture of the Mx protein in which many portions may be involved in self-aggregation. This conclusion is

supported by the findings of Garber *et al.* (1993), who identified multiple sites that impacted biological activity of mouse Mx1. Toyoda and colleagues (1995) have shown that the GTPase sequence may also be involved in normal aggregation of Mx1, but probably, in light of the finding of Schwemmle *et al.*, (1995b) discussed above, have simply demonstrated that full carboxy sequences are necessary for both oligomerization and GTPase activity. The relative importance and relationship of these domains with regard to self-aggregation has recently been addressed for the case of human MxA (Schumacher and Staeheli, 1998; Di Paolo *et al.*, 1999). In these most recent reports, it appears that the leucine zipper motifs cause an intramolecular association, or back folding which allows two other domains (amino acids 362-415 and 415-576) to play a role in intermolecular oligomerization. These interior domains, referred to some as the central interactive region, can be sterically disrupted with antibodies, thus disrupting GTPase and antiviral activity (Flohr *et al.*, 1999).

As mentioned previously, mouse Mx1 segregates to the nucleus. This is a characteristic of mouse Mx1 and rat Mx1 and has been shown to be dependent on basic residues in the 19 carboxy terminal amino acids of the protein (Noteborn *et al.*, 1987). Human MxB is found both in the cytoplasm and the nucleus (Melén *et al.*, 1996). The location of human MxB is dependent on the presence of an amino terminal nuclear targeting sequence, and whether or not this sequence is included depends on which of two ATGs is used for translation initiation (Melén *et al.*, 1996). The different forms of MxB are able to oligomerize via their leucine zippers, such that the smaller non-nuclear targeted form can be translocated by the by the larger form (Melén and Julkunen, 1997). The sequences responsible for nuclear accumulation of duck Mx (Bazzigher *et al.*, 1993) and

rainbow trout Mx2 (Trobridge *et al.*, 1997) have not been elucidated but are likely not mediated by a basic carboxy termini, similar to those seen in the rodent nuclear Mx1 proteins.

Electron microscopy has been used to address the nature of cellular and Mx protein interaction (Nakayama, *et al.*, 1993; Melén, 1996). In the case of mouse Mx1, it tends to form nuclear aggregates *in vivo*. Human MxB has been shown to localize to the heterochromatin region along the interior aspect of the nuclear membrane. MxB was also found associated with cytoplasmic membrane structures, as well as diffusely in the cytoplasm. There is evidence for the interaction of mouse Mx1 proteins as part of nuclear bodies (Chelbi-Alix *et al.*, 1995). Recently, using a yeast two-hybrid screen, an Mx1 interacting serine/threonine kinase, also associated with nuclear bodies has been identified, although the functional significance of this kinase interaction is unknown (Trost *et al.*, 2000).

Biological Activity of Mx Proteins

Mechanism of Action at the Whole Animal Level

Analyses of the mouse as a model for the mechanism of action of the Mx protein in the context of its native promoter provide an illustration of the mechanism of antiviral action at the whole animal level. Horisberger and de Staritzky (1989) demonstrated that with experimental interferon induction using NDV, Mx appeared in many tissues within 4 hours, peaked at 24 hours, and persisted over two weeks. The average biological half life was 3 days. When challenged with influenza A viruses having either liver or lung tropism, Mx1 accumulated in the target tissues of viral replication. Chang and colleagues (1990a)

examined tissue distribution of Mx production in response to interferon and interferon inducers. Interestingly, Mx protein was evident in control animals in the epithelia of the uterus and duodenum. Transcription of Mx was observed within 4 hours post interferon treatment, with highest responses seen in the liver, lungs, and uterus. Using *in situ* hybridization, the cells expressing Mx in the liver were determined to be Kupffer cells and the cells of the vasculature and sinusoids, rather than hepatocytes. The picture that emerges is one where systemic interferon induces Mx expression, thereby preventing or minimizing viremia, and hence disseminated disease. The nucleated components of the blood have been assessed for their ability to produce Mx. Polymorphonuclear cells do not produce measurable Mx in response to interferon (Heinz *et al.*, 1994), while monocytes and macrophages appear to be efficiently induced to produce Mx. In the case of viral induction of Mx, it is likely that tissue production of interferon has a paracrine effect, inhibiting the spread of virus within a tissue. A successful systemic interferon response is however, very likely necessary. An illustration of this is provided by an observation of the first reports of Mx (Lindenmann, 1962; Lindenmann *et al.*, 1963). In these studies it was found that very high doses of influenza virus (10^{-1}) led to survival, as did very low doses and (10^{-6}) while intermediate doses caused occasional death (10^{-4}), a so-called prozone effect. Interpretation of this result postulates a certain balance between strength of the initial virus inoculum and induction of a systemic response. With very week virus inoculum a paracrine effect of interferon and Mx may contain the infection. At very high virus dose, systemic interferon response is key to survival. At the intermediate level of virus dose, virus did not stimulate an adequate systemic response, and virus dose was too high to be controlled locally, leading to animals occasionally succumbing. However, it has

been noted, that mice resistant to influenza consistently produce less interferon than controls, and yet survive (Arnheiter and Staeheli, 1983). In the authors words it "appears that during the first rounds of viral replication, interferon is made in hardly measurable but sufficient amount to protect the highly interferon-sensitive animals against fulminate infection." As conclusion and reiteration of the previous comment, at the whole animal level three criteria must be met to obtain Mx-mediated resistance. The infecting virus has to induce at least some level of interferon response, the host has to be able to appropriately produce an Mx protein and the Mx protein must have an antiviral effect. Mx proteins have varied molecular mechanisms for inhibiting viral replication, many details of which are still poorly understood. The mechanism of resistance depends on the virus and host in question, and will be dealt with below as part of the discussion of viruses sensitive to Mx protein.

Antiviral Activity: Spectrum of Resistance

The most notable characteristic of Mx proteins is the antiviral effect that some possess. To date, antiviral activity has been evaluated for Mx proteins from humans, rats, mice, chickens, ducks and trout. A number of Mx cDNAs from other species have not been evaluated for antiviral activity of the proteins. Among these species are pig, sheep, horses, flounder, and salmon. Of those species whose Mx proteins do possess antiviral activity, *i.e.* human, rat, and mouse, not all proteins within a species have been confirmed to have antiviral activity. In the case of the human, MxA has antiviral activity but MxB does not. In the case of the rat, Mx1 and Mx2 are antiviral but Mx3 is not. Both mouse proteins have antiviral activity, though mutations in both genes leave many lines of mice unable to produce any Mx protein. One or more of the antivirally-active Mx proteins have

been shown to inhibit replication *in vitro* of vesicular stomatitis (Meier *et al.*, 1990; Pavlovic *et al.*, 1990; Zürcher *et al.*, 1992a), influenza A (Staeheli *et al.*, 1986b; Meier *et al.*, 1990; Pavlovic *et al.*, 1990), Thogoto (Frese *et al.*, 1995; Haller *et al.*, 1995; Hefti *et al*, 1999), Dhori (Thimme *et al.*, 1995), Batken (Frese *et al.*, 1997), measles (Schnorr *et al.*, 1993; Schneider-Schaulies *et al.*, 1994), and parainfluenza type 3 (Zhao *et al.*, 1996) viruses, plus several members of the Bunyaviridae (Frese *et al.*, 1996; Kanerva *et al.*, 1996; Hefti *et al.*, 1999). All of these viruses listed above are negative sense RNA viruses, and with the exception of antiviral activity of Human MxA against Semliki forest virus, a single stranded, positive sense RNA virus (Landis *et al.*, 1998; Hefti *et al.* 1999), antiviral activity of Mx proteins has been demonstrated only against negative sense viruses. The activity of Mx proteins appears to be discrete, in that not all proteins active against influenza A virus will be active against vesicular stomatitis virus. Nor will antiviral characteristics *in vitro* translate to a similar resistance *in vivo*. For example, rat Mx 2 and Mouse Mx2 have antiviral activity *in vitro*, but have not been shown to have a parallel phenotype *in vivo*.

Orthomyxoviridae

Using congenic strains of mice, Staeheli *et al.* (1984) reported compelling evidence for Mx1 as the sole mediator of antiviral activity against influenza virus. Clear proof of Mx as the sole mediator of resistance awaited the development of cells which constitutively expressed a mouse Mx1 cDNA (Staeheli *et al.*, 1986b). Cells, which had been stably transfected with the Mx1 cDNA, under the control of a constitutive promoter, were found to be refractory to influenza infection. Mice transgenic for constitutive production of Mx1 are also resistant to influenza infection (Kolb *et al.*, 1992). The only

other Mx proteins, which has been shown to have anti-influenza A virus activity are human MxA (Pavlovic *et al.*, 1990) and rat Mx1 and Mx2 (Meier *et al.*, 1990). The latter's activity was seen only if translocated to the nucleus through the inclusion of a nuclear targeting sequence (Johannes *et al.*, 1993).

Although reports exist which implicated translation of viral transcripts as a mechanism (Meyer and Horisberger, 1984), further evidence suggested that primary transcription was the target of Mx anti-influenza activity (Krug *et al.*, 1985). The possibility that Mx1 interfered with parental virion nucleocapsid transport to the nucleus or in the availability of cellular capped RNA was addressed and refuted by Broni *et al.* (1990). Pavlovic *et al.* (1992) provided evidence that Mx1 interferes with the transcription of the genes for the influenza polymerase proteins PB1, PB2, and PA. This conclusion is supported by the findings of Huang *et al.* (1992) and Stranden *et al.* (1993) that over expression of PB2 can titrate out the anti-influenza A virus activity of Mx1. Mouse Mx1 is only active in the nucleus. If it is targeted to the cytoplasm, antiviral activity against influenza virus is lost (Zürcher *et al.*, 1992c).

The mechanism of anti-influenza A virus activity of human MxA is not known with certainty. It is clear however that there is no defect in viral primary transcription. Viral polyadenylated transcripts were efficiently produced and transported to the cytoplasm. The defect is thought to interrupt viral transcript transport within the cytoplasm, viral transcript translation, or protein translocation to the nucleus (Pavlovic *et al.*, 1992). In an anomalous finding, Zürcher and colleagues (1992b) found that a nuclear-targeted mutant of human MxA was still able to restrict influenza virus. During a similar translocation rat Mx2 gained anti-influenza activity. It is most odd, that to accomplish this, human MxA

had to interfere at a different step in influenza A virus production. When translocated to the nucleus it functioned as did mouse Mx1, by limiting the accumulation of viral primary transcripts. In this regard, MxA is less restricted a form of Mx, since when mouse Mx1 was translocated to the cytoplasm, it lost all activity. Pavlovic *et al.* (1995) have documented improved resistance to influenza A virus infection in mice constitutively expressing the human protein. Though resistance was improved over littermates, as was seen with VSV, resistance was not total and the vast majority of challenged mice did eventually succumb to challenge. A mechanism for the anti-influenza activity of nuclear-targeted rat Mx2 has not been reported.

Although influenza was the virus that helped define the resistant phenotype that became known as Mx^{-}, a different member of the orthomyxoviridae is showing itself to be a more sensitive target. Now classed among the Thogoto-like virus group of orthomyxoviridae, Thogoto virus is a tick borne virus first isolated in the Thogoto forest of Kenya (Haig *et al.*, 1965). Evidence of infection of humans, ticks, livestock, and rodents, has been documented in Africa, Europe, and Asia (Moore *et al.*, 1975; Darwish *et al.*, 1979; Johnson *et al.*, 1980; Calisher *et al.*, 1987; Filipe and de Andrade, 1990). In livestock, Thogoto has been associated with febrile illness and abortions (Davies *et al.*, 1984). Thogoto virus has been implicated in neurological disease in man (Moore *et a.*, 1975). Human MxA and mouse Mx1 have both been shown to confer resistance to Thogoto virus (Frese *et al.*, 1995; Haller *et al.*, 1995). In the case of mouse Mx1, resistance was against lethal challenge at the whole animal level using a variety of both Mx^{-} and Mx^{-} mouse lines, as well as transgenic mice with constitutive Mx1 expression. The resistance was considered more dramatic than that seen with hepatotropic isolates of

influenza A virus. Cellular resistance was also documented. These findings are also notable in that, in contrast to the resistance to influenza, mice were resistant to a non-mouse adapted isolate of Thogoto virus. Though nothing is known of natural infections of mice with Thogoto virus, it has been isolated from rats. In the case of human MxA, resistance was seen with normal and mutant (MxA-Glu645Arg) protein. When MxA-Glu645Arg was targeted to the nucleus, it retained antiviral activity. In this respect, MxA has a similar spectrum of resistance to influenza as it does to Thogoto virus. Transgenic mice that constitutively express human MxA are resistant to lethal challenge from Thogoto virus (Pavlovic *et al.*, 1995) and resistance is profound, even in mice that are non-responsive to interferon (Hefti *et al.*, 1999). Virus yield in MxA transgenic mice was undetectable. The mechanism of resistance to Thogoto virus appears to involve GTP-dependent interference with the viral ribonucleoprotein component of the nuclear capsid, thereby preventing nuclear importation (Kochs *et al.*, 1998; Kochs and Haller, 1999a; Kochs and Haller, 1999b; Weber *et al.*, 2000).

Mouse Mx1 confers resistance to lethal infection with two tick borne Thogoto-like viruses, Batken and Dhori virus (Thimme *et al.*, 1995; Frese *et al.*, 1997). Resistance was complete in that 100% of animals with either intact Mx1 genes or constitutive expression of transgenic Mx1 survived lethal challenge. As was seen with Thogoto virus, Dhori virus yield from organs was below the limit of detection (Thimme *et al.*, 1995). Dhori virus resistance specific to Mx1 producing cells was also shown. In contrast to the case with Thogoto virus, human MxA was shown not to confer resistance to Dhori virus either *in vivo* (Thimme *et al.*, 1995), or *in vitro* (Frese *et al.*, 1995).

Paramyxoviridae

Human MxA has been evaluated for its antiviral activity against measles virus in two different cell lines, a human monocytic cell line (Schnorr *et al.*, 1993), and a human glioblastoma cell line (Schneider-Schaulies *et al.*, 1994). These studies were of note for the fact that, using the same Mx protein and the same virus, different stages of interference in viral replication were documented. In both studies viral yields were reduced ~100 fold. In the glioblastoma cells the mechanism appeared similar to that seen with VSV, *i.e.* attenuation of viral RNA synthesis, which impacted the distal measles virus transcripts most. In the case of the monocytic cell line, there appeared to be inhibited production of viral glycoproteins F and H. Measles virus transcription and transcript accumulation appeared not to be limited. These studies were of significance, not just for the activity they illustrated against a new class of virus, but because they indicate that Mx activity and mechanism of action may be influenced by cell type. Prior to these experiments the understanding was that Mx antiviral activity was manifest independent of other genes and proteins.

Human MxA has also been shown to have antiviral activity against human parainfluenza virus-3 (Zhao *et al.*, 1996). While the mechanism of action was not rigorously investigated, there was no inhibition of viral primary transcription, though NP and P transcript levels were much reduced.

Rhabdoviridae

The mechanisms of antiviral activity of four viruses, namely VSV, influenza A, Thogoto and measles viruses, have been studied in greater detail than other viruses susceptible to the antiviral effects of Mx. Human MxA has antiviral activity against VSV

(Pavlovic *et al.*, 1990). Pavlovic *et al.* (1995) have also documented improved resistance to VSV infection in mice constitutively expressing the human protein. Though improved over littermates, resistance was not complete and all challenged mice eventually succumbed. Staeheli and Pavlovic (1991) provided strong evidence that the mechanism of action involved a step in the transcription of the viral genome. This interference was thought to occur by one or more of the following mechanisms: interference of transcription initiation, attenuation of transcription at the intergenic sequences of the viral genome, or interference of RNA polymerase directed chain elongation. The VS L transcript appeared to be the most severely limited when evaluated in Swiss 3T3 mouse fibroblasts. Since the translation product of the VS L transcript is the viral RNA polymerase, it was considered that this step had the strongest effect on limiting VSV replication. No evidence was found for an antiviral role exerted by Mx at the steps of absorption, internalization or uncoating of the viral particles, or by interference of polyadenylation of viral transcripts. More recent work has shown *in vitro* that MxA acts by interfering with transcription initiation (Schwemmle *et al.*, 1995b). This inhibition required an intact GTPase site, though GTP hydrolysis was not necessary, suggesting that Mx need only bind GTP to exercise its antiviral activity. Also in the case of human MxA an amino acid substitution in the extreme carboxy portion (Glu645Arg) of the protein obliterates anti-VSV activity, while maintaining anti-influenza activity (Zürcher *et al.*, 1992b). An adequate model to explain these results does not exist but in the light of later observations (Schwemmle *et al.*, 1995a) one might speculate that the substitution disrupts GTP binding. Schuster *et al.* (1996) have reported that the polymerase of VSV is hyperphosphorylated in a human glioma tumor cell line (U-87) which constitutively

produces human MxA. It is intriguing to speculate on whether a recently isolated Mx associated kinase may be responsible (Trost *et al.*, 2000).

Rat Mx1 and Mx2 also have antiviral activity against VSV (Meier *et al.*, 1990). That rat Mx1, a nuclear protein, should inhibit VSV, a cytoplasmic virus is particularly puzzling. Regarding the anti-VSV activity of rat Mx2, the sequence of rat Mx2 and Mx3 differ by only 8 amino acids, which has allowed examination of the critical residues. Antiviral activity is dependent on the residues at 588 (Arg) and 630 (His) of rat Mx2 (Johannes *et al.*, 1997). Rat Mx2 must be in the cytoplasm to exercise its anti-VSV activity, since construction of nuclear targeted mutants of the protein lead to loss of anti-VSV activity (Johannes *et al.*, 1993), which, given that VSV replicates in the cytoplasm, is not unexpected. What was unexpected is that moving rat Mx2 to the nucleus conferred anti-influenza activity to the protein, whereas it had not had such an activity when it was found in the cytoplasm. Mouse Mx2 is similar to rat Mx2 in that it is a cytoplasmic protein active against only VSV (Zürcher *et al.*, 1992a; Jin *et al.*, 1999).

Bunyaviridae

Inhibition of La Crosse virus replication in cultured cells by human MxA was first reported by Frese *et al.* (1996), who found that MxA limited transcription and hence viral protein accumulation. Hefti (1999) reported a dramatic 45% survival rate of MxA transgenic mice to lethal IP challenge with La Crosse virus. All challenged mice had a defective interferon response system ($INFAR1^{-/-}$) and the experimental group expressed human MxA in a constitutive manner. With respect to spread of virus in the challenged animals, it appears that MxA was able to restrict virus spread, as none was detected in the $MxA^{+/+}$ animals brains, versus very heavy yield in those of the controls.

Replication of two phleboviruses, Rift Valley fever and sandfly fever Sicilian viruses, and three Hantaviruses, Tula, Puumala and Hantaan viruses, are inhibited by the human MxA protein (Frese *et al.*, 1996; Kanerva *et al.*, 1996). Hantaan virus and the two phleboviruses showed a several order of magnitude reduction in plaque formation. Phlebovirus yield from MxA cells approached the level of inhibition seen for influenza A virus. Hantaan virus transcription was not evident in MxA cells. The inhibition of Puumale and Tula virus was less dramatic, primarily because of technical difficulties in assaying virus.

Togaviridae

Landis and colleagues (1998) provided the first evidence that an Mx protein can inhibit replication of a positive sense RNA virus. They reported antiviral activity of human MxA against Semliki forest virus (SFV), a member of the togaviridae. Evidence suggests that resistance was mediated at a step between nonstructural gene translation and structural gene transcription. Hefti *et al* (1999) demonstrated 40% resistance to lethal challenge with SFV in mice constitutively expressing MxA. Virus replicated in all tissues tested but yields were two to three orders of magnitude lower than was seen in controls.

Viral Inhibition of, or Insensitivity to Mx

It is a familiar theme in the battle of host and virus that each will evolve mechanisms to overcome the other. The interferon system is no different. Several viruses are documented to have strategies to escape the effects of interferon (Young *et al.*, 2000; Cebulla *et al.*, 1999). Rosmorduc and colleagues (1999) reported that hepatitis B virus capsid protein mediated attenuation of the response of MxA to interferon, and that this was mediated by a *trans* acting suppression by the capsid protein of the MxA promoter.

This finding is likely a non-MxA specific effect on interferon response elements in the promoter, since hepatitis B virus is a DNA virus. It would seem odd in the extreme for this virus to develop a strategy to elude a protein which, in all cases, is active against only RNA viruses. Pavlovic and colleagues (1993) have reported a failure to detect influenza variants resistant to the antiviral effects of Mx . Dessens and Nuttall (1998) have reported that Thogoto virus can undergo nonviremic transmission if A2G mice are inoculated via infected ticks or are injected with tick salivary gland extract, containing virus, in contrast to injection of virus alone. This finding, if true, is likely the result of an anti-interferon, rather than an anti-MxA effect of tick saliva.

Respiratory syncytial virus (Paramyxoviridae) was found to be resistant to both the inhibitory effect of MxA under constitutive production as well as to interferon in general (Atrya and Kulkarni, 1999). Patients with confirmed rhinovirus infections were evaluated for Mx in their PBMCs as a means of assessing systemic interferon response to rhinoviral infection (Mäkelä *et al.*, 1999). No evidence of MxA induction was found in patients with rhinoviral infections, while those with influenza had MxA induction. As a positive sense RNA virus, one would not have thought that rhinoviruses should be susceptible to Mx inhibition. However the report listed earlier of inhibition of Semliki forest virus by Mx has cast this bit of dogma into doubt. While the study of Mäkelä and colleagues (1999) assessed a systemic interferon response, it would be of greater interest, from the standpoint of Mx studies, to examine the local MxA response, or the direct inhibition of rhinovirus by MxA .

Miscellaneous Properties of Mx

Potential Toxicity of Constitutive Expression of Mx

Mx proteins normally are synthesized only upon stimulation of cells with type I interferons, although some epithelial surfaces have been shown to produce Mx in what appears to be a constitutive manner (Chang *et al.*, 1990a). However mice kept gnotobiotically were not seen to express interferon in this pattern (Chang *et al.*, 1990b), thus implicating environmental exposure to microbes. Several reports suggest that constitutive expression of certain Mx proteins is toxic and,. in whole animals, incompatible with normal growth and development. Researchers were unable to obtain stably transfected clones for cells that expressed chicken Mx (Bernasconi *et al.*, 1995) or rat Mx1 (Meire *et al.*, 1990), and had difficulty establishing such cell lines with mouse Mx1 (Staeheli, 1990). Initial efforts to generated transgenic mice that expressed Mx1 from an SV40 early promoter yielded only mice that had rearranged, inactive transgenes (Kolb *et al.*, 1992). Similar difficulty was obtained making mice that constitutively expressed human MxA (Arnheiter *et al.*, 1996). Finally, a large amount of work was expended attempting to make pigs that expressed mouse Mx1 from a strong viral promoter, but no such transgenic pigs were produced (Müller *et al.*, 1992a). Collectively, these results indicate that prolonged or high level, constitutive expression of at least several Mx proteins is not compatible with cell survival.

Use of Mx in Clinical and Research Applications

There has long been an interest in using a type I interferon-induced protein as a marker for what are often transient elevations in plasma interferon (Schattner *et al.*, 1981). Mx proteins have also attracted attention for the specificity of the their response to

interferon, a quality which saw practical research application early on (Gresser *et al.*, 1988). These characteristics have prompted many to pursue use of Mx as such a marker, so that a number of assay systems for Mx proteins or Mx responses now exist. Production of Mx in cell lines has been used both to quantify interferon levels as well as to measure type I interferon neutralizing antibodies (Files *et al.*, 1998; Pungor *et al.*, 1998). An Mx promoter driven bioassay has also been described (Canosi *et al.*, 1996), and two assay systems have been described for use on whole blood samples (Towbin *et al.*, 1992; Oh *et al.*, 1994).

The inducibility of Mx by interferon and its stability make it an excellent marker of endogenous interferon responses to viral and infectious diseases and has received increasing use as such (von Wussow *et al.*, 1990a; Forster *et al.*, 1996; Halminen *et al.*, 1997; Fernández *et al.*, 1997; Chieux *et al.*, 1998). Mx has also been used to monitor response to interferon therapy for a variety of clinical conditions (Jakschies *et al.*, 1990a; Jakschies *et al.*, 1990b; von Wussow *et al.*, 1991a; von Wussow *et al.*, 1991b; Antonelli *et al.*, 1999; Kracke *et al.*, 2000). As a maker for the presence of type I interferon in research applications Mx responses have become the standard of assessment (Schiller *et al.*, 1990; Roers *et al.*, 1994; Jackshies *et al.*, 1994; Brod *et al.*, 1999; Fujii *et al.*, 1999; Keskinen *et al.*, 1999; Yuasa *et al.*, 1999). In clinical investigations of mechanisms of immune mediated disease or in diseases for which a viral agent or trigger have been postulated Mx has seen important application (von Wussow *et al.*, 1989; Fäh *et al.*, 1995; Yamada, 1995; Li and Youssoufian, 1997; Floege *et al.*, 1999). Mx promoters, due to their specific inducibility, have seen use as a tool in the immortalization of primary cell

lines (Winn *et al.*, 1999), as well as in the development of transgenic animals resistant to viral disease (Ono *et al.*, 1999).

The Interferon and Mx Systems In Cattle

Cattle have four sub-groups of type I interferon (α, β, ω, and τ), found on at least 32 genes located on bovine chromosome 8 (Ryan and Womack, 1993; Ryan and Womack, 1997). Both native and recombinant type I interferons have been evaluated in cattle as antiviral and immune modulating molecules (Babiuk *et al.*, 1985; Fulton *et al.*, 1986; Schwers *et al.*, 1989; Peel *et al.*, 1990). The ability of various viruses to induce an obvious interferon response have also been investigated in cattle (Savan *et al.*, 1979; El-Azhary, *et al.*, 1981). However, with the exception of 2', 5'-oligoA synthetase, the mechanisms of antiviral action, in terms of interferon induced proteins, has not been addressed in cattle (Perino *et al.*, 1990; Holland *et al.*, 1991).

At the initiation of the studies presented herein, the only information available on the bovine Mx system was from the report of Horisberger (1988). His work, based on immunoblots of interferon-stimulated MDBK cells, indicated the presence of two bovine Mx proteins with slightly different electrophoretic mobilities. This is in contrast to the sheep where there is only one protein identified, the cDNA of which has been cloned. By synteny with SOD1 and ETS1 and numerous other type I loci, the chromosomal location of Mx1 can be predicated to reside on *Bos taurus* (BTA1).

Conclusions

Despite a wealth of information on the molecular genetics of the Mx system in multiple species, essentially no work has been reported for any species that critically

addresses the potential role of Mx proteins in natural resistance to viral disease. Cattle would seem to represent an ideal species for pursuit of such an investigation. The dairy and beef industries have become quite sophisticated, which would facilitate molecular epidemiological investigations and potential links with Mx genotypes. A number of the economically-important diseases of cattle, including shipping fever complex, are caused by, or involve infection with, negative sense RNA viruses, which may be susceptible to inhibition by Mx proteins. Within the beef industry particularly, due to the fact that susceptibility to infection is typically seen after animals are removed from the gene pool, *i.e.* when in feed lots, the possibility exists that little selection pressure for functional Mx genes has occurred, thereby increasing the chance of finding significant diversity. Finally, if bovine Mx proteins are found to confer resistance to important viral disease agents and cattle populations manifest significant heterogeneity in Mx genes, it would be economically feasible to institute genetic screening program to select for disease-resistant animals.

In the research and clinical setting, a quick and economical whole blood diagnostic for Mx could find use in monitoring the strength of the interferon response to virus, or as a step in a diagnostic algorithm for abortion, which can have viral or bacterial causes. If Mx is found in the systemic blood as a result of interferon-τ production by the implanting fetus, use of an Mx test could find application in intensively managed herds as a pregnancy diagnostic. The best strategy for establishing a foundation to pursue the above described goals is the isolation and evaluation of a bovine Mx cDNA and characterization of the bovine Mx protein, which is the subject of this dissertation.

CHAPTER II

CLONING AND CHARACTERIZATION OF *BOS TAURUS* MX PROTEIN CDNAS AND LINKAGE MAPPING OF THE GENE TO *BOS TAURUS* CHROMOSOME 1

Introduction

The Mx system was discovered by Lindenmann (1962) as a heritable trait in A2G mice that conferred resistance to influenza A virus. Resistance was inherited as a simple autosomal dominant trait (Lindenmann, 1964), and the dominant allele was eventually designated Mx^r to denote myxovirus-resistance. The mouse gene responsible for resistance to influenza virus, Mx1, and a second mouse Mx gene (Mx2) have been cloned and sequenced (Staeheli *et al.*, 1986b; Hug *et al.*, 1988; Staeheli and Sutcliffe, 1988; Jin *et al.*, 1999). Additionally, homologues have been found in humans (Aebi *et al.*, 1989; Horisberger *et al.*, 1990a), rats (Meier *et al.*, 1990), horses (Chesters *et al.*, 1997), sheep (Charleston and Stewart, 1993), swine (Müller *et al.*, 1992b), chickens (Bernasconi *et al.*, 1995), ducks (Bazzigher *et al.*, 1993), trout (Trobridge and Leong, 1995; Trobridge *et al.*, 1997), salmon (Robertsen *et al.*, 1997), and flounder (Lee *et al.*, 2000). All Mx proteins are members of a superfamily of GTPases (Bourne *et al.*, 1991), which includes, among others, dynamin (Obar *et al.*, 1990), the yeast proteins VPS1 (Rothman *et al.*, 1990) and Mgm1 (Jones and Fangman, 1992), and the *Arabidopsis* protein aG68 (Dombrowski and Raikhel, 1995). In addition to GTPase activity, which is required for antiviral action (Pitossi *et al.*, 1993), Mx proteins possess a sequence that functions as a self-assembly

domain, known as the dynamin family signature, as well as leucine zipper motifs which lead to oligomerization of the protein (Melén *et al.*, 1992). Strong sequence conservation of Mx proteins among vertebrates argues for an important role for this gene, but a non-redundant, constitutive, or developmental function has not been found. Furthermore, all but two of the many inbred strains of mice examined have inactivating mutations in both Mx genes (Staeheli and Sutcliffe, 1988; Staeheli *et al.*, 1988), mutations which do not appear to have a deleterious effect on normal growth and reproduction.

Potent, though restricted, antiviral activity is the most striking feature of many Mx proteins, and has been demonstrated in cultured cells that constitutively express human MxA (Pavlovic *et al.*, 1990; Schnorr *et al.*, 1993; Schneider-Schaulies *et al.*, 1994; Frese *et al.*, 1995; Frese *et al.*, 1996; Zhao *et al.*, 1996; Kanerva *et al.*, 1996; Landis *et al.*, 1998), mouse Mx1 and Mx2 (Staeheli *et al.*, 1986b; Zücher *et al.*, 1992a; Haller *et al.*, 1995; Thimme *et al.*, 1995; Frese *et al.*, 1997; Jin *et al.*, 1999), and rat Mx1 and Mx2 (Meier *et al.*, 1990). Some Mx proteins, including human MxB, duck Mx, and chicken Mx, are apparently devoid of antiviral activity (Bernasconi *et al.*, 1995; Bazzigher *et al.*, 1993; Pavlovic *et al.*, 1990). Except for the demonstration that human MxA inhibited replication of the alphavirus Semliki forest virus (Landis *et al.*, 1998; Hefti *et al.* 1999), the antiviral spectrum of Mx proteins appears restricted to negative-sense RNA viruses. One or more Mx proteins have been shown to inhibit replication *in vitro* of vesicular stomatitis (Meier *et al.*, 1990; Pavlovic *et al.*, 1990; Zürcher *et al.*, 1992a), influenza A (Staeheli *et al.*, 1986b; Meier *et al.*, 1990; Pavlovic *et al.*, 1990), Thogoto (Frese *et al.*, 1995; Haller *et al.*, 1995; Hefti *et al*, 1999), Dhori (Thimme *et al.*, 1995), Batken (Frese *et al.*, 1997), measles (Schnorr *et al.*, 1993; Schneider-Schaulies *et al.*, 1994), and

parainfluenza type 3 (Zhao *et al.*, 1996) viruses, plus several members of the Bunyaviridae (Frese *et al.*, 1996; Kanerva *et al.*, 1996; Hefti *et al.*, 1999).

Despite the wealth of nucleotide sequence data and information on *in vitro* antiviral effects, essentially nothing is known about the role of Mx proteins in natural resistance to viral disease. Mice that carry wild-type forms of Mx1 are resistant to lethal challenge with influenza A and Thogoto viruses (Haller *et al.*, 1995; Arnheiter *et al.*, 1990; Arnheiter *et al.*, 1996), but influenza A is not considered a normal pathogen of mice and Thogoto virus has not been characterized well enough to know its status as a significant murine pathogen. The information on wild type and null mutant alleles in mice has been documented in strains founded from wild caught animals (Haller *et al.*, 1986; Haller *et al.*, 1987; Jin *et al.*, 1998; Jin *et al.*, 1999), but sadly no field epidemiological data exist for mice. The human MxA protein has been very well characterized with regard to antiviral properties, but *in vivo* studies addressing the importance of human Mx genes or proteins, relative to viral disease resistance, have not been reported. However, human MxA has been the focus of clinical investigation as a marker for the presence of interferon in disease states (Halminen *et al.*, 1997; Li and Youssoufian, 1997; Jakschies *et al.*, 1994), and to evaluate therapeutic use of interferon (Jakschies *et al.*, 1994). Among agriculturally important animals, cattle may provide an excellent opportunity to address the question of Mx activity *in vivo* as they are naturally infected with several viruses known to be sensitive to one or more Mx proteins, including vesicular stomatitis, parainfluenza type 3, and Rift Valley fever viruses. Additionally, if Mx alleles with differing antiviral properties exist in cattle, as they do in wild mice (Haller *et al.*, 1986; Haller *et al.*, 1987), Mx might be exploited as a marker for development of disease

resistant cattle. As a first step toward characterization of the bovine Mx system, several bovine Mx cDNAs were cloned and the sequences obtained. Following a strategy used to clone the ovine Mx cDNA (Charleston and Stewart, 1993), a bovine endometrial cDNA library, prepared using RNA from endometrium of early pregnancy, was screened. As expected, Mx clones were found in abundance in the endometrial library, owing to the fact that the ruminant embryonic trophoblast produces interferon–τ in great quantities (Farin *et al.*, 1990; Hansen *et al.*, 1999). The two full-length bovine cDNAs isolated showed a high degree of identity to Mx genes from other species, and represent two alleles of a bovine Mx homologue. A polymorphism in the 3'-UTR permitted linkage mapping of the Mx1 gene locus (designated MX1) distally on *Bos taurus* chromosome 1 (BTA1). Treatment of Madin-Darby bovine kidney (MDBK) cells with type I interferon induced expression of the Mx mRNA at times and levels consistent with a type I interferon-induced gene. Evaluation of bovine Mx proteins has been described once in the published literature (Horisberger, 1988). To validate and expand these initial findings, production of bovine Mx protein was evaluated *in vitro* using MDBK cells treated with type I interferon. The monoclonal antibody 2C12 (Staeheli *et al.*, 1985) bound a cytoplasmic protein in interferon-induced cells. The mobility of this protein on SDS/PAGE was consistent with a protein of approximately 75 kDa. The kinetics of *in vitro* bovine Mx induction as a dose response to type I interferon are consistent with what is known of Mx in other species. A previous report of immunological characterization of bovine Mx proteins indicated that two species of bovine Mx protein exist which we were unable to convincingly confirm using the monoclonal antibody mentioned above, (Horisberger, 1988).

Materials and Methods

Cells, Interferon, and Interferon Assays

MDBK (Madin and Darby, 1958), fetal lamb kidney, and 2C12 (Staeheli *et al.*, 1985) cells were cultured at 37°C in 5% CO_2 using Dulbecco's modified Eagle medium supplemented with calf serum to 8%, and penicillin and streptomycin. The 2C12 hybridoma cell line was the kind gift of Dr. Otto Haller of the Universität Freiburg, and was supplied by Dr. Heinz Arnheiter of the NIH. Mx synthesis was induced in the ovine cells by exposure to recombinant bovine interferon-β, which was produced by transducing D17 cells with retroviral particles carrying a bovine interferon-β gene under the control of a constitutive promoter (Haskell, 1995). Mx synthesis was induced in the bovine cells by exposure to recombinant human interferon-α-2b (INTRON-A, Schering Corp., Kenilworth, New Jersey). Interferons were assayed by micro-plaque reduction (Langford *et al.*, 1981), using MDBK cells as indicators and vesicular stomatitis virus (VSV, Indiana serotype) as the challenge. All interferon units of INTRON-A refer to units of activity on MBDK cells. Briefly, MDBK target cells were seeded into 96 well micro titration plates at a concentration of 35,000 cells per well. The following day, medium was removed and interferon applied to the cells. Interferon samples were diluted using media with 1.0% fetal bovine serum (1.0% FBS), and serial 0.5 log dilutions were made directly in the wells. Cells were allowed to remain in culture for 24 hours and then exposed to 50 plaque forming units of VSV/well. Virus was applied to the wells after interferon samples had been removed and the wells rinsed twice with Saline-A. Virus was diluted with 1% FBS to a concentration of 2,000 PFU/ml and 25 µl added to each well. The 96 well plates were returned to the cell culture incubator and virus was allowed to be absorbed for

1 hour with intermittent agitation, after which the inoculation medium was discarded and wells washed as above. Cells were then overlain with methycellulose-containing culture media, returned to culture, fixed and stained with crystal violet in methanol 24-36 hours post virus exposure, and plaques counted by visual inspection using a dissection microscope. Samples were run in duplicate, and both positive (untreated, infected) and negative (untreated, uninfected) controls were run as triplicates. One unit of interferon, by convention, is equal to the highest dilution which is able to reduce the plaque number/well by greater than 50%, relative to control wells. The limit of sensitivity of this assay is 9 U/ml.

Library Screening, cDNA Isolation, and DNA Sequencing

A bovine endometrial library prepared using lambda ZapII phage and tissues collected at day 17 of pregnancy (Mathialagan *et al.*, 1996), was kindly provided by Dr. Thomas. R. Hansen (University of Wyoming). The tissues used to generate this library originated from four Angus-Gelbvieh crossbred cows. Phage were plaqued on XL1-blue *E. coli* bacteria, and lifts prepared on nylon membranes (Hybond N+, Amersham Life Sciences Inc., Arlington Heights, IL) were hybridized to ovine or bovine Mx cDNA probes labeled with ^{32}P by random priming. Hybridizing phage were purified to homogeneity and the phagemid obtained by *in vivo* excision.

A fragment of the ovine Mx cDNA corresponding to positions 209 to 989 of the published sequence (Charleston and Stewart, 1993) was obtained by polymerase chain reaction (PCR). Total RNA was isolated from fetal lamb kidney cells that had been exposed for 6 hours to bovine interferon-β, reverse transcribed, and amplified using primers oMX1 (5' CTGCATTGACCTCATCGACT) and oMX2 (5' CGGCACTTGACG-

ATCATGTA). The resulting product was sub-cloned into pKS(-) (Stratagene Inc., La Jolla, CA), and its nucleotide sequence obtained to confirm identity.

DNA sequencing was conducted by Macromolecular Resources at Colorado State University using an Applied Biosystems Model 377 DNA sequencer. The two full-length bovine Mx clones (Genbank AF047692 and U88329) were sequenced bi-directionally, whereas other sequence data referred to were uni-directional. Nucleotide and deduced protein sequences were aligned using the CLUSTALW program of the European Bioinformatics Institute (Thompson *et al.*, 1994).

Northern Blot Analyses

Total RNA was isolated by guanidinium-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) from MDBK cells harvested at 0^{+}, 1.5, 3, 6, 9, 12, and 24 hours after exposure to recombinant human interferon at a concentration of 1,000 U of bovine activity per ml. Total RNA (20 μg) was electrophoresed through a 1% formaldehyde-agarose gel (Sambrook *et al.*, 1989), transferred to nylon membrane, and hybridized overnight to a PCR generated antisense ^{32}P labeled probe (bp 1988-2243 of bovine). Final washing was conducted at 50°C in 0.5X SSC/0.1% SDS, and autoradiographs were prepared using Reflection autoradiography film (New England Nuclear, Boston, MA). The membrane was stripped and hybridized to a human γ-actin random-primed ^{32}P-labeled probe, and exposed to film. Values from scanning densitometry (Hoefer Scientific Instruments, San Francisco, CA) of the Mx signals were normalized for loading error using data from the γ-actin autoradiograph.

Genomic DNA PCR

DNA was isolated using standard techniques (Sambrook *et al.*, 1989), from endometrial tissue of the four cows that contributed to the cDNA library (generously supplied by Dr. Thomas R. Hansen, University of Wyoming), and from tissue or blood collected from other cattle. These samples were subjected to PCR using primers BMX3.1 (5' CCGTAGTCTCTGCTGTCTCT) and BMX3.2 (5' ACCCTTCTACAGTGCTGTGT), which amplified a fragment of the 3'-UTR of bovine Mx. Each 50 µl reaction contained 500 ng genomic DNA, 10 pmol of each primer, 0.8 mM dNTPs, 1.7 mM $MgCl_2$, and 0.25 U Taq DNA polymerase in the supplied buffer (Life Technologies, Gaithersburg, MD). Amplification was performed using a Stratagene Robocycler for 35 cycles (95°C for 1 min, 58°C for 1 min, and 72°C for 1 min). Products were electrophoresed in 4% agarose, transferred to nylon membrane, and hybridized to an antisense PCR-labeled probe (bp 1988-2243 of bovine Mx1). Following washing, autoradiographs were prepared as described above.

Genotyping of bovine Mx1 by PCR for linkage mapping was performed by Dr. Sheila M. Schmutz and colleagues at the University of Saskatchewan, using genomic samples of cattle from linkage mapping families. The PCR reactions followed the protocol above with the following changes: reaction volume was 15 µl containing 50-100 ng of DNA, 0.7 mM dNTP, 1.5 mM $MgCl_2$, and 0.1 U *Taq* DNA polymerase. Cycling conditions were as follows; 4 min at 95° C, followed by 30 cycles of 30 sec at 95° C, 30 sec at 58° C, 15 sec at 72 ° C, and finishing with a 4 min extension at 72° C. Products were evaluated by electrophoresis in a 4% agarose gel, stained with ethidium bromide.

Linkage Mapping

Linkage mapping used the Canadian beef cattle reference families (designated CCA, for Canadian Cattlemen's Association): 17 full-sibling cattle families from 5 sires and 16 dams, comprising the following breeds; Angus, Belgian Blue, Charolais, Hereford, Limousin, and Simmental (http://sask.usask.ca/~schmutz). All founders of the CCA families and a selection of unrelated bulls were genotyped to assess the degree of polymorphism of MX1. Four families possessed the polymorphism and were used for linkage. Additional families included four of the USDA gene mapping families (http://sol.marc.usda.gov/genome/cattle/). Linkage analysis involved computation of the maximum log of odds score (LOD) for linkage of the MX1 gene to 4 microsatellites on BTA1; BMS4043 (Sonstegard *et al.*, 1997), BM1824, BM6506, and BR2724 (Bishop *et al.*, 1994). Initial computation of LODs proceeded by 5 cM increments up to a distance of 45 cM. Microsatellites markers which yielded significant LOD scores (≥3) were then calculated using 1 cM increments.

Bovine Mx Production: Induction and Transfection

Transient transfection of Vero cells with an expression plasmid (pTARGET™, Promega, Madison, WI) containing the cDNA of bovine Mx1 used calcium phosphate precipitation and followed a conventional protocol (Sambrook *et al.*, 1989). Cells were fixed and processed as described below 48 hours post transfection. All other protein studies used MDBK cells induced to produce Mx with INTRON-A. For immunocytofluorescence, MDBK cells were seeded sparsely onto glass chamber slides and incubated as described above with the exception that the culture medium contained either

1,000 U/ml of interferon (treated cells) or no added interferon (control cells). Cells were further incubated for 48 hours. Chamber slides were then washed in Dulbecco's saline A and fixed for 5 minutes with a 4% paraformaldehyde solution. Chamber slides were rinsed briefly with fluorescent antibody wash buffer (FAWB) and cell membranes permeabilized by incubation in FAWB with Triton X-100 (0.05%) for 1 minute. Chamber slides were washed three times for 10 minutes with FAWB. Chamber slides were then exposed to the primary antibody (2C12, as an undiluted hybridoma supernatant) diluted 1:3 in FAWB, and incubated overnight in a 4° C humidifying chamber. Chamber slides were washed three times for five minutes each with FAWB and incubated in a room temperature humidifying chamber for 45 minutes with a FITC-conjugated anti-mouse IgG antibody diluted 1:100 in FAWB. Final washing was in FAWB for three times five minutes each, and slides were coverslipped with 9:1 (v/v) glycerol:FAWB. Cells were examined using a Nikon eclipse E800 microscope (Melville, NY) and images were captured digitally using a 3CCD camera (Dage-MTI, Inc. Michigan City, IN) and Image-Pro® Plus software (Media cybernetics, Silver Spring MD).

Validation of the monoclonal 2C12 for ability to cross-react with a bovine Mx by immunoblot analysis employed cells exposed to 1,000 U/ml for 24 hours. Negative control cells were handled identically except they were not induced with interferon, but were incubated with normal media. For dilutional studies, MDBK cells were induced for five days with 5,000 U/ml. Dose response studies were conducted as follows. Culture dishes (six cm) containing MDBK monolayers were exposed separately to 1.0 $\log_{10}$ dilutions of interferon at concentrations from 10,000 U/ml to 0.1 U/ml. Cells were harvested at 60 hours post induction and stored as 50 µl aliquots at an estimated

concentration of 20,000 cells/μl. A standard was prepared which consisted of MDBK cells exposed to 1,000 U/ml for 24 hours, and was included in the dose response SDS/PAGE gels as serial two-fold dilutions. To harvest cells, the monolayers were washed with Dulbecco's saline A and disassociated with trypsin. An aliquot was used to calculate cell concentration using a counting chamber, while the remaining cells were collected by centrifugation. Cells were resuspended in lysis buffer (50 mM Tris HCl, pH 7.4) at a concentration, unless otherwise stated, of 20,000 cells/μl, and stored at -70° C.

Total cell lysates were subjected to SDS/PAGE using the Laemmli (1970) buffer system. Total protein concentrations of the dose response cell lysates were measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA), and 5 μg of protein from each sample loaded into lanes on a SDS/PAGE gel. Gels were prepared and electrophoresed using a Bio-Rad Mini Protean cell, and proteins were transferred to nitrocellulose membrane using a Bio-Rad mini Trans-Blot cell. After transfer, membranes were washed with phosphate buffered saline containing Tween-20 (0.1%; PBS-T), for 15 minutes followed by a second, five minute wash. Blocking of non-specific binding sites was accomplished by incubating the blot in 5% non-fat dried cows milk in PBS-T at 4° C, overnight. The primary antibody (2C12, undiluted hybridoma supernatant) was used at a 1:3 dilution in PBS-T, and incubated at 4° C for 1.5 hours. Two rinses, one of 15 and one of five minutes using PBS-T, were followed by incubation for 1.5 hours at 4° C with the secondary, anti-mouse IgG antibody (diluted 1:1,000 in PBS-T), supplied with the chemiluminescent imaging kit (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were then washed five times in PBS-T, once for 15 minutes and four times for five minutes. Chemiluminescent autographs were generated using ECL reagents and

Reflection autoradiography film (New England Nuclear, Boston, MA). Optical densities of the chemiluminescent autograph bands for the maximally induced MDBK cells were quantified by densitometric scanning (Hoefer Scientific Instruments, San Francisco, CA). Densitometric scanning of the immunoblot autograph of the Mx dose response to interferon employed an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA). Integration values of the area under the curve of three scans per lane were averaged. The values of the dilution immunoblot were plotted as a percent of the maximal value. Comparison of immunoreactive chemiluminescent signals of the dose response samples and standard, as measured by densitometric scanning, was used to calculate the amount of Mx, as measured in Mx units. One Mx unit was arbitrarily set as the amount of Mx in 6,000 MDBK cells induced for 24 hours with 1,000 units of interferon. The dose response immunoblot data were plotted as Mx units on the y-axis and a log scale of interferon units/ml on the x-axis. Both standard curve values were linear with high correlation between the estimated number of cells contributing to the total cell lysate and the resulting densitometric reading ($r = 0.98$). The limit of detection for the dose response assay was 0.2 Mx units, based on the values of the highest dilution of the standard. The relationship of the log-transformed values of the interferon unit concentration and the Mx units was linear over 3 orders of magnitude, with a high correlation ($r = 0.99$).

Results

Isolation of Bovine Mx cDNAs

The endometrial library was screened initially using a fragment of ovine Mx cDNA, which identified several partial-length bovine Mx cDNA clones. To efficiently

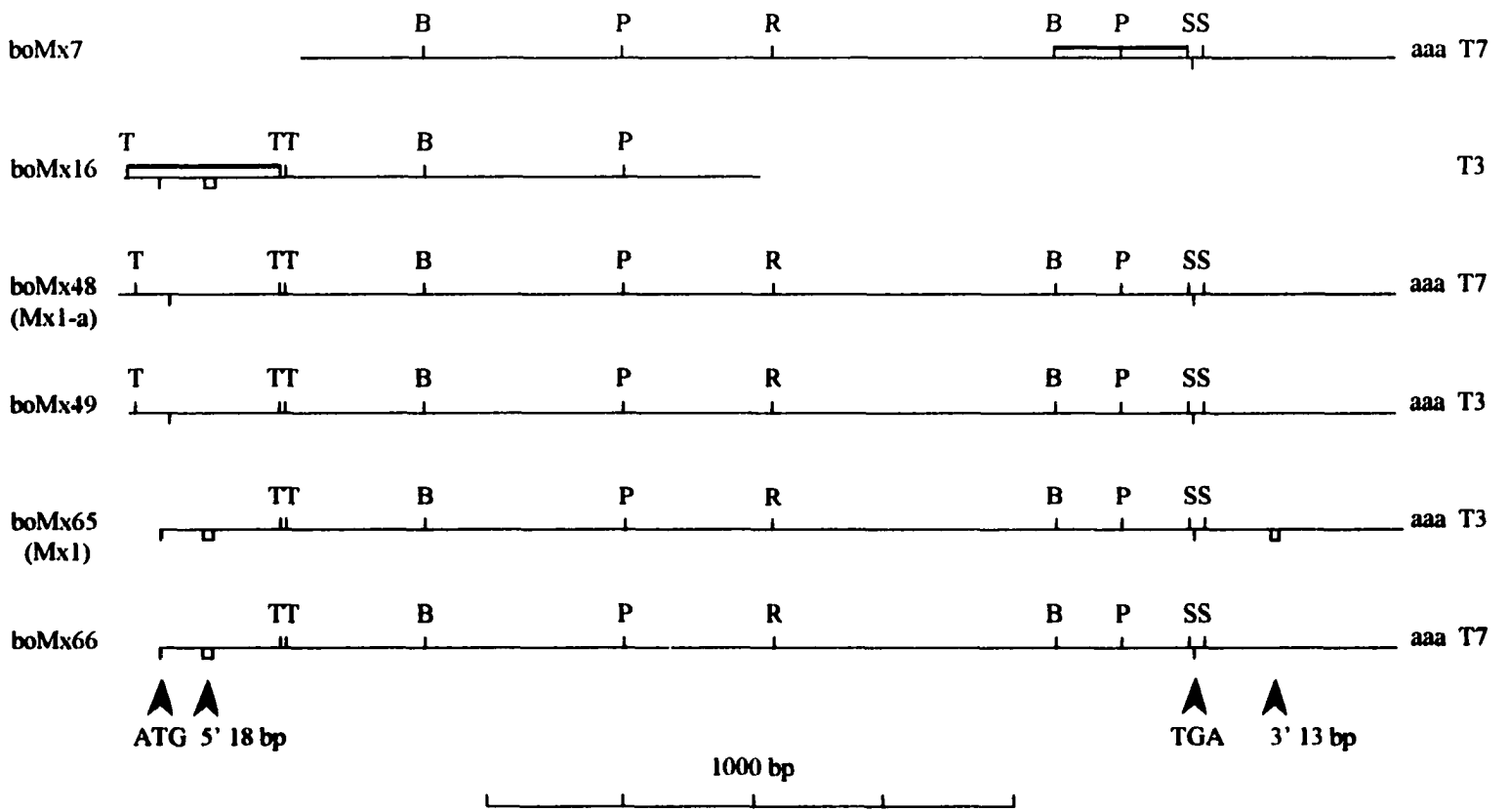


Figure 2.1. Phagemid cDNA clones of bovine Mx1. Restriction sites *SstI* (T), *EcoRI* (R), *BglII* (B), *PstI* (P), and *SmaI* (S) are indicated by upward ticks. T3 and T7 indicate orientation in the phagemid vector. Bold lines indicate probes used to isolate full length clones. Initiation and stop codons are indicated by downward ticks and arrows. An 18 bp segment in the 5' coding region and a 13 bp segment in the 3' UTR are shown as shaded boxes and indicated by arrows.

isolate full–length clones, subsequent screening of the library employed fragments at the extreme ends of two bovine Mx cDNAs (Figure 2.1). Fragments from both BoMx7 and BoMx16 were sequentially hybridized to primary lifts, and only those plaques hybridizing to both probes were purified. As anticipated, the library contained a large number of Mx clones, estimated to be approximately 0.1% of all phage. Eleven hybridizing clones were at least partially characterized, of which four appeared to be full length. Analysis of nucleotide sequences indicated that at least two species of bovine Mx cDNA had been isolated. The first species is represented by clones boMx48 and boMx49 (Figure 2.1). These clones appeared to be separate cDNAs of the same transcript, as judged by restriction digestion, sequence data, and their orientation in the phagemid. Clone boMx48 was sequenced in its entirety and is referred to as bovine Mx1-a (Genbank U88329). Neither boMx48 nor boMx49 contained an 18-bp sequence identified in the 5'-coding region of clone boMx16 and also present in the ovine Mx cDNA, suggesting that a second species of bovine Mx was present in the library. Further screening resulted in isolation of clones boMx65 and boMx66, both of which contained an *HphI* restriction site indicative of the 18-bp segment in the 5'-coding region. Based on restriction analyses, these two clones also appeared to be full-length and differed in their orientation within the vector. Clone boMx65 was fully sequenced and is referred to as bovine Mx1 (Genbank AF047692).

The bovine Mx1-a cDNA comprises 2424-bp followed by a polyadenylate tail. An open reading frame of 1947-bp is flanked by 98-bp and 379-bp of 5'-UTR and 3'-UTR, respectively (Figure 2.2). The predicted amino acid sequence of 648 residues yields a calculated mass of 74,728 Da and a theoretical pI of 5.61. The translation start site, AG-

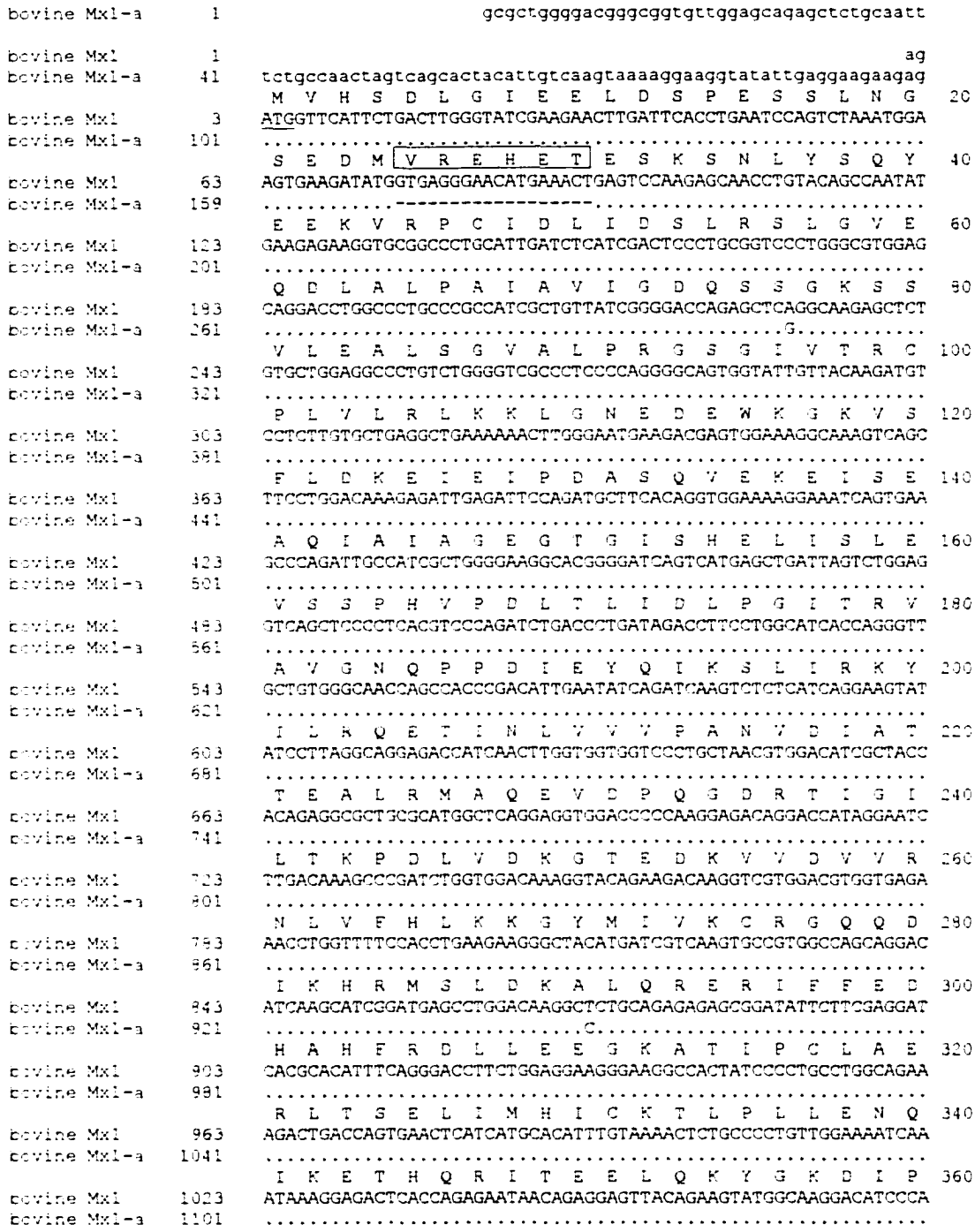


Figure 2.2. Bovine Mx1 and Mx1-a cDNA alignment (Genbank accession numbers AF047692 and U88329). Dots designate identical nucleotides and dashes designate gaps. The translation sequence is of bovine Mx1. Boxed residues are absent in bovine Mx1-a. The Pro_{370} of Mx1 is altered to Leu_{364} in Mx1-a. Initiation and stop codons, and the potential polyadenylation signal are underlined. Arrows indicate primer sites for PCR amplification of 3'-UTR polymorphism.

Figure continued on next page

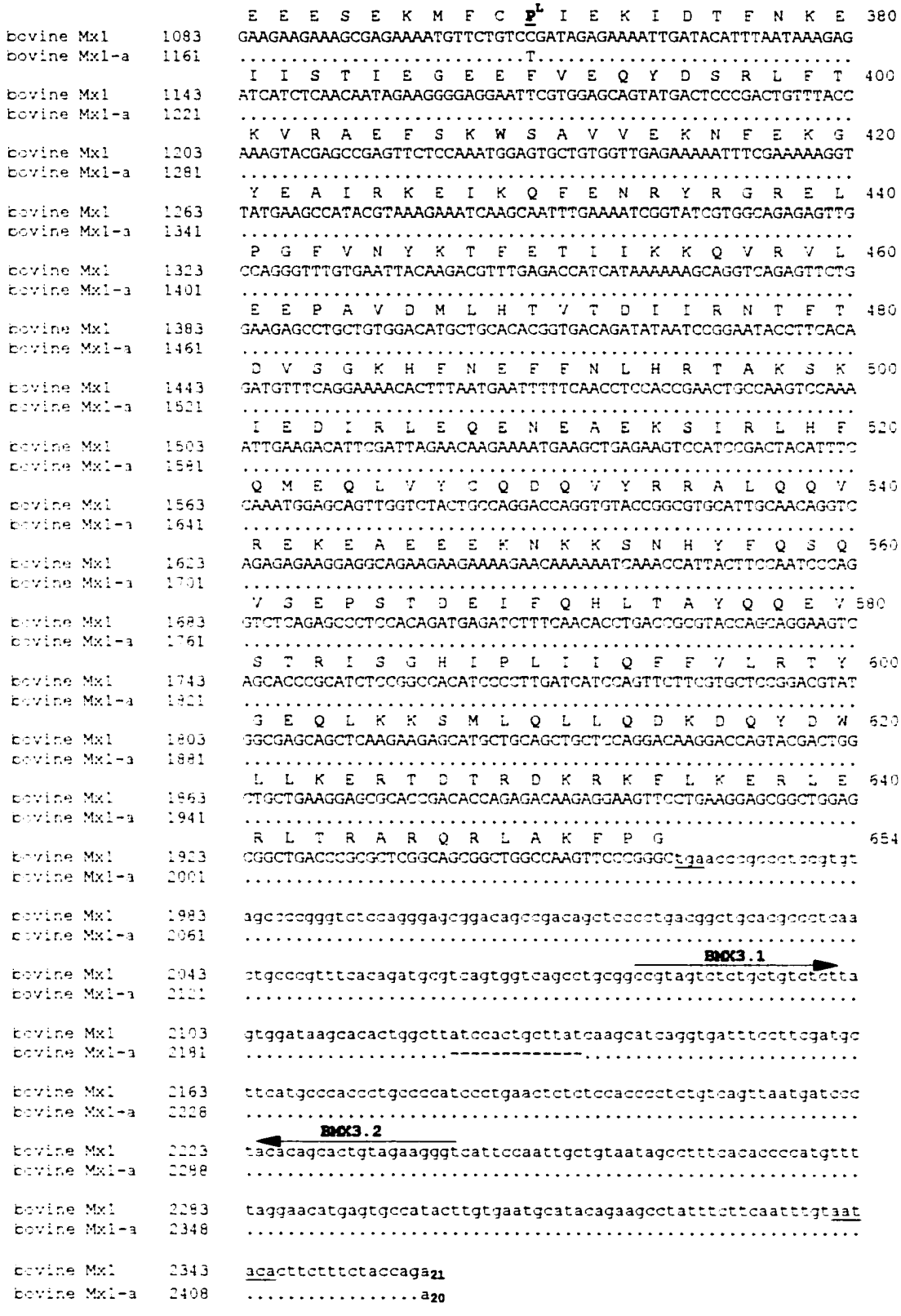


Figure 2.2. *Continued*

GAAGAAGATGG, conforms to the sequence proposed by Kozak (1987), having a purine at –3 and a G at +4, and is nearly identical to the consensus sequence derived from other Mx genes, AGAN$^{G}/_{A}$GAAGATGG (Müller *et al.*, 1992b). The 379-bp 3'-UTR does not have the consensus polyadenylation signal AATAAA, but rather a potential polyadenylation signal AATACA beginning 19-bp before the polyadenylate tail. Based on *in vitro* experiments, this sequence would be expected to function inefficiently as a polyadenylation signal (Sheets *et al.*, 1990), raising the possibility this is not the true signal but rather a sequence upstream of an A-rich region which was primed by the oligo-dT primer.

The bovine Mx1 cDNA contains 2,359-bp followed by a polyadenylate tail. The open reading frame of 1,965-bp is preceded by 2 bp and followed by 392-bp of 3'-UTR (Figure 2.2). Considering the short length of putative 5'-UTR sequence in the clone, assignment of the ATG at position 3 as the initiation codon was based on the near identity of the coding region of Mx1 and Mx1-a. Additionally, the sequence of clone boMx16, which shares with bovine Mx1 the 18-bp segment (Figure 2.1), is identical to bovine Mx1-a for 60-bp upstream of the beginning of the deduced coding region. The predicted amino acid sequence of 654 residues gives a predicted mass of 75,541 Da with a predicted pI of 5.60. The putative polyadenylation signal of bovine Mx1 conforms to that of bovine Mx1-a.

The sequences of bovine Mx1 and bovine Mx1-a differ in six locations (Figure 2.2). Within the 5'-coding region, bovine Mx1 contains an 18-bp segment absent in bovine Mx1-a, but present in the ovine sequence. Four other nucleotide differences in the coding region give rise to only one difference in the amino acid sequence; proline$_{370}$ in

Mx1 is leucine$_{364}$ of Mx1-a. The 5' 18-bp segment of bovine Mx1 is located at a position corresponding to the boundary of exon 2 and 3 of mouse Mx1 (Figure 2.3). Additionally, the GT at the 5' end of the 18-bp segment is consistent with the invariant GT of an intron splice donor site. Given that this 18-bp segment is present in the ovine sequence, the lack of this segment in Mx1-a may be the result of mutations about the splice donor site.

Primary Protein Structure Compared to Other Mx Proteins

The deduced protein sequences of bovine Mx1 and Mx1-a were aligned to a selection of other Mx sequences (Table 2.3). The highest degree of identity was to the ovine protein (93%), followed in order by porcine Mx1, human MxA, equine MxA, mouse Mx2, rat Mx2, rat Mx3, and rat Mx1. Bovine Mx1 and Mx1-a share with all other Mx proteins the tripartite GTPase domains (GDQSSGKS, DLPG, and TKPD), dynamin family signature (LPRGSGIVTR), and the carboxy-terminal leucine zipper motif (Figure 2.12). The

TABLE 2.1. COMPARISON OF BOVINE MX1 AND MX1-A WITH VARIOUS MX PROTEINS

Bovine Mx1 & 1-a comparisons to	*Protein length*	*Genbank Accession #*	*% identity to*	
			Bovine Mx1	*Bovine Mx1-a*
Bovine Mx1	654	AF047692	-	99
Bovine Mx1-a	648	U88329	99	-
Ovine Mx	654	X66093	93	93
Porcine Mx	663	M65087	79	80
Human MxA	662	M30817	73	74
Human MxB	715	M30818	57	57
Equine MxA	660	U55216	70	71
Mouse Mx1	631	M12279	63	63
Mouse Mx2	655	AB029920	69	69
Rat Mx1	652	X52711	63	64
Rat Mx2	659	X52712	69	69
Rat Mx3	659	X52713	68	68
Trout Mx1	621	U30253	53	53
Salmon Mx1	623	U66475	53	53
Chicken Mx	705	Z23168	45	46
Duck Mx	721	Z21550	49	50

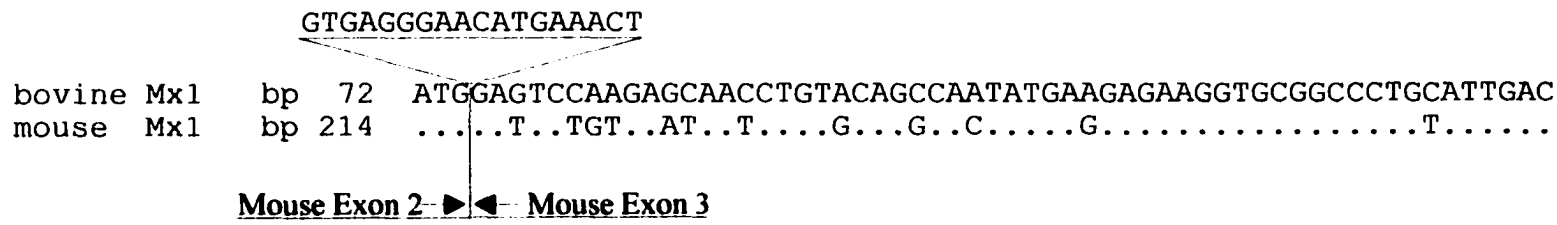


Figure 2.3. The 5' 18-bp segment of bovine Mx1 and its relationship to the boundary of exons 2 and 3 of the mouse Mx1 cDNA. Identity of mouse sequence to the bovine is indicated by dots, while differences are shown. The ATG ending mouse exon 2 is the initiation codon.

protein sequence of bovine Mx1 and Mx1-a lack the nuclear targeting sequence in the carboxy terminus of mouse and rat Mx1, and in the amino terminus of human MxB.

Polymorphism and Linkage Mapping of the MX1 Gene

In comparison to bovine Mx1, the Mx1-a cDNA contains a 13 bp deletion in the 3'-UTR. To address the significance of this difference, primers were designed to amplify that portion of the 3'-UTR (Figure 2.2), yielding either a 150-bp (lacking 13-bp) or a 163-bp (containing the 13-bp) amplicon. The 13-bp segment is present in clone boMx65 (Mx1) but absent in all other phagemid clones tested. This finding was unexpected given that boMx66, which shares with Mx1 the 5' 18-bp segment (Figure 2.1), would have been predicted to contain the 13-bp 3'-UTR segment. To confirm that the differences in the 3'-UTR of our clones reflected true genomic differences, the genomic sequence of the animals whose tissues contributed to the library was evaluated, as was the genomic sequences of other unrelated cattle. The 13-bp containing 3'-UTR segment was identified in one of the four animals represented in the library (animal 304, Figure 2.4). This animal appeared to be a heterozygote that possessed both the 150-bp and 163-bp amplicons. Of the four additional cattle tested, a Hereford bull also appeared to be a heterozygote (animal 6343).

To confirm the allelic nature of the suspected polymorphism in the 3'-UTR, and if possible to linkage map the MX1 gene, cattle of the Canadian Beef Cattle Reference Families, and a selection of unrelated bulls from other mapping studies, were genotyped for the polymorphism by Dr. Sheila M. Schmutz and colleagues at the University of Saskatchewan (Table 2.2). Among the 34 unrelated cattle genotyped, the MX1 gene was found to be polymorphic, with the allele containing a 13-bp segment in the 3'-UTR (Mx1)

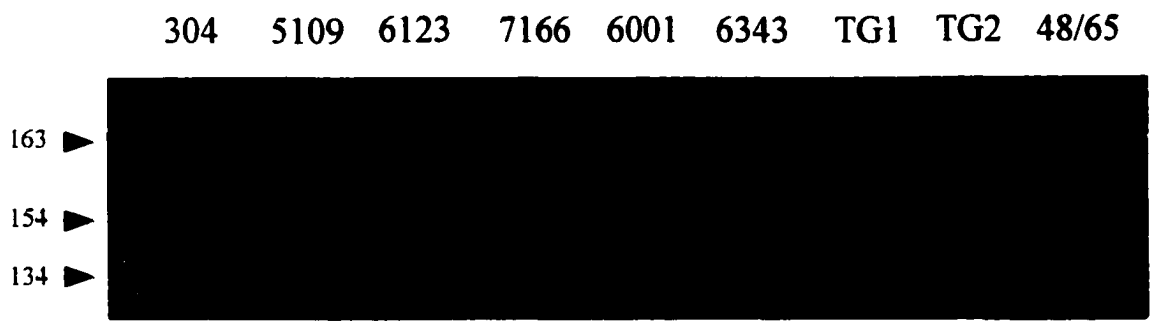


Figure 2.4. PCR analysis of the polymorphism in the 3' UTR. Arrows indicate position of DNA molecular markers (154 and 134) and the position of the larger of the two polymorphisms (163). Hybridization signals near the 154 bp maker are consistent with the predicted length of amplicon from the sequence of Mx1-a (150 bp), while signals at aproximately 163 bp are consistent with an amplicon from the sequence of Mx1. Animals 304, 5109, 6123, and 7166 are Angus-Gelbvieh crossbred cows whose tissues contributed to the phage library. Animal 6001 is an Angus bull and 6343 is a Hereford bull. Animals TG1 (bull) and TG2 (heifer) were sired by a Chianina bull out of crossbred dams. Lane 48/65 is a positive control amplification containing equimolar concentrations of plasmids boMx48 (Mx1-a) and boMx65 (Mx1).

having a frequency of 12%. Among these cattle, the 163-bp allele was detected only in animals of Hereford or Belgian Blue descent, and the frequency of this allele in this group was 57%. Subsequent evaluation of a number of Hereford cattle (Schmutz, personal communication) showed an allele frequency of 24/120 haploid genomes or 15%, which is equivalent to the frequency seen in the original population. Segregation consistent with codominant inheritance was seen in both the CCA and USDA mapping herds. Figure 2.5 illustrates family CCA 5, showing both hetero- and homozygous offspring from a cross between a homozygote dam (163-bp/163-bp) and a heterozygote sire (163-bp/150-bp). Four families of the CCA herd and four from the USDA herd contributed data for linkage mapping MX1 with at least one other marker (Table 2.2). Two-point linkage analysis used 4 microsatellites on BTA1 to position the MX1 locus on BTA1 (Table 2.2). The

TABLE 2.2. MX1 GENOTYPE OF CCA FOUNDERS AND SELECTED BULLS

Sires	Breed	MX1	Dams	Breed	MX1
Extrordinaire	Charolais	150/150	1	Angus	150/150
Krugerand	Charolais	150/150	2	Angus	150/150
Polled Star Palm	Simmental	150/150	5	Limousin	150/150
Skyliter	Charolais	150/150	7	Limousin	150/150
Winfield	Charolais	150/150	9	Charolais	150/150
Ken Gillies	Limousin	150/150	10	Charolais	150/150
Yukon Jack	Limousin	150/150	11	Charolais	150/150
Charlie Applause	Simmental	150/150	**13**	Hereford	**163/163**
King Pin 066D	Simmental	150/150	**14**	**Hereford**	**150/163**
King Pin 055C	Simmental	150/150	**15**	**Hereford**	**163/163**
Master	Simmental	150/150	17	Belgian Blue	150/150
Mastermind	Simmental	150/150	**18**	**Belgian Blue**	**150/163**
Red Enformer	Simmental	150/150	**19**	**Belgian Blue**	**150/163**
4	Angus	150/150	21	Simmental	150/150
8	Limousin	150/150	22	Simmental	150/150
16	**Hereford**	**150/163**	23	Simmental	150/150
24	Simmental	150/150			

MX1 gene was mapped between flanking markers, 10 cM from BM1824 (Log of odds = 10.24), which is at 108.6 cM, and 14 cM from BMS4043 (Log of odds = 3.96), which is at 128.7 cM from the centromere on the USDA sex-averaged map of BTA1 (Kappes *et al.*, 1997). This places the MX1 gene between 114.7 and 118.6 cM from the centromere of BTA1 (Figure 2.6).

TABLE 2.3. TWO POINT LINKAGE MAPPING OF MX1 AND TWO MICROSATELLITES

Locus Pair	Families	Non	Rec	Phase	Z max	*cM at Z max*	*Marker Position (cM)*	*MX1 Position (cM)*
BM1824/MX1	CCA 5*	5	0	Known	1.276			
	CCA 8*	11	0	Known	2.808			
	CCA 11*	7	2	Known	0.389			
	CCA 14*	2	2	Known	-0.887			
	USDA 702	6	1	Unknown	0.532			
	USDA 848	13	0	Unknown	3.018			
	USDA 874	6	2	Unknown	-0.167			
	USDA 1029	14	0	Unknown	3.272			
					10.240	*10*	*108.6*	*118.6*
BMS4043/MX1	USDA 702	5	2	Unknown	1.972			
	USDA 848	12	1	Unknown	-0.227			
	USDA 874	7	1	Unknown	0.795			
	USDA 1029	12	2	Unknown	1.420			
					3.960	*14*	*128.7*	*114.7*

* CCA families. 5: Hereford dam, Hereford sire. 8: Belgian Blue dam, Simmental sire. 11: Belgian Blue dam, Limousin sire. 14: Belgian Blue dam, Hereford sire.

Northern analysis

To confirm hybridization of bovine Mx cDNAs to a type I interferon-induced mRNA, total RNA from MDBK cells exposed to interferon was subjected to Northern analysis (Figure 2.7). Bovine Mx mRNA was not detected at the 0* hour (approximately 5 minutes post-induction), but was evident by one and one half hours, and peaked between three and nine hours post induction. By 24 hours, Mx mRNA levels had fallen to roughly 60% of the six-hour sample. Based on the position of the ribosomal and the γ-actin bands, the size of the Mx mRNA was calculated to be approximately 2,500 bases.

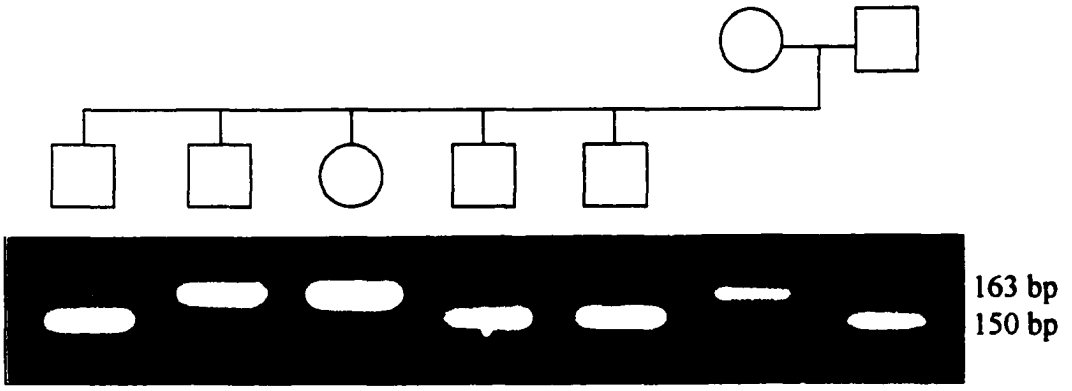
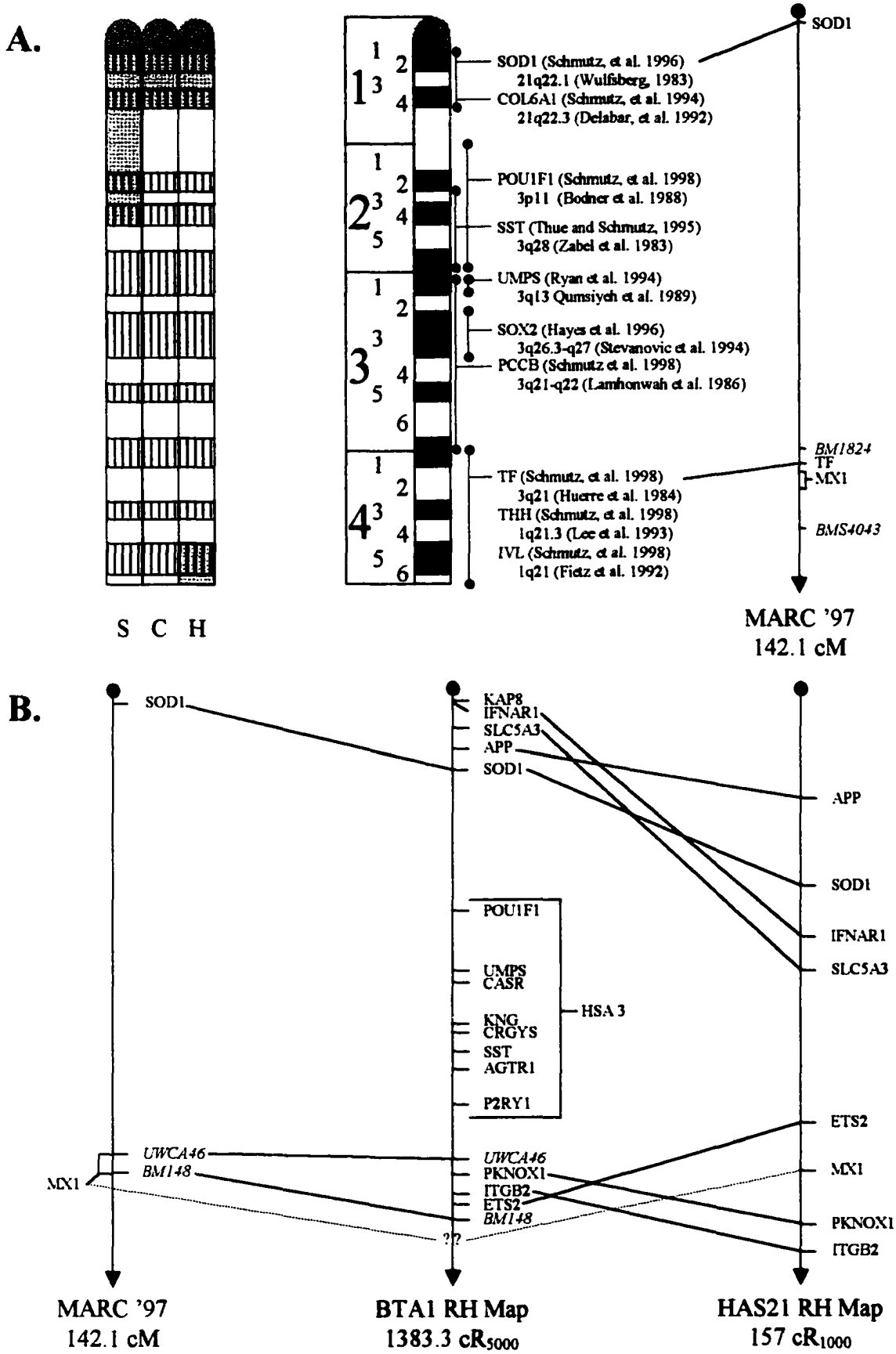


Figure 2.5. Photograph of an ethidium stained electrophoretic agarose gel (4%). PCR amplicons of the 3'-UTR of Mx1 alleles illustrate segregation consistent with codominant inheritance in a family of Hereford cattle consisting of a homozygous dam (CCA dam 13) crossed to a heterozygous bull (CCA sire 16). The more slowly migrating band is consistent with 163-bp amplicon predicted for an animal that possess the 13-bp seen in the Mx1 allele. The more quickly migrating band is consistent with a 150-bp amplicon predicted for an animal that that does not possess the 13-bp sequence.

Figure 2.6. A series of schemata of BTA1. As a matter of convention bovine gene symbol nomenclature follows human and in cases where the human homologues have been identified, the nomenclature used reflects that of the Human Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/). **A.** On the left are three ideograms of R-banded BTA1 (Di Berardino *et al.*, 1989) summarizing the Zoo-FISH findings of three studies (S=Solinas-Toldo *et al.*, 1995; C=Chowdhary *et al.*, 1996; H=Hayes, 1995). Shaded areas indicate reported hybridization to HSA 21, while non-shaded areas indicate hybridization to HSA 3. The ideogram in the center indicates placement and references of genes physically mapped to BTA 1, with their human homologue assignments noted and referenced. On the right is a schematic of the USDA Meat Animal Research Center 1997 (MARC '97) BTA1 linkage map (Kappes *et al.*, 1997) with the two previously mapped type I loci; transferrin (TF) and superoxide dismutase 1 (SOD1). Also indicated are the positions of MX1 and the flanking microsatellite markers (*in italics*) used in the pairwise linkage analysis. **B.** Alignments of two maps of BTA1 and one of HSA21 (Kappes *et al.*, 1997; Rexroad *et al.*, 1999; Rexroad and Womack, 1999). On the left is the MARC '97 BTA linkage map with the following genes noted; type I loci shared between MARC '97 and the RH map of HSA21, which includes SOD1 and MX1. Microsatellites that are both common to the BTA1 RH map and the MARC '97 map, and also closest in proximity to MX1 on the MARC '97 map are indicated in italics. The BTA1 RH map shows the human HSA3 homologues bracketed, while the relationship to HSA21 homologues are traced to the HSA21 RH map. The placement of MX1 on HSA21 is according to the findings of the Stanford Human Genome Center (http://www-shgc.stanford.edu/Mapping/rh/MapsV2/) RH map, volume 2.0 .



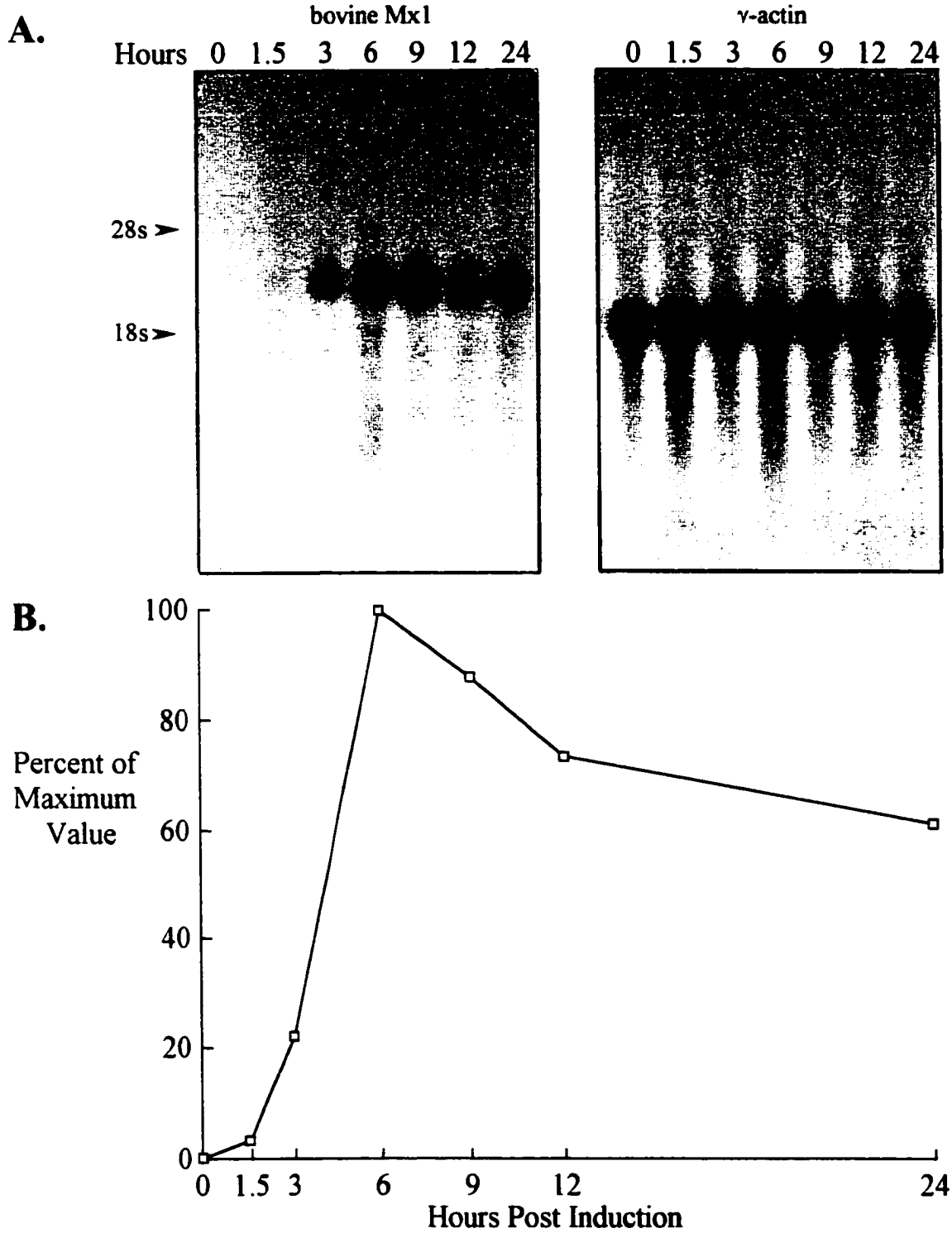


Figure 2.7. Induction of bovine Mx1 mRNA in MDBK cells by recombinant human IFN-α2b. **A.** Northern blot, sequentially hybridized to bovine Mx1 and human γ-actin probes. The positions of the 28s and 18s ribosomal bands are indicated by arrows. **B.** Levels of Mx1 mRNA over time, normalized against γ-actin values and expressed as a percentage of the peak value.

Protein Studies

The monoclonal antibody 2C12, generated against murine Mx1, cross-reacts with bovine Mx1 transiently expressed in Vero cells. Bovine Mx1 staining was clearly confined to the cytoplasm in these cells, a finding confirmed in the interferon treated MDBK cells (Figure 2.8). Immunoblot analysis, identified a protein, in whole cells lysates from interferon treated MDBK cells, of approximately 75 kDa, judged by mobility relative to the protein molecular weight standards (Figure 2.9). The 75 kDa band was absent in untreated controls. In addition to the Mx band there were three smaller bands present, which were also seen in the uninduced cells. The ECL system used for chemiluminescent quantification of Mx was found to be quite sensitive in the identification of Mx protein in fully-induced MDBK cells (5 day culture, 5,000 U/ml). Plotting of the Mx protein densitometry signal of serial dilutions of these cells yielded a plot with a linear range that extended over an eight-fold increase in concentration (Figure 2.10). A detectable band at approximately 75 kDa was found in a dilution equivalent to 625 cells/lane (Figure 2.10).

A protein with a predicted mass of 75 kDa was induced by type I interferon in a dose dependent manner, as would be expected for a bovine Mx homologue (Figure 2.11). The lowest concentration of interferon used was 0.1 U/ml and this resulted in a detectable Mx signal. At a concentration of 1,000 U/ml and above there was no detectable increase in the level of Mx protein identified. At the lower concentrations of interferon, a distinct second band, migrating just below that of the larger signal was seen. The intensity of this smaller band did not increase in tandem with the primary Mx band. Plotting the Mx signal as Mx units, against a log scale of the interferon units/ml results in a linear plot over three orders of magnitude of interferon unit concentration.

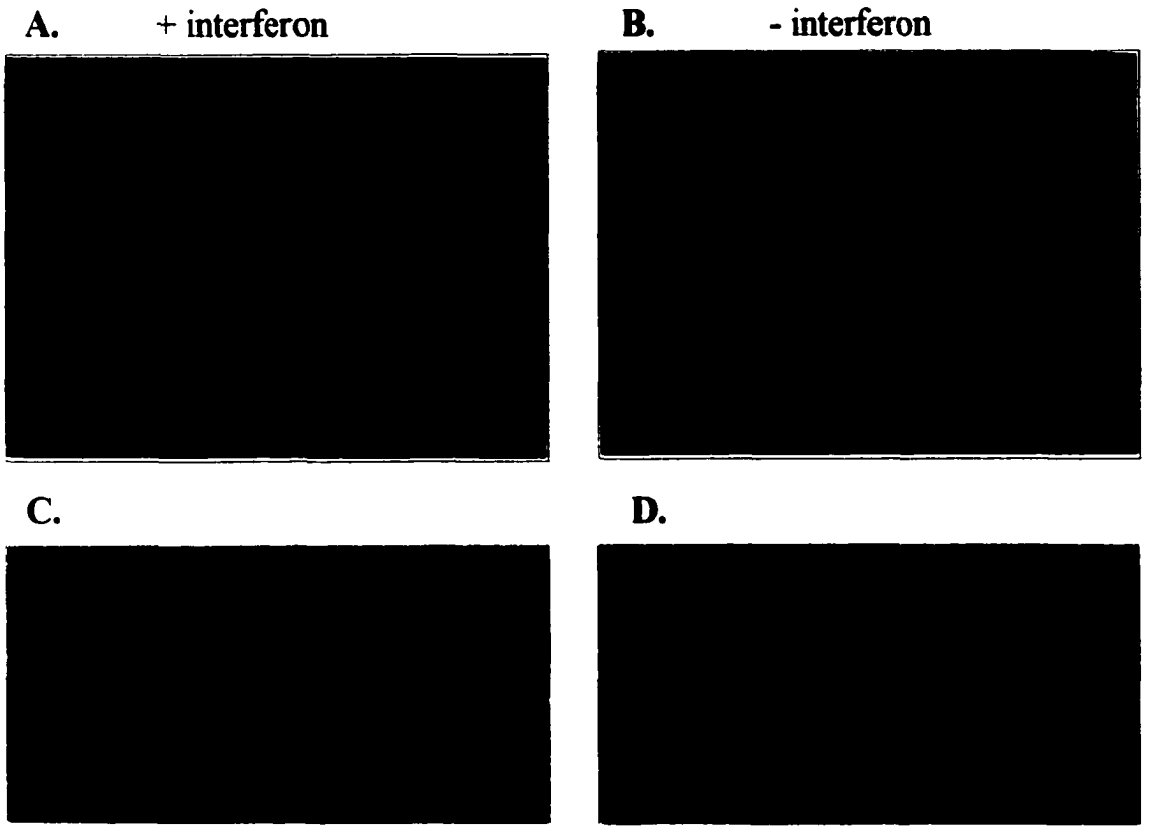


Figure 2.8. MDBK cells treated (**A.** + interferon), or untreated (**B.** -interferon) with 1,000 U/ml, type I interferon for 24 hours. Vero cells transiently transfected with cDNA clone of boMx1 (**C.** and **D.**). After fixing, cells were incubated with a primary antibody (monoclonal 2C12) and then incubated with anti-mouse IgG conjugated to FITC.

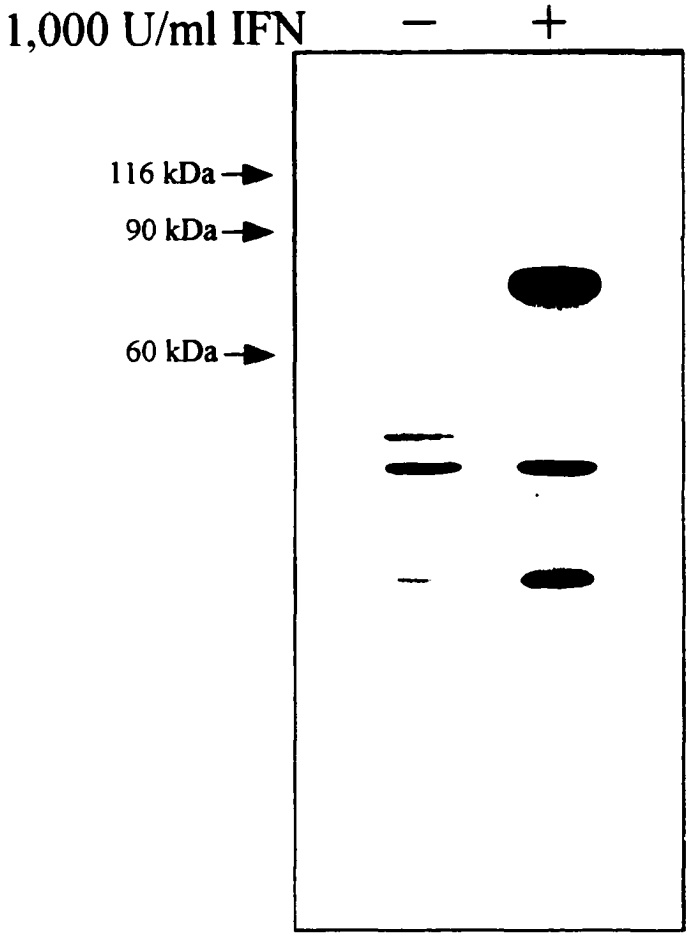


Figure 2.9. Western blot of SDS/PAGE fractionated total cell lysates of MDBK cells. Cells were either treated with 1,000 U/ml of recombinant human interferon-α2b, or remained untreated. Treatment consisted of continual incubation with or without interferon in the media. Cells were harvested 24 hours after incubation with interferon had begun. The antibody used was the 2C12 monoclonal, and the autograph was made using ECL chemiluminescent reagents.

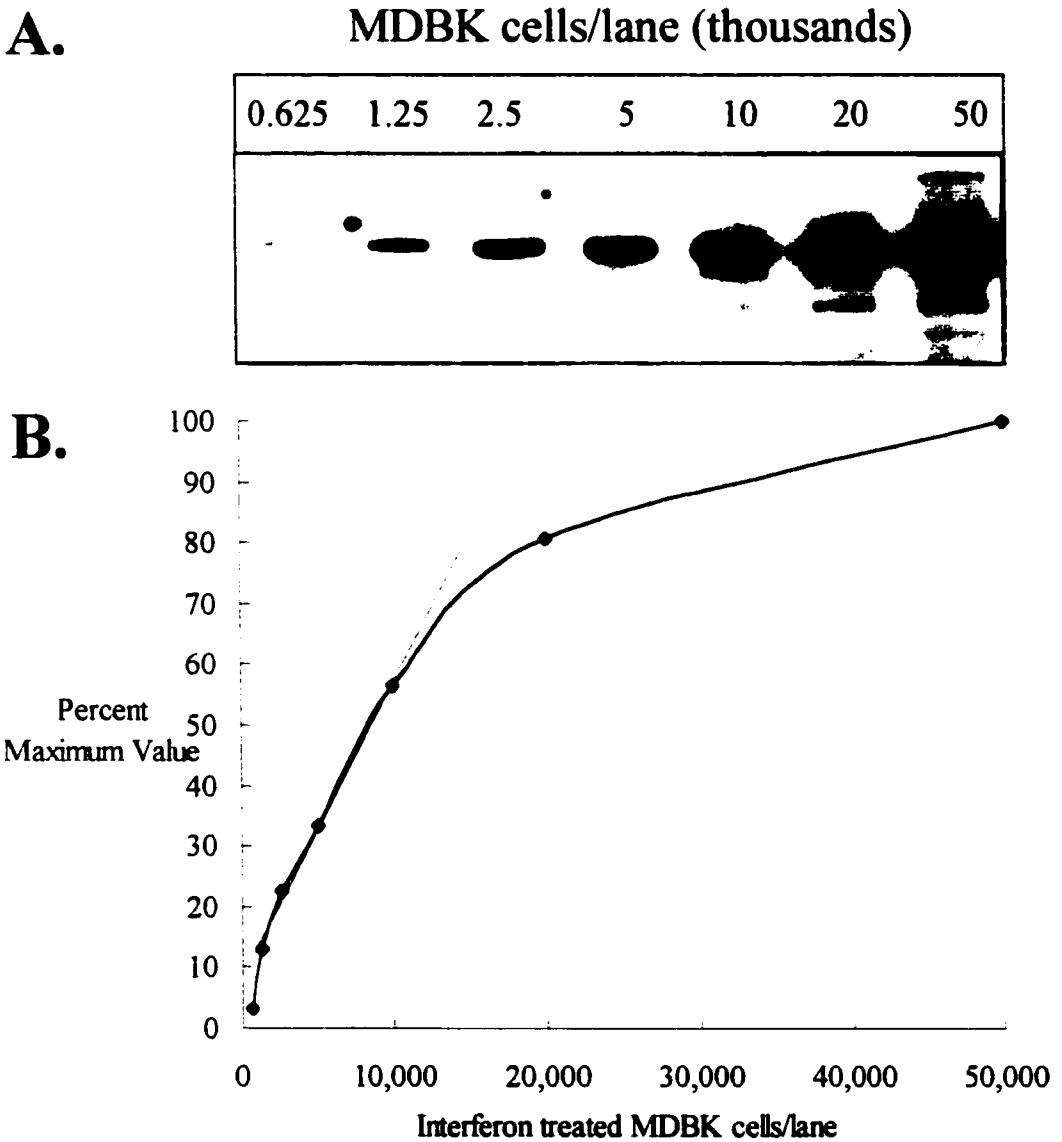


Figure 2.10. **A.** Western blot indicating dilutions of maximally induced cells. **B.** Graph shows densitometric measures as a percentage of highest value.

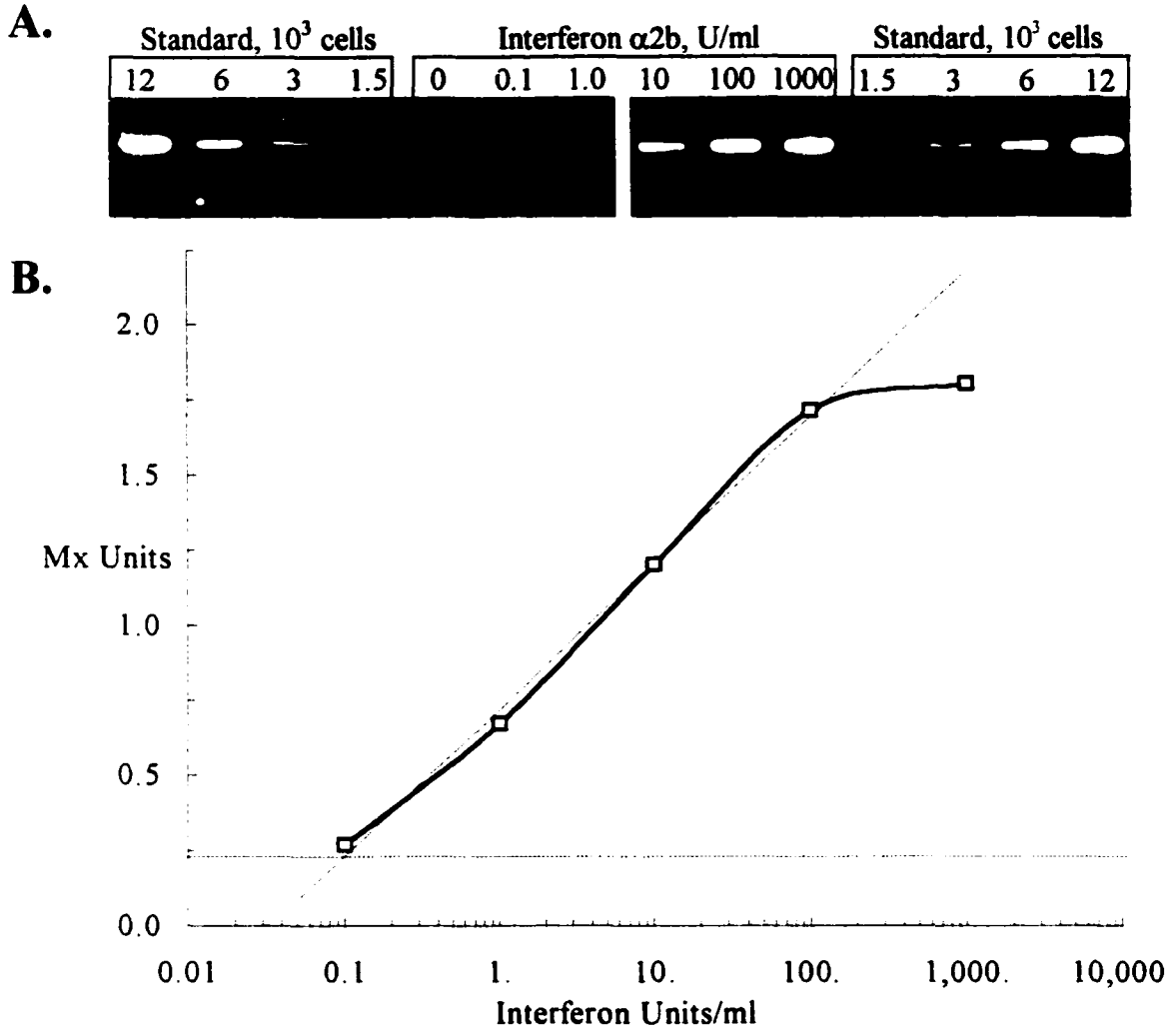


Figure 2.11. A. A Western blot of Mx dose response to varying concentrations of interferon, shown as a negative image. Samples were run on two separate minigels with standard curve samples included on each gel. Thousands of cells/lane are indicated for the standard samples, as are interferon concentrations of the dose response samples. **B.** A graph of the desitometric values of Mx protein as induced by varying concentrations of interferon. Mx signal was converted to Mx units, which are arbitrarily defined as the amount of Mx signal induced in 6,000 MDBK cells by 1,000 U/ml over a 24-hour period. Interferon concentrations are shown on a log scale. The dashed line indicates the Mx units of the lowest point in the standard curve.

Discussion

Two full-length cDNAs of a bovine Mx homologue were isolated which represent alleles of one gene and are designated Mx1 and Mx1-a. The deduced protein sequences of these cDNAs are highly similar to Mx proteins from other species (Table 2.3) and contain the canonical GTPase and leucine zipper domains, as well as the dynamin family signature found in all other Mx proteins (Figure 2.12).

Several findings support the conclusion that the Mx1 and Mx1-a cDNAs are derived from alleles rather than from two distinct genes. First, the coding regions of the two cDNAs differ at only four nucleotides and by the presence of an additional 18-bp segment in Mx1. Second, the 3'-UTR sequences of the two bovine cDNAs are identical over 379 bases except for a single 13-bp segment present only in Mx1. This degree of identity in both the coding and non-coding regions is far higher than is seen in any other species having multiple Mx genes except for the salmon Mx1 and Mx2 cDNAs, which were also considered probable alleles by their discoverers (Robertsen *et al.*, 1997).

Additional proofs confirm the allelic relationship of bovine Mx1 and Mx1-a. The polymorphism in the 3'-UTR of Mx1, which consisted of a 13-bp insert relative to the other sequence, was identified in the genome of only one heterozygous animal whose tissues contributed to the cDNA library. The polymorphism was also found in only one of four unrelated cattle tested, again a heterozygote (Figure 2.4). With respect to inheritance by descent, we have shown that the allele segregates in a manner consistent with codominant inheritance (Figure 2.5). Both cows and bulls were identified that possessed both the 150 and 163-bp alleles in homozygous forms (Figures 2.4 and 2.5, Table 2.1).

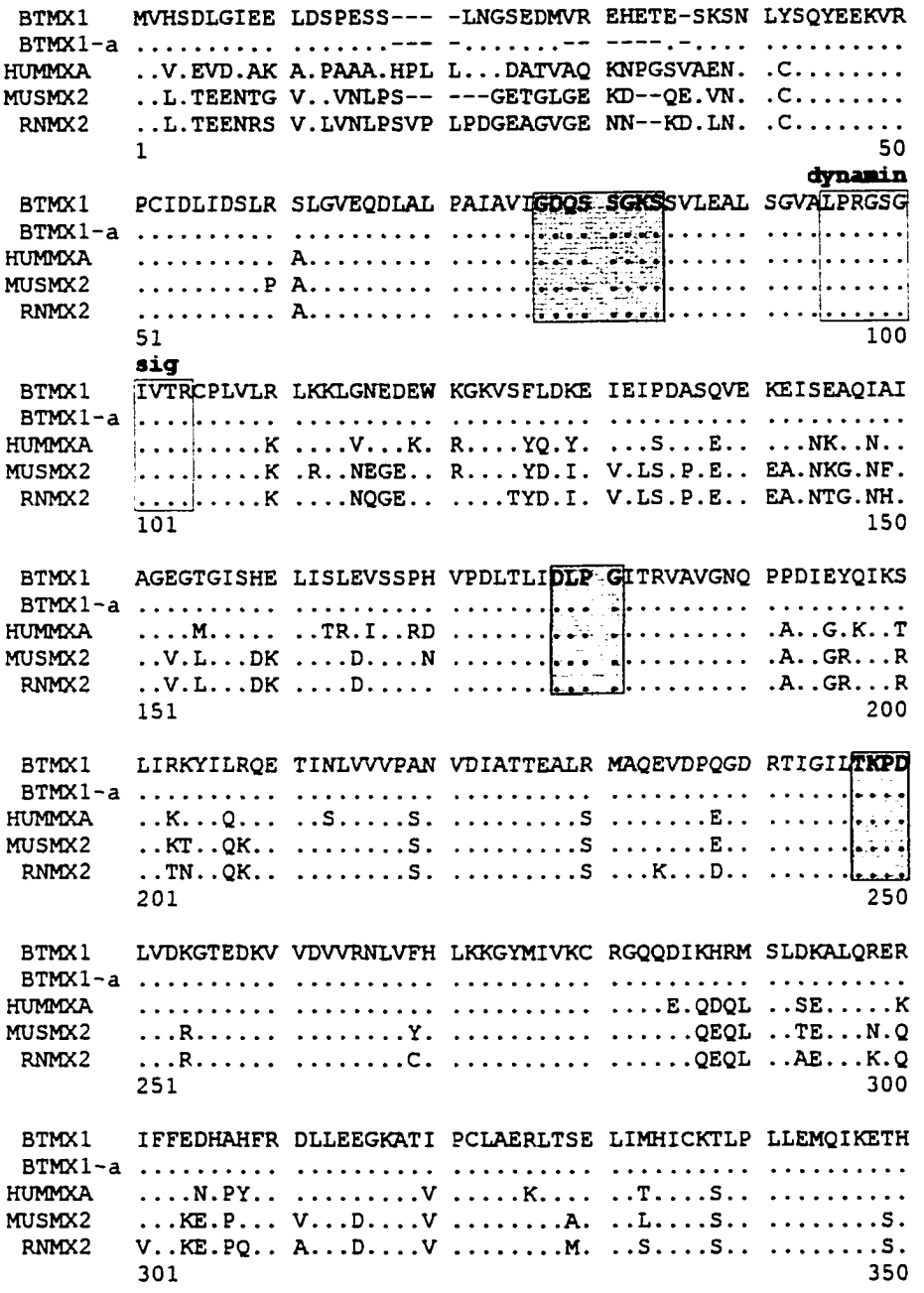


Figure 2.12. Alignment of bovine Mx1 and Mx1-a protein sequences with cytoplasmic Mx proteins known to have antiviral activity; human MxA, mouse Mx2, and rat Mx2. Tripartite GTPase domains are boxed and shaded. Dynamin family signature is boxed and indicated, as are sequences recognized by the monoclonal antibody 2C12. Single residues of interest are boxed and indicated: leucines (**L**) contributing to the leucine zipper structure; a proline (**P**) in Mx1, which occupies the position of an otherwise universally conserved leucine; an arginine (**R**) and an histidine (**H**) which are necessary for antiviral activity of rat Mx2 against vesicular stomatitis virus (Zürcher *et al.*, 1992); a glutamate (**E**) which is necessary for antiviral activity of human MxA against vesicular stomatitis virus (Johannes *et al.*, 1997). *Figure continued on next page*

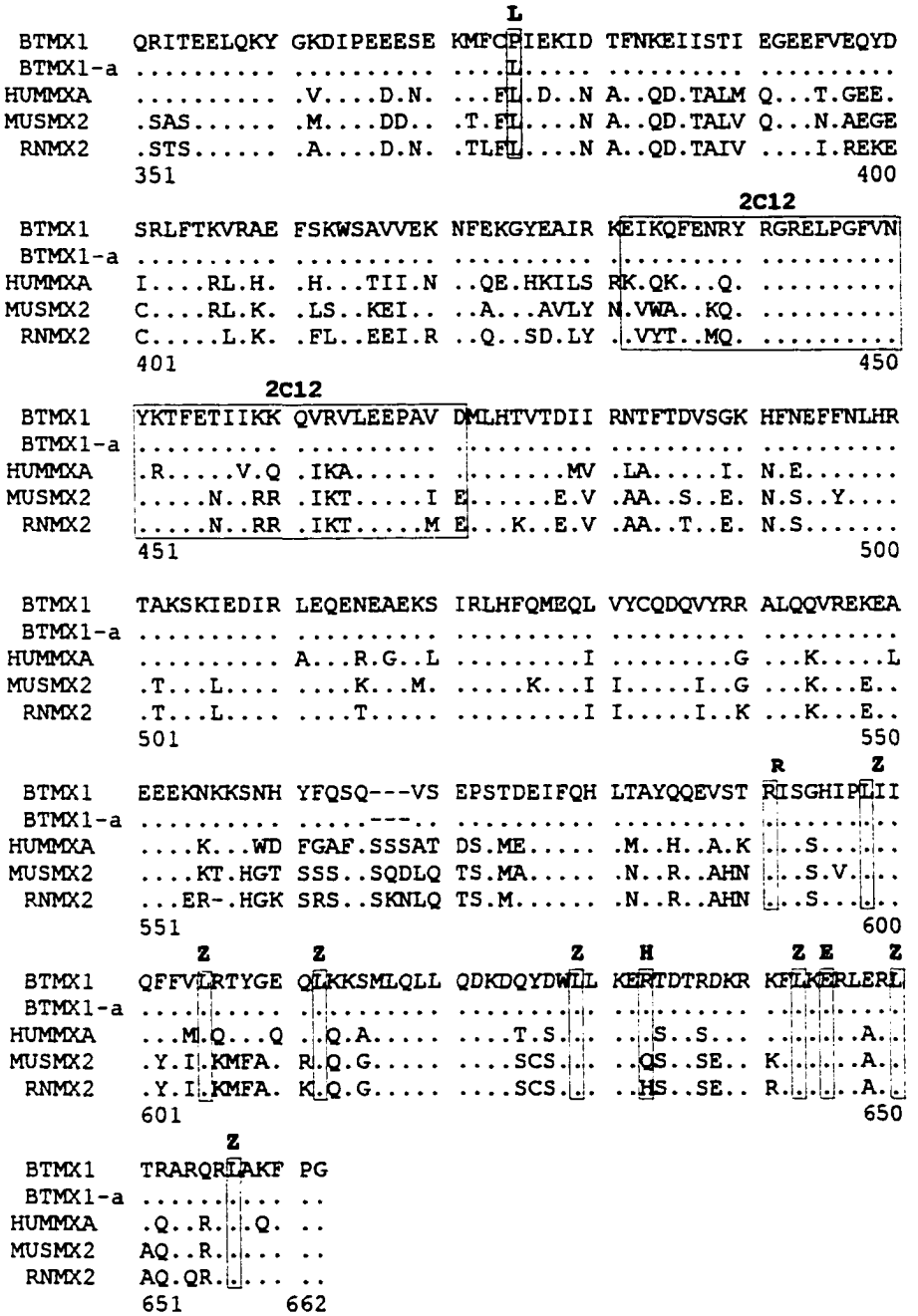


Figure 2.12. *Continued*

Mapping of MX1 to BTA1

The alleles allowed for linkage mapping of the MX1 gene, which placed it on BTA1 at between 114 and 118 cM from the centromere of the USDA sex-averaged linkage map. The mapping of MX1 to BTA1 is consistent with syntenic information that thus far has placed all type I loci from human chromosome 21 on BTA1 by in situ hybridization (Schmutz *et al.*, 1994 and 1996), somatic cell hybrid analysis (McAvin *et al.*, 1988; Skow *et al.*, 1988), linkage mapping (Barendse *et al.*, 1993 and 1997; Kappes *et al.*, 1997), radiation hybrid mapping (Rexroad and Womack, 1999; Rexroad *et al.*, 1990), and Zoo FISH (Hayes, 1995; Solinas-Toldo *et al.*, 1995; Chowdhary *et al.*, 1996). Though the USDA linkage map has the greatest density of loci mapped, it is poor in type I loci. The MX1 gene is one of only three type I loci mapped on the USDA linkage map, the other being SOD1 and TF (Figure 2.6.A.). The IBPR map of BTA1 has nine type I loci (Taylor *et al.*, 1998), however, the only gene from HSA21 which maps to the distal portion of BTA1 is CRYA1. The CRYA1 gene is non-recombinant on the female IBRP BTA1 map with the microsatellite marker BM148 which is itself in close proximity to MX1 (Figure 2.6.B). The finding of MX1 and other HSA21 genes linked to the telomeric region of BTA1 supports the Zoo FISH findings (Figure 2.6.A.) of Hayes (1995). Solinas-Toldo *et al.*, (1995), and Chowdhary *et al.*, (1996) failed to identify HSA21 hybridization to the telomeric end of BTA1. Given the variable sensitivity of the Zoo FISH technique, this failure is not unreasonable. For example, all three efforts at Zoo FISH failed to identify any HSA1 hybridization to BTA1, even though two HSA1 genes have now been placed on BTA1 (Figure 2.6.A.) by *in situ* hybridization (Schmutz *et al.*, 1998).

A syntenic relationship has long been noted between genes on HSA3, HSA21, genes of BTA1, and genes of MMU16, MMU17, and MMU10 (Threadgill and Womack, 1991; Threadgill *et al.*, 1991; Barendse *et al.*, 1993) and these relationships have been extended to other species (Chowdhary *et al.*, 1998). The most recent effort in elucidating this syntenic relationship involved parallel mapping of numerous type I loci (Rexroad and Womack, 1999) on the radiation hybrid panels of BTA1 (Rexroad *et al.*, 1999), HSA3, and HSA21. The human MX1 gene has been placed on the radiation hybrid panel of the Stanford human genome center's map of HSA21 (Stewart *et al.*, 1997), which is the panel used by Rexroad and colleagues (http://www-shgc.stanford.edu). Figure 2.6.B. illustrates the relative positions of the type I and II loci shared between the RH maps, both bovine and human, and the USDA linkage map. The gene for MX1 has been designated a reference anchor loci for comparative genetic mapping (O'Brien *et al.*, 1993), and mapping of MX1 to the BTA1 radiation hybrid panel should now be possible. Regardless of the exact location of MX1 on BTA1, because of its placement on HSA21 between ETS2 and PKNOX1, eventual mapping of MX1 on the BTA1 radiation hybrid panel will help elucidate the more subtle elements of gene sequence conservation between BTA1 and HSA21.

Significance of Mx1 cDNA Polymorphisms

By far the largest difference in the coding sequences of Mx1 and Mx1-a is an 18-bp sequence in the 5' terminus seen only in Mx1, a sequence which is conserved in an ovine Mx cDNA (Charleston and Stewart, 1993). Because of the conservation of the ovine sequence, it seems likely that bovine Mx1 represents a form of the gene ancestral to Mx1-a, and this was the rationale for the designation clone boMx65 as the wild type Mx1

and clone boMx48 as Mx1-a. However, the fact that Mx1 has an uncommon polymorphism in the 3'-UTR and a proline at position 370, where all other Mx proteins, including bovine Mx1-a, have a leucine, raises the possibility that the sequence of bovine Mx1 may represent a slightly altered allele of an as yet unidentified wild-type.

In summary, limited sequence and restriction analysis show that there are at least two and perhaps three forms of the Mx1 gene in cattle. The first two forms are represented by Mx1 and Mx1-a. A third possible form of Mx1 is represented by the boMx66 clone (Figure 2.1), which possesses the 5' 18-bp segment, but lacks the 3'-UTR 13-bp segment. However, since the 5' 18-bp segment is associated with an intron/exon boundary, the possibility exists that varying transcripts may derive from the same genomic sequences. Definitive clarification of the nature of all forms of bovine Mx1 awaits isolation and analysis of further cDNA as well as genomic clones.

Is Bovine Mx a Member of a Multigene Family?

In the species that have been investigated most rigorously (human, mouse, rat, pig, salmon, and trout), two and sometimes three Mx genes have been identified. In a previous study of bovine Mx proteins, two distinct species of the protein were identified by two dimensional gel electrophoresis (Horisberger *et al.*, 1988). It is unlikely that the small differences in mass (813 Da) and charge (calculated pI of 5.61 vs. 5.60) between Mx1 and Mx1-a would result in such resolution, suggesting that a second bovine Mx exists. At very low levels of induction of MDBK cells (0.1 and 1.0 U/ml, Figure 2.13), a faint, yet visible band on the Western autograph can be seen, which migrated slightly faster than the major band. This band did not appear to increase with higher concentrations of interferon, but rather may be obscured under such conditions. Lower level of induction of

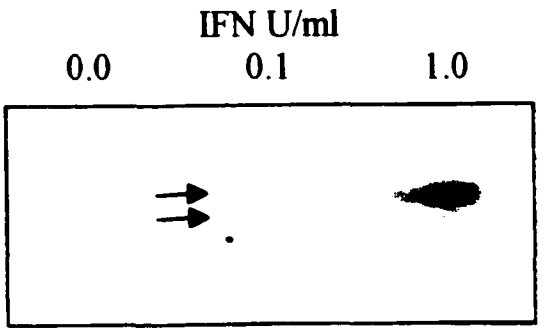


Figure 2.13. A positive image enlargement of a portion of the Western immunoblot from Figure 2.11. Bovine Mx bands are evident in the interferon treated lanes. A more quickly migrating band is evident just below the band that shows a dose dependent increase in intensity. The second band may reflect the existence of a second bovine Mx protein.

second Mx genes is consistent with what is known of the accumulation of human MxB (Aebi *et al.*, 1989; Horisberger *et al.*, 1990) and porcine Mx2 (Müller *et al.*, 1992b). An alternative explanation of the lighter second band is that it may be a technical artifact. It is also theoretically possible that there is an alternate initiation site for bovine Mx1, similar to the alternate initiation site utilization seen with human MxB (Melén *et al.*, 1996). Inspection of the second methionine in the open reading frame of bovine Mx1 finds that it is in an appropriate context for initiation of translation, having a purine at −3 and G at +4. Though the second ATG lies at the border of the 5' 18-bp segment, which is not evident in all cDNAs, the second ATG has a G at +4 regardless of the whether the 5' 18-bp segment is present or not (Figure 2.3). Interestingly, the second ATG is in alignment with the initiating ATG of the mouse Mx1 cDNA. However, even though the context of both ATG sequences is adequate, it is the first ATG that has been shown to have preference (Kozak, 1995). Lacking experimental information on bovine Mx1, some insight may be gained by assessment of the alternate initiation of human MxB, in which initiation from the second ATG accounts for 75% of the protein product. In the case of human MxB the first ATG has a C at -3 and a T at +4, neither of which meet the criteria for optimal initiation. The second at least meets half the requirements for an optimal transcription initiation context, having a G at -3, though an A at +4, so that, unlike bovine Mx1, it seems more reasonable that the human MxB initiation favors the second ATG as it meets at least one criterion. In any event, regardless of the context of the ATG in bovine Mx1, the ultimate clarification of whether the Mx1 gene is initiated from two sites would require experimental evaluation. Evidence to support an alternate site for translation initiation still leaves open the question of a potential second bovine Mx gene. Horisberger (1988) did

address and refute the possibility that the two bovine Mx proteins he identified resulted from post-translational modification of a singular product. Clearly, resolution of this question awaits isolation and analysis of future clones.

Bovine Mx mRNA Induction

As a general observation, Mx gene transcription is stringently induced by type I interferon. Northern analysis was used to confirm that the bovine Mx1 mRNA was induced by type I interferon. Hybridization to a bovine Mx1 cDNA probe was not observed with RNA from cells collected immediately after stimulation by type I interferon, but was clearly present 1.5 hours. The kinetics of induction over the first 24 hours were consistent with what has been reported in other mammals, including mouse (Staeheli *et al.*, 1986), rat (Meier *et al.*, 1988), and human (Aebi *et al.*, 1989). The observed transcript size of approximately 2,500 bases is in good agreement with the size of our Mx1 and Mx1-a cDNAs.

The abundance of Mx clones in the endometrial cDNA library is another indication that bovine Mx1 genes are induced by type I interferon. The early ruminant embryo secretes copious quantities of interferon–τ, which functions as the signal for maternal recognition of pregnancy (Farin *et al.*, 1990; Hansen *et al.*, 1999). Mx expression in pregnant ruminants is, to our knowledge, the only situation described where the Mx protein is expressed as part of normal growth and development, rather than in response to virally induced interferon production. It is not clear whether Mx expression in the endometrium of ruminants is simply the result of embryonic secretion of interferon-τ or whether it serves another important role in pregnancy, perhaps as an antiviral compound.

Protein Production: Localization and Interferon Response

Type I interferon induced in cultured bovine cells a cytoplasmic moity which is recognized by the monoclonal antibody 2C12 (Figure 2.8). This antibody recognizes a moity determined by amino acid 430-469 of rat Mx3 (Johannes *et al.*, 1997) and amino acids 432-471 of human MxA (Flohr *et al.*, 1999), which correspond to amino acids 427-466 of bovine Mx1 and amino acids 421-460 of Mx1-a (Figure 2.12). The identity of the regions in the bovine Mx1 cDNAs that correspond to the region recognized by 2C12 in rat Mx3 is 67% and 71% in human MxA. In transfected Vero cells and MDBK cells the Mx protein recognized by 2C12 localized to the cytoplasm. This is consistent with the fact that the bovine Mx1 amino acid sequence lack the nuclear targeting sequences seen in other Mx proteins, both the basic carboxy terminal sequences that have been shown to cause nuclear localization of mouse (Noteborn *et al.*, 1987) and, by extension, rat Mx1 (Meier *et al.*, 1990), as well as the characteristic amino terminal sequences implicated in the nuclear localization of human MxB (Melén *et al.*, 1996). Furthermore the finding of bovine Mx as a cytoplasmic protein confirms a previous report (Horisberger, 1988).

A chemiluminescent immunoblot technique, utilizing MDBK cells and the 2C12 monoclonal, produced a signal at approximately 75 kDa that was evident only in interferon treated cells (Figure 2.9). Dilution of a stock of MDBK cells with high levels of Mx protein yielded a linear range, which extended over an eight-fold increase in concentration (Figure 2.10). This same immunoblot indicated that in these highly induced cells, as few as 625 cells/lane gave a positive signal for Mx. The per cell protein concentration of the total cell lysates was approximately 220 pg. Hence, assuming that the last dilution was the limit of detection for this system, that the average total cell protein content is 220 pg, and

that a fully induced Mx concentration per cell is 1.0-0.5% of total cellular protein (Horisberger, 1992a), the limit of the immunoblot to detect Mx may be in a range of 1.3-0.7 ng/lane. Regardless of the limit of detection, the linear range of the immunoblot indicates that this system can function as a semi-quantitative assay of Mx protein.

The Mx immunoblot, when used to assay the concentrations of type I interferon, yielded a clear and convincing dose response curve. When the results of the scanned autograph were plotted with interferon units on a log scale, the relationship between the interferon concentration and the Mx units was linear over three orders of magnitude. As an assay for interferon, this system has the potential for much greater sensitivity over the conventional micro plaque reduction assay, *i.e.* two orders of magnitude, since the limit of detection with the latter assay is approximately 10 U/ml. Much of the process described above is cumbersome for a routine bioassay. However, application of Mx assays in humans have proved rapid, reliable, and sensitive (Oh *et al.*, 1994), and nothing in the findings above rule out the possibility of development of such an assay for cattle.

Potential for Antiviral Activity of Bovine Mx1

The antiviral activity of bovine Mx proteins is not known. We have been unable to establish permanently transfected clones of cells that express bovine Mx1 or Mx1-a, suggesting that constitutive expression of these proteins may be toxic. Similar difficulties have been reported in expressing rat and chicken Mx proteins (Meier *et al.*, 1990; Bernasconi *et al.*, 1995). Scant information is available on sequences in other Mx proteins critical for antiviral activity. Limited mutational analysis of human MxA (Zürcher *et al.*, 1992b) showed that the antiviral activity against vesicular stomatitis virus is dependent upon Glu_{645}, which is conserved in both bovine Mx1 sequences (Figure 2.9). The Arg_{588}

residue of rat Mx2, which is also present in both bovine Mx sequences, was shown to be one of two residues necessary for antiviral activity against vesicular stomatitis virus (Johannes *et al.*, 1997). The second rat Mx2 residue, His_{630}, appears in both bovine Mx1 proteins as an arginine. This arginine residue in bovine Mx1 would not necessarily be expected to ablate antiviral activity since human MxA, also active against vesicular stomatitis virus, has an arginine at this position (Figure 2.10). In summary, and in light of the experimental data from other species, none of the findings of the primary protein sequence of Mx1 and Mx1-a predict an absence of antiviral activity, though only *in vitro* assessment will provide definitive answers. However, some findings exist on sequence analysis of bovine Mx1 that are so stark, such as the existence of a completely non-conserved amino acid (*i.e.* the $proline_{370}$ in Mxl), that the possibility exists that a non-functional bovine Mx protein may already have been found. This adds support for the need to confirm the antiviral activity, since it appears that the establishment of stable and expressing clones, at least by conventional methods, is not a viable means to assess antiviral activity.

CHAPTER III

GENERAL DISCUSSIONS AND CONCLUSIONS

As a first step in the process of investigating the Mx system in cattle, cDNAs were isolated from a phage library constructed using endometrial tissue from early pregnancy. Sequence characterization of these *Bos taurus* cDNAs revealed them to be Mx cDNAs by a host of criteria. Analysis of translation of the cDNAs predict proteins of 654 and 648 amino acids, a size consistent with known Mx proteins. Further analysis found the bovine Mx sequences to possess the tripartite GTPase domain, the self-assembly domain known as the dynamin family signature, and leucine zipper motifs conserved in all known Mx proteins. The bovine protein sequences show highest identity to ovine Mx (93%) and are substantially similar to human MxA (73%) and mouse Mx1 (63%). Induction of an appropriately-sized single species of RNA by type I interferon, which hybridized to the isolated Mx1 cDNA, was confirmed by Northern analysis. By virtue of sequence identity and biological response to interferon induction, we conclude these cDNAs to be genuine Mx cDNAs and have designated them Mx1, to reflect the probable existence of additional Mx genes.

Close scrutiny of the cDNAs revealed slight sequence inconsistencies. These inconsistencies led us to hypothesize that the differing cDNAs, rather than representing sequencing error or cloning artifact, actually represented allelic variation of the gene for bovine Mx1. The allelic nature of one of the polymorphisms was confirmed in a number

of ways. The 3-UTR polymorphism with cDNAs differing by the presence or absence of a 13-bp segment was investigated by PCR screening. Homozygotes for either the 150-bp or the 163-bp allele, as well as heterozygotes, were found among both sexes. Segregation of the alleles in a manner consistent with codominant inheritance was confirmed. Furthermore, the polymorphism was used to successfully linkage-map the MX1 gene to between 114.7 and 118.6 cM from the centromere on the USDA map of BTA1.

Production of a bovine Mx protein by was assessed by immunocytofluorescence and immunoblot analysis using the monoclonal antibody 2C12, which cross-reacts to Mx proteins from a variety of species. A cytoplasmic protein was identified within both interferon induced MDBK cells as well as cells which had been transiently transfected with bovine Mx1 cDNAs. Using Western analysis of interferon-induced MDBK cells, we demonstrated a protein with a molecular mass of 75 kDa that was found to be present only in interferon-treated MDBK cells, and whose induction showed dose-dependency on type I interferon.

Directions for Further Inquiry

As discussed briefly in chapter II, we have been unsuccessful in establishing permanently transfected cell lines, which express either the bovine Mx1 or Mx1-a cDNAs. We used a variety of promoters and plasmids, all without success. Conventional calcium phosphate precipitation co-transfection with a selectable plasmid was used with plasmids containing Mx under the control of constitutive viral promoters. As a screening tool we used immunocytofluorescence staining. None of the drug resistant clones stained positive for Mx protein. We were not able to discern the nature of the problem. Failure to isolate Mx positive clones could be a result of transcriptional problems or perhaps there is a

selective advantage to the generation of transcripts that have flaws, leading to no translation or to the production of inactive or at least non-immuno-reactive protein. The difficulty in getting stable transcripts is a phenomenon that has been reported in other species including mouse (Staeheli, 1990) and chicken (Bernasconi *et al.*, 1995). There is no correlation between antiviral activity and ease of generating stable clones. The Mx cDNAs from some species, including human MxB and duck Mx, have no antiviral activity *in vivo*, despite successful constitutive expression (Pavlovic *et al.*, 1990; Bazzigher *et al.*, 1993). There are no reports of cell lines stably transfected to express cDNAs from several other species, including sheep, horse, and swine (Charleston and Stewart, 1993; Chesters *et al.*, 1997; Müller *et al.*, 1992b). This absence of data prompts speculation as to whether similar, though unreported, difficulties in establishing constitutive expression are a feature of these Mx cDNAs. Interestingly, mouse Mx1 was reported to be refractory to the establishment of constitutively expressing clones (Staeheli, 1990), but only when the promoter used was the early promoter from SV40 (Staeheli *et al.*, 1986). Stably transfected clones expressing mouse Mx1 were eventually established when the promoter used was the eukaryote housekeeping gene for 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Pavlovic *et al.*, 1990). Though not assessed as a control *per se*, concurrent with our difficulties in establishing bovine Mx1 cell lines, we were able to successfully isolate constitutively expressing cell lines which produce human MxA without difficulty. The plasmids used (the gift of Dr. Peter Staeheli, Universität Freiburg, supplied by Dr. Heinz Arnheiter, NIH) contained the same eukaryotic promoter described above (Pavlovic *et al.*, 1990).

A more plausible explanation of our difficulty in obtaining stable expression of

bovine Mx1 is that the bovine protein is toxic when produced constitutively. Possible tests of this hypothesis might include use of an inducible vector system for bovine Mx such as is possible with the Tet-On™/Tet-Off™ gene expression system (CLONTECH Laboratories, Inc. Palo Alto, CA). This would allow one to discretely turn on Mx transcription and monitor cells for a general decline in transcriptional or translational activity, or for apoptosis. If bovine Mx demonstrates cytotoxic activity, one may wish to analyze hybrids of Mx, derived from bovine and mouse or human sequences.

Such arduous studies would be of interest, but it would be more important to document antiviral activity. Generation of an inducible system, as described above could be used to accomplish this given the difficulty in establishing cells lines by more conventional means. Other avenues to pursue might include infection of transiently transfected cells, followed by analysis by double immunofluorescence. This method has been used for rat and chicken Mx cDNAs (Meier *et al.*, 1990; Bernasconi *et al.*, 1995) to identify antiviral activity, and while effective, it is cumbersome, requiring individual and qualitative scoring of cells for fluorescence in two spectra. Using flow cytometry would avoid the need to manually count and score hundreds of individual cells. Flow cytometry has been used to measure Mx in leukocytes, and was an aid in confirming that Mx production in peripheral blood leukocytes was primarily limited to monocytes (Ronni *et al.*, 1995). Ideally one would want to use adherent cell cultures for double immunofluorescence. Since adherent cells can be successfully subjected to flow cytometry (Maier *et al.*, 1998), this technique should prove both practical in development and powerful in application.

Significance of Alleles

There are two disparities found in the primary protein sequence of the Mx1 alleles. One of these is a proline in Mx1 at 370, which is found to be a leucine at 364 in Mx1-a. This proline is not found in any other of the Mx proteins. Because this proline does not exist in other Mx proteins, one should first test Mx1-a for biological activity. If the Mx1-a allele were indeed shown to have antiviral activity *in vitro*, full assessment of Mx1 would become a priority. In spite of the fact that the leucine to proline change is a conservative one, in that both amino acids are neutral and hydrophobic, one might predict that a residue which is universally conserved in all Mxs, as the leucine is, would be important for function. Documenting such an alteration to confer no antiviral activity *in vitro*, and to reflect a true alteration of genomic sequence (*i.e.* not be the result of a cloning artifact) in pedigreed cattle, would be a significant finding.

The second sequence disparity, a six-residue section of the amino terminus, is of somewhat less concern. The additional six base pairs are found in the extreme amino-terminus of the protein, an area which shows much heterogeneity between species, and which, with the exception of the nuclear targeting sequence seen in human MxB, is an area that is not known to influence biological function. The sheep Mx protein sequence also contains these six residues seen in Mx1 and it was this conservation that led us to conclude that the Mx1 allele was ancestral to Mx1-a. This six-residue section is found at a location that is equivalent to the boundary of mouse exons two and three (Figure 2.3). The nucleotides on the 5' end of the 18 base pairs that encode the six-residue addition are compatible with the intron splice donor site. We did not investigate the possibility that the Mx1 5' discrepancy is the result of differential splicing. There are a host of possibilities

that could lead to such a difference and the most conclusive answers will be provided by combining genomic sequencing of the areas flanking the 18 bp segment and using S1 nuclease to confirm the nature and frequency of the mature transcripts. It is of interest to note that the 5' end of mouse Mx1 and human MxA cDNAs have alternately used non-coding exons (Hug *et al.*, 1988; Horisberger *et al.*, 1990a). Cells from CBA mice have been shown to use alternative slicing to give an additional exon in all cDNA clones assessed (Hug *et al.*, 1988). This alternate exon exists in the genome of A2G mice, but is included in only 10% of transcripts (Hug *et al.*, 1988). Staeheli made the case that this differential use of the alternate exon was a strain specific phenomenon (1990), however CBA cells were reported to yield insufficient high quality Mx1 mRNA unless cultured with cycloheximide (Staeheli *et al.*, 1988). Rather than reflecting mouse strain differences, the frequency of alternate exon usage may be related to cycloheximide treatment. In any event, a controlled analysis of the splicing of the alternate exon in mouse Mx1 has not received a critical test if usage of the exon is sequence dependent or whether it is dependent on ancillary protein involved in the splicing process. It is intriguing that in general there is a great deal of heterogeneity in the 5' end of the Mx proteins and in at least two distant mammalian species, *i.e.* human and mice, there appears to be differential exon usage in the 5' end of the cDNA.

The confirmed alleles of Mx1 in cattle appear to segregate according to breed. In the linkage studies, the 3'-UTR 163-bp allele was found exclusively in Hereford and Belgian Blue cattle (Table 2.1). Very limited screening found the 163-bp allele to exist in one of the four animals whose tissues contributed to the library, and in one of four unrelated animals (Figure 2.4). The library contributor was purported to have been an

Angus-Gelbvieh cross breed, while the other positive animal was a Hereford bull. To draw the most conservative conclusion regarding the distribution of this allele among breeds, at the very least one can say that the 163-bp allele appears to be found only in cattle descended from breeds of Great Britain (Angus and Hereford) or Belgian blue. Belgian blue are known to share polymorphisms with Shorthorned cattle (Seitz *et al.*, 1999). This could be a result of the fact that Shorthorned cattle contributed to the development of Belgian Blue cattle (http:/www.ansi.okstate.edu/breeds/cattle/), which could explain why Belgian Blue are the only non-British cattle which have this Mx allele. However the number animals assessed form each breed are too few to draw significant conclusions. While a protein polymorphism (*i.e.* the proline for leucine substitution in Mx1) that co-segregates with the 3'-UTR polymorphism remains undocumented, the breed-associated differences seen offers the possibility of further polymorphisms to be found by investigation of individual breeds. This could be pursued in both *Bos taurus* as well as *Bos indicus*, which have been shown to have a large number of polymorphisms relative to *Bos taurus* (Lagziel and Soller, 1999).

The chromosomal location of bovine Mx1, coupled with the density of highly polymorphic satellite markers on the linkage map of BTA1, make the screening for linkage to antiviral activity that is significant at the whole animal level a practical procedure. Indeed, preliminary linkage data now exist for an RFLP polymorphism at the trout Mx locus and resistance to rhabdoviral disease (Trobridge *et al.*, 2000).

Pursuit of Additional Mx Genes

There is only one documented reported of two Mx proteins in cattle (Horisberger, 1988). The polymorphism identified in chapter II would seem unlikely to account for the

differences that they report. Polymorphism in Mx proteins has been reported for human MxB, which results from utilization of alternate initiation codon, downstream of the initial methionine. The contexts of the first two in frame methionines of bovine Mx1 are in equally optimal contexts, though initiation is known to favor the first such codon. It is intriguing that this second methionine, in an alignment with murine Mx1 is cognate with the initiation methionine of mouse Mx1, perhaps reflecting the possibility that the mouse gene has, within an evolutionary time frame, lost coding sequence at the 5' end.

The question of differentially spliced transcripts as a source of protein polymorphism aside, one would expect multiple Mx genes simply from what is known in those species which have been most carefully and thoroughly characterized, *i.e.* human, rat, mice, swine, trout, and salmon, which all have multiple Mx genes. Evaluations of swine (Müller *et al.*, 1992b) and human (Aebi *et al.*, 1989; Horisberger *et al.*, 1990) found transcripts of one gene (huMxB and poMx2) to exist at approximately one fifth to one tenth the level of the other Mx gene transcripts. Assuming a similar phenomenon may exist in bovine Mx gene transcription, *i.e.* only 10% of Mx transcripts represent the second bovine Mx gene, thoroughly screening 30 clones would be required to assume, at a 95% confidence interval, that the existence of a second gene had been ruled out. Use of a 5' probe, from the highly conserved region of the tripartite GTPase elements, could be used to screen a library at low stringency. Determining the DNA sequence of the less conserved 3' ends of cDNAs would be more sensitive than simply restriction mapping clones. Following such a plan should yield a successful outcome. If the option exists, cycloheximide treatment of interferon induced cells in culture, which has led to successful

cloning of transcripts with questionable stability in the past (Staeheli *et al.*, 1988), may be useful.

Mx Genes: Mapping and Genomics

In the linkage families studied, the polymorphism segregated two groups of *Bos taurus*; Hereford and Belgian Blue cattle possessed the both the 163-bp and 150-bp alleles, while representatives of different continental breeds possessed only the 150-bp allele. The mapping of the Mx1 gene to BTA1 is expected, given that the Mx gene is syntenic with HSA21 genes, all of which have mapped to BTA1. Cattle, however, have the HSA21 genes distributed as two discrete groups, one on the proximal portion and one at the distal portion of BTA1, with the balance of BTA1 being syntenic with human chromosome 3. The association of a syntenic group that corresponds to genes from HSA3 and HSA21 is also found in other ruminants. For example, the q arm of sheep chromosome 1 (OAR1) is equivalent to BTA1, shares synteny with HSA3 and HSA21, and includes the split distribution of HSA21 seen on BTA1 (Iannuzzi *et al.*, 1996). The p arm of OAR1 is equivalent to BTA3, and is syntenic with HSA1. Postulating a single ancestral ruminant chromosome, from which BTA1 and BTA3 arose, would provide some evolutionary explanation of the existence of HSA1 homologous genes on BTA1. Mapping of bovine Mx1 on the RH map of BTA1 will help to clarify the order of conserved genes shared between BTA1 and HSA21. When Mx genes and its neighbors, are mapped on other ruminant chromosomes, it will also be possible to postulate the structure of an ancestral ruminant chromosome from which BTA1 derived, and to speculate in an informed manner about the location of Mx on such an antecedent. The form of such an ancestral ruminant chromosome is likely to confirm a close syntenic

relationship between HSA3 and HSA21 genes, owing to the antiquity of this relationship. A syntenic relationship, similar to that seen between BTA1 and HSA3 and HSA21 is found the European shrew (Dixkens *et al.*, 1998) that diverged from other mammals as part of the epitherial radiation of the eutheria, which occurred approximately 115 million years ago (Shoshani *et al.*, 1998). Primates maintained the association of this primitive syntenic group until the radiation that saw the lemurs, in whom both HSA3 and HSA21 hybridize to the q arm of lemur chromosome 1 (Apiou *et al.*, 1996), split from other primates.

The organization of the Mx genes relative to each other has not been determined. Recombination between mouse Mx1 and Mx2 has not been seen in the animals that were used to generate the congenic Mx^{+} mice. Identification of a second bovine Mx would allow for sequence analysis and mapping, which would broaden our understanding of the evolution of Mx genes. The most conservative assumption is that the Mx genes were evident as duplicates before extensive mammalian speciation.

Assay Development

We have shown that bovine Mx can be used as an indirect assay of interferon in cell culture. As an interferon assay, Mx is 100 fold more sensitive to interferon levels when compared to conventional bioassays. An Mx assay has the potential advantage of being refractory to significant concentrations of interferon-γ, which simplifies the purification steps necessary. An assay could also potentially accommodate samples contaminated with virus by selecting harvest time points and target cells so that one would not have interference from virally stimulated endogenous interferon. One can also use the Mx promoter in the context of an easily assayed reporter gene, as has been done for

human type I interferons (Canosi *et al.*, 1996). The possible uses of such an assay, aside from the advantage of a one-day turn around without need of challenge virus, could include clinical as well as research application. Use of Mx assays as markers for interferon could be part of an algorithm assessing animals for unknown infectious agents, either routinely, for example as part of an abortion work-up, or in unusual settings, such as novel infectious diseases of unknown etiology. Simple and practical systems that involve whole blood samples and chemiluminescent immunoassays have been used in humans for quantification of Mx and reach a level of sensitivity of 20 ng/ml (Oh *et al.*, 1994). Mx assays may have use as markers for pregnancy as well, if elevation of peripheral blood leukocyte Mx levels can be documented at this time. Use in research studies would likewise be as a marker for a successful interferon response in target tissues. It is entirely possible that any assay that is developed for cattle could find utility in sheep as well.

Concluding Remarks

I have detailed the various levels significance of the findings of the research presented in this dissertation in the sections previous to this conclusion, and will end with a summation of directions that cattle Mx research should take.

Development of an easy, quick, specific, and sensitive bovine Mx assay has significant potential. As assay that uses whole blood or cells in culture can be an extremely valuable research tool, even if bovine Mx is eventually found to have no antiviral activity, since it may find its greatest utility as a marker in peripheral blood of recent interferon exposure and in cell culture as a type I interferon bioassay.

Pursuit of antiviral activity of bovine Mx should not be abandoned if the clones in this report are eventually proven to be inert. We have demonstrated breed-associated

Mx1 alleles, and there are other cattle of both *Bos indicus* and *Bos taurus* breeds whose Mx proteins could be evaluated. Use of double immunofluorescence and flow cytometry could allow quantification of levels of Mx staining and correlations with diminished viral protein expression. This would allow investigation of transient transfections of proteins which seem to have some long term toxic effect. However results of such studies may not be conclusive since alterations of Mx staining levels relative to viral protein production could be caused by an altered biological activity, altered immunoreactivity, or both.

Assumption of non-antiviral activity from the results of one group of clones would be a mistake. For whatever reason, the Mx genes have demonstrated heterogeneity, as seen in the mice, with inactive and active genes found in both domestic and non-domestic lines. A telling example of the non-activity of Mx genes is seen in mouse Mx2, which was assumed to be inactive in both wild and domestic populations for over a decade until the question was re-examined. Hence, a conclusion that cattle Mx lacks antiviral activity should be postponed until a study using an appropriate sample size derived of genetically diverse animals is concluded. Clearly there is still much to explore in Mx studies in agricultural animals.

CHAPTER IV

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