POLYMORPHISM IN CHICKEN IMMUNE RESPONSE GENES AND RESISTANCE TO DISEASE

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POLYMORPHISM IN CHICKEN IMMUNE RESPONSE GENES AND RESISTANCE TO DISEASE

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POLYMORPHISM IN CHICKEN IMMUNE RESPONSE GENES AND RESISTANCE TO DISEASE

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VITA

Ann Marie O'Neill, daughter of Francis Crawford and Mary Crawford, was born in Belfast, Ireland on September 11th 1967. She graduated from the University of Ulster in 1989 and pursued a career in Human Resource management until 1997, when she moved with her family to Australia. She graduated from Edith Cowan University in Perth, Western Australia with a Bachelor of Science in Human Biology in 2003. She was granted a Cellular and Molecular Biology fellowship at Auburn University in fall 2003. In fall 2004 she joined the Department of Pathobiology as a graduate research assistant. She is married to Martin O'Neill and they have four children Conor, Eoghan, Sorcha and Ruarc.

DISSERTATION ABSTRACT

POLYMORPHISM IN CHICKEN IMMUNE RESPONSE GENES AND RESISTANCE TO DISEASE

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Genetic resistance to a number of viral pathogens has been linked to polymorphisms in genes of the immune system. Among these are the genes of the major histocompatibility complex (MHC) and those induced by the type I interferons (IFN). The chicken MHC contains two classical loci, *BF1* and *BF2*, with *BF2* reported as the dominantly expressed gene that functions to present antigen to cytotoxic T lymphocytes (CTL). The BF1 molecule has recently been shown to function as negative regulator of Natural Killer (NK)-like cells.

In mammals, the level of expression of MHC molecules influences their function. Furthermore selective pressures exerted by pathogens are thought to drive diversification of MHC expression. Little is known in chickens about the effects of allelic differences among BF1 molecules on expression or in regulatory elements that control transcription.

The first two objectives during this investigation were to quantify the level of expression of the two alleles of the MHC class I in the chicken in several haplotypes and then to attempt to provide a molecular explanation for the differences in expression by sequencing of the promoter and signal peptide regions and also to sequence the entire gene of one BF1 allele with very low transcript levels.

This study confirmed by quantitative reverse transcription PCR the lower level of expression of *BF1* relative to the *BF2* gene. In addition, nucleotide sequences were obtained that showed extensive polymorphism in promoter regions between alleles with conservation in identified regulatory elements. One BF1 allele had very low transcript abundance, presumably caused by a mutation that abolished a 3' splice site in intron 7.

Interferon induced antiviral activity is an important line of defense against viral pathogens. Among the genes induced by interferon, the GTPase Mx is one of the best characterized in mammals, and polymorphism at residue 631 determines antiviral activity in chickens. The role of chicken Mx has been assessed against vesicular stomatitis virus and influenza virus in transfection experiments. However, the role of chicken Mx against infectious bursal disease virus, an important pathogen of chicken, has not been examined. In the third part of this investigation the antiviral activity of interferon and the IFN-induced protein Mx were examined against infectious bursal disease virus in cell cultures differing in Mx genotype. This study confirmed that IFN has antiviral activity against IBDV infection in this system in a dose dependent manner. Collectively, the results presented in this dissertation may contribute to understanding the immune mechanisms involved in response to disease and genetic factors that determine susceptibility or resistance.

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I. INTRODUCTION

Innate immunity is the first line of defense in response to viral infections. It is characterized by a non-specific rapid response that is short-lived. This response is induced by the recognition of virally derived components by pattern recognition receptors on host cells. This leads to the release of interferon (IFN), expression of IFN-stimulated genes (ISGs) and establishment of the antiviral state in host cells (Medhovitz and Janeway 2000).

The major histocompatibility complex (MHC) is a cluster of genes that encodes proteins that are mainly associated with both innate and adaptive immune responses and regulation. Among these are membrane glycoproteins that present pathogen derived peptides to effector cells of the immune system. The MHC of chickens is smaller and more compact than its mammalian counterpart. One of the most striking features of the chicken MHC is the influence it has on disease susceptibility, in particular to Marek's disease virus and Rous sarcoma virus (Bacon 1987; Briles et al. 1983; Brown et al. 1984). This region in the chicken, in comparison to mammals, is small and compact with two closely linked class I loci, *BF1* and *BF2*. While it is well documented that BF2 functions to present antigen to cytolytic T lymphocytes (CTL) in an MHC restricted manner, the function of BF1 was unknown until recently. Because the BFI molecule resembles the human HLA- C in terms of level of expression and patterns of polymorphism it was hypothesized that it may function as a negative regulator of natural killer (NK) cells

(Livant et al. 2004). Indeed, unpublished experiments by collaborators demonstrated that while BF1 is not involved in presentation to CTL, expression of BF1 molecules on target cells inhibits NK-like killing.

In addition to the genes of the MHC, other genetic regions are involved in determining host response to pathogens, in particular, those ISGs that encode proteins involved in establishing the antiviral state. Among the ISGs, one of the best characterized is the GTPase-encoding *Mx* gene. The Mx protein has been shown to exhibit antiviral activity in humans, mice and fish. Two functionally different alleles have been reported in the chicken, with the *Mx*Asn631 allele showing antiviral activity against both influenza virus and vesicular stomatitis virus in transfection experiments whereas *Mx* Ser631 lacks antiviral activity (Ko et al. 2002, 2004b). However to date the chicken *Mx* Asn631 and Ser631 alleles have not been evaluated for antiviral activity against infectious bursal disease virus (IBDV), an important virus of poultry.

In light of the functional specialization of *BF1* and *BF2* genes, these studies evaluate five MHC haplotypes for the level of class I gene expression using quantitative PCR. In order to investigate molecular reasons for differential expression, nucleotide sequences of nine different *BF1* alleles were obtained and comparison made with known transcription regulatory regions in the 5' upstream region of mammalian MHC class I, and also between haplotypes, through the entire gene into the 3'UTR. Finally, chicken IFNα, and the two alleles of the IFN-induced chicken *Mx,* were examined for differential antiviral effects following IBDV infection in cell culture.

II. LITERATURE REVIEW

Major Histocompatibility Complex

A cluster of genes on human chromosome 6 and mouse chromosome 7 encodes the major histocompatibility proteins. This cluster of genes is therefore called the major histocompatibility complex (MHC). The MHC was first described in mice (*H-2* complex) and later in humans, the human leukocyte antigen (*HLA*) system, as a result of transplantation experiments (Kelley et al. 2005). In the chicken these genes were originally identified as a blood group antigen system (Briles et al. 1950). Genes encoded by the MHC are mainly associated with immune response and regulation (Knapp 2005). In the mammal, the MHC is divided into three regions, MHC class I, MHC class II and MHC class III. MHC class I proteins present intracellularly derived peptides to CD8+ T cells and are ligands for natural killer (NK) cells (Williams et al. 2002) while MHC class II proteins present exogenously derived peptide to CD4+ T cells (Villadangos 2001). The MHC III region separates the MHC I and MHC II regions and contains a heterogeneous array of genes that have roles in both the innate and adaptive immune response; however the function of many genes in this region have still not been defined (Xie et al. 2003). One of the hallmarks of the MHC is its extreme polymorphism, in particular in the MHC class I and II genes. It is speculated that selection for resistance to pathogens has been the impetus behind this variety as most of the variation is associated with peptide binding (Trowsdale and Parham 2004).

MHC class I molecules are constitutively expressed on almost all nucleated cells with levels of expression varying by cell type; immune cells have the highest level of expression while expression is lowest in neural and germline cells (Daar et al. 1984; Le Boutellier 1994; Singer and Maguire 1990). MHC class I genes can be divided into classical and non-classical. The classical MHC class I proteins in mammals have 2 major functions : to bind intracellularly-derived antigenic peptides, such as viral, and present them to CD8+ T cells in an MHC-restricted manner thus inducing an adaptive immune response (Germain and Margulies 1993), and to function as regulators of the innate immune response in that they are ligands for killer inhibitory receptors (KIR) on natural killer (NK) cells (reviewed in Lanier 1998).

The MHC class I molecule is a heterodimer and consists of a polymorphic heavy chain that comprises a cytoplasmic domain, a transmembrane region and 3 extracellular domains, α1, α2 and α3, and a β2 microglobin – a conserved protein that facilitates the cell surface expression of the molecule; it is not encoded in the MHC (Grey et al. 1973; Orr et al. 1979; Peterson et al. 1974). The peptide binding groove is made by pockets in the α1 and α2 domains of the heavy chain and this area is highly polymorphic mainly as a result of multiple single residue substitutions, with many alleles at each locus (Bjorkman and Parham 1990; Le Boutellier 1994; Parham 1994). Each protein is capable of binding a wide repertoire of related antigenic peptides, thus conferring diversity in antigen presentation to CTLs (Lawlor et al. 1990). In humans, there are three classical MHC class I loci, *HLA-A, B* and *C*. HLA-A and B are the main molecules that are involved in inducing a CTL response whereas HLA-C is important in modulating the innate response and NK cells (Cohen et al. 1999; Littaua et al. 1991; Ljunggren and Kärre 1990).

Overall, *HLA-A* and *HLA-B* alleles are more polymorphic than *HLA-C*. Additionally, a number of conserved residues are present in the α1 helix of HLA-C that are highly variable among HLA-A and HLA-B (Zemmour and Parham 1992); these conserved residues are contacts interacting with inhibitory receptors on NK cells (reviewed in Vilches and Parham 2002).

MHC class I molecules form complexes with endogenously derived peptides and are expressed on the cell surface. MHC class I molecules are assembled in the endoplasmic reticulum (ER) and antigenic peptides are delivered to the ER from the cytoplasm by TAP (transporter associated with antigen processing) and loaded onto the empty MHC class I molecules (Heemels and Ploegh 1995). These peptides are generated by the actions of proteasomes that cleave proteins targeted for degradation via the ubiquitination pathway into peptides suitable to fit the peptide binding groove (Rock and Goldberg 1999). Following this cleavage, the peptides bind TAP and are transported across the lumen of the ER and loaded onto the MHC (Androlewicz et al. 1993). These loaded peptides are further cleaved before they reach the cell surface to ensure high affinity complexes for T-cell receptor (TCR) engagement (reviewed in Williams et al. 2002).

MHC class II glycoproteins, on the other hand, are expressed on the surface of cells of the immune system, for example B lymphocytes, dendritic cells and macrophages Steimle et al. 1994). Structurally, the MHC class II molecule comprises two transmembrane chains, α and β, both encoded in the MHC locus. The peptide binding groove is formed by the α1 and β1 domains, this region displaying high polymorphism (Fremont et al. 1996; Parnes 1989). MHC class II molecules function to present peptide

derived from exogenously synthesized proteins to CD4 helper T cells (Parnes 1989; Teh et al. 1988). These peptides vary in length but generally are 13-17 amino acids long (Lippolis et al. 2002).

Regulation of MHC I expression

Expression of MHC class I is mainly transcriptionally regulated, in part by *cis*-acting sequences in the promoter region of the gene that interact with *trans*-acting factors (Gobin et al.1997). The Enhancer A region is a conserved sequence in the upstream region of the *MHC I* gene and it refers to 2 NFκB binding sites, NFκB2 and NFκB1 (Burke et al. 1989; Kimura et al. 1985). Enhancer A controls transcription of the class I gene by interaction with nuclear proteins from the NFκB/Rel family (Burke et al. 1989; Gobin et al. 1998). The NFκB binding motif GGGGATTCCCC is highly conserved and the dyad symmetry of this element is critical to NFκB binding (Mansky et al. 1994). In particular, this element is most conserved in both *HLA-A* and *HLA-B*, such that NFκB mediated activation of classical MHC class I is restricted to these loci (Cereb et al. 1994; Gobin et al. 1998). However, lack of symmetry in the NFκB1 site of *HLA-B* requires binding of other transcription factors to this site (Gobin et al. 1998). CREB/ATF, AP-1 and SP-1 have all been described as binding here (reviewed in Singer and Howcroft 2003).

Expression of MHC class I genes is also under the control of Type I and Type II interferons (IFNs) (Johnson and Pober 1994). This is mediated by the interferon stimulated response element (ISRE), a DNA sequence located downstream of Enhancer A that binds members of the IFN regulatory factor (IRF) family, specifically IRF1, IRF2

and IRF3 (Singer and Maguire 1990). These proteins are induced by the IFN stimulated gene factor (ISGF)3 complex following activation by pathogens (Chang et al. 1992; Harada et al. 1989).

A number of other short sequences, homologous to other regulatory elements, are also present in the promoter of the MHC class I gene. The SXY regulatory module is present in the MHC class I gene and was first described in the promoter of MHC class II genes. It comprises 4 elements: the S box, the X1 box, the X2 or cAMP response element (CRE) and the Y box /Enhancer B (van den Elsen et al. 1998). This module is bound by a multi-protein complex comprising regulatory factor X (RFX), CRE binding protein (CREB)/activating transcription factor (ATF), nuclear factor Y (NFY) and class II transactivator (CIITA) (Gobin et al. 2001). As with the majority of eukaryotic genes that are transcribed by RNA polymerase II, the common promoter elements of the TATA box and the CCAAT box have also been described in MHC class I genes (Singer and Maguire 1990).

Cell surface expression of HLA-C is approximately one-third to one-tenth that of *HLA-A* or *HLA-B* (McCutcheon et al. 1995). This reduced expression may be explained in part by the difference in transcriptional regulation and lack of NFκB binding sites in Enhancer A of *HLA-C* (Gobin 1998). Other possible explanations for this reduced expression include the high turnover of HLA-C mRNA (McCutcheon et al. 1995; Zemmour and Parham 1992) or, at the protein level, prolonged interaction with TAP due to the restricted peptide binding ability (Neisig et al. 1998).

Chicken MHC

The chicken MHC, called the *B* complex, was originally described as a system controlling blood group antigens (Briles et al. 1950). Hemagglutination assays were used to define haplotypes serologically and these were numbered consecutively from B1 to B27 (Briles and Briles 1982). The classical MHC complex (*B* region) in chickens is simple and compact, with 19 genes in approximately 92 kb (Kaufman et al. 1999). It can be subdivided into three regions: *B-F, B-LB* and *B-G*, with the *B-F* and *B-LB* regions corresponding to MHC class I and MHC class II respectively (Guillemot et al. 1988; Pink et al. 1977) (Figure 1). In particular, the *B-F/B-L* region is remarkably compact, with eleven genes in 44 kb with an average size of 1.3 kb (Kaufman 2000). This region contains no repetitive elements, gene fragments or pseudogenes and distances between adjacent genes are short; thus recombination between the *B-F* and *B-L* regions is extremely rare, with no recombination events having been observed in over 6000 experimental matings (Skjødt et al. 1985); consequently alleles at these loci are inherited together resulting in stable and distinct MHC haplotypes (Briles and Briles 1982; Hala et al. 1981a, b).

The gene content and organization of the chicken MHC differs from its mammalian counterparts. A number of mammalian orthologues that are also on the mammalian MHC are located in this region, including classical class I and class IIB genes close together on the locus, *TAP, DMα, DMβ, RING3, histone H3, leu-tRNA* and *tapasin* (Kaufman et al. 1999b). However, unlike the mammalian MHC there are no classical class IIα genes, no *LMP* genes and the class III region has only one gene, *C4* (Kaufman et al. 1999b). There are also genes present that would not be expected to be

there, as they are not present on the mammalian MHC, including a c type lectin and a putative NK receptor gene. Additionally, this region also contains genes for which no mammalian equivalent has been identified (Kaufman et al. 1999b).

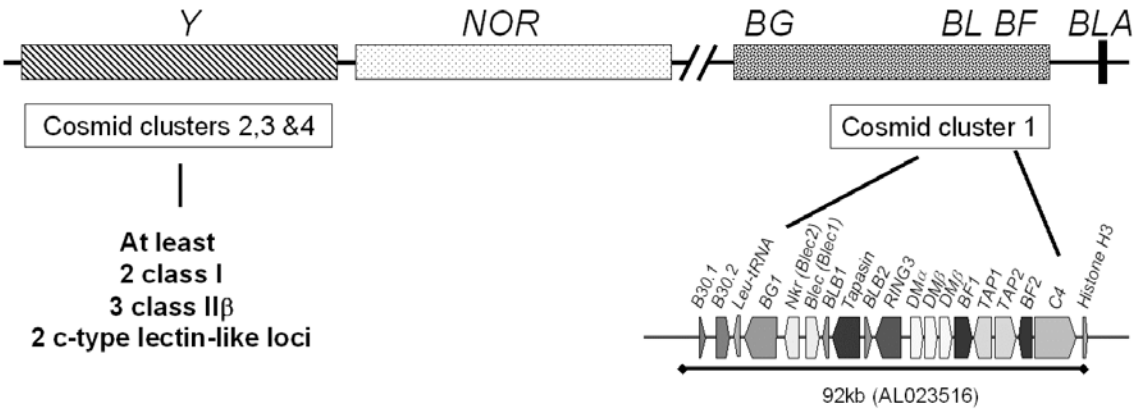


Figure 1. Map of the chicken MHC on chicken chromosome 16. (From Miller et al. 2004)

The B complex encodes two genes that are homologous to mammalian MHC Class I genes, *BF1* and *BF2*. These genes flank the two *TAP* genes and lie in opposite transcriptional orientation (Kaufman 1999b) (Figure 1). Structurally, the BF molecules are predicted to resemble MHC class I with most of the polymorphic residues located in the in the α1 and α2 domains (Hunt and Fulton 1998).

Like their mammalian counterparts, expression of chicken MHC class I and MHC class II molecules is controlled primarily at the transcriptional level. A number of conserved sequences and putative regulatory elements have been reported in the *BF2*

allele of the B12 haplotype (Kroemer et al. 1990). These include Enhancer A regions, and ISRE, X1 Box, CRE, Y Box and CCAAT Box. No TATA box was identified but rather an SP1 binding where the TATA box would be predicted to be, based on the situation in humans and mice (Briggs et al. 1986). However, one porcine MHC molecule is expressed despite the lack of a TATA box in the promoter region, so it is likely that it is not required (Ehrlich et al. 1987; Singer and Maguire 1990). Of these elements, to date only the ISRE has been studied with regard to function in chickens and was shown to activate transcription *in vitro* in response to chicken IFN (Zöller et al. 1992).

BF2 are the dominantly expressed class I molecules and present antigen to CTL in an MHC-restricted manner (Fulton et al. 1995; Omar et al. 1998; Thacker et al. 1995). BF1 molecules are expressed at a lower level than BF2 and some haplotypes lack expression entirely having only one intact MHC class I locus, *BF2* (Kaufman et al. 1999a). The function of BF1 has only recently been elucidated; it has been shown to be incapable of presenting viral antigen to CD8+ T cells, but it does prevent NK–like killing when expressed on the surface of target cells (Hunt et al. unpublished).

MHC and Disease Resistance

One of the most striking characteristics of the chicken *B* complex is the influence it has on disease resistance or susceptibility in chickens, the two best documented examples being Rous Sarcoma Virus (RSV) and Marek's Disease virus (MDV) (Bacon 1987; Briles et al. 1983; Brown et al. 1984). MDV is a herpesvirus with a large genome that encodes around eighty proteins. Disease progression following infection is long, eventually leading to lethal T cell tumors (Witter and Schat 2003). MDV is the most

prominent of the naturally occurring avian diseases for which MHC-associated influence has been described; indeed, across any species it represents the strongest association between MHC and disease resistance (Plachy et al. 1992). Several MHC genes are candidates for mediating susceptibility or resistance to MDV due to their known roles in immune responses. The identity of the MHC gene(s) involved in determining resistance or susceptibility are not known; however cell mediated immune responses are important in genetic resistance to MDV, with both NK cells (Garcia-Comacho et al. 2003) and CTLs (Markowski-Grimsrud and Schat 2002) having been proposed as mediators. Among MHC haplotypes, *B*21 is the most resistant to MDV infection, while *B*2, *B*6 and *B*14 are moderately resistant with *B*19 being most susceptible (Bacon 1987). RSV is a small retrovirus with four genes that causes rapidly developing tumors in infected chickens that progress in some haplotypes but regress in others, with MHC being a major determinant for regression (Collins et al. 1977; Plachy et al. 1992; Svoboda et al. 1992). The *B*12 haplotype shows regression of the tumors while in the *B*4 and *B*15 these tumors progress (Cutting et al. 1981; Plachy and Vilhelmova 1984).

A number of hypotheses exist in an attempt to explain the control of susceptibility or resistance to disease exerted by the MHC. First, the peptide binding motifs of the BF2 molecule, that is the predominantly expressed MHC class I in the chicken, may ultimately determine whether or not a strong CTL response is made towards the pathogen. Second, the low level of MHC class I expression in some haplotypes may reduce the threshold of activation of NK cells. Third, the BF1 molecule may function as a negative inhibitor of NK cells and fourthly, other genes encoded in the chicken MHC

that resemble those for NK regulators in mammals may be important in resistance or susceptibility.

Cytotoxic T lymphocytes, which are CD8+, target virally infected cells that display antigen via the MHC class I molecule in an MHC restricted manner (Marrack and Kappler 1987) and CD8+ Tcell-mediated killing of target cells has been demonstrated in chickens (MacCubbin et al. 1986; Weinstock et al. 1989). Of the chicken MHC class I molecules, the BF2 molecule is the most highly expressed and has been shown to present peptide to CTLs in an MHC-restricted manner (Fulton et al. 1995; Omar et al. 1998). Schat and co-workers have developed a system utilizing transformed cell lines expressing MDV genes that has been used in assays to ascertain CTL involvement in MDV resistance (Omar and Schat 1996; Schat and Xing 2000); they have shown that CD8+ cells are involved in MDV resistance, with B19 (susceptible) chickens having a reduced response to cells expressing ICP4, an MDV-encoded protein, compared to the B21 (resistant) chickens (Markowski-Grimsrud and Schat 2002). The "minimum essential MHC" hypothesis proposed by Kaufman and co-workers (1995) attempts to explain MHC-controlled disease resistance in terms of the BF2 molecule and peptide binding specificity. In mammals, the redundancy of the MHC confers more or less equal protection against pathogens across haplotypes. However, the small compact MHC of the chicken and the dominant expression of a single MHC class I molecule (BF2) results in differential susceptibility to pathogens among haplotypes so that no single haplotype responds optimally to all diseases (Kaufman 2000). This was illustrated with RSV, where it was shown that the binding motifs of the single, dominantly expressed MHC class I molecule can determine resistance or susceptibility to disease (Hoffman et al.

2003). It has been shown by peptide binding assays that the susceptible B4 haplotype is incapable of presenting viral peptide to CD8+ T cells via the MHC class I molecule. Therefore no T cell response is elicited whereas a response is elicited in the resistant *B*12 haplotype, which can bind several RSV peptides (Hofmann et al. 2003). Consequently, it has been proposed by Kaufman that MHC-associated disease susceptibility to viruses with small genomes may be due to the inability of some *BF* alleles to present viral antigen to CD8+ T cells.

The MHC-mediated control of MDV may be due to increased activity of NK cells (Kaufman et al. 1995). In mammals, NK cells are important in the control of viral infections and their activity is regulated by both inhibitory and activating receptors (reviewed in French and Yokoyama 2003). The importance of the role of NK cells is reinforced by the viral mechanisms that have evolved to evade NK-mediated killing with some herpesviruses encoding MHC class I mimics (reviewed in French and Yokoyama 2003). Mammalian NK cells are inhibited by cell surface MHC class I molecules (Moretta et al. 2002); loss of cell surface MHC in virally infected cells is common and releases NK cells from inhibition (reviewed in French and Yokoyama 2003).

Cells that functionally and phenotypically resemble mammalian NK cells have been described in the chicken (Chai and Lillehoj 1988; Göbel et al. 1994; 2001; Sharma and Okazaki 1981). Therefore, the low expression of cell surface MHC class I molecules in the MDV resistant *B*21 haplotype is theorized to reduce inhibition and allow for easier activation of NK cells (Kaufman et al. 1995). Furthermore, *B*21 chickens, which are genetically resistant to MDV, show increased levels of NK-like cells in the spleen following infection, while in the susceptible *B*19 haplotype there is a decrease in

NK-like activity (Garcia-Comacho et al. 2003; Sharma 1981). Antibody staining and flow cytometry were used to determine the cell surface expression of MHC class I molecules on chicken cells of various MHC haplotypes. What emerged was that the pattern of cell surface expression of MHC class I molecules correlated inversely to disease resistance – the resistant *B*21 haplotype has low levels of cell surface expression, with susceptible *B*19 having the highest level (Kaufman and Salmonsen 1997). This reduced level of expression in *B*21 cells is not due to transcription, translation or association with β2 microglobulin but is likely to be related to mechanisms that facilitate transport to the cell surface (Kaufman et al. 1999a, b).

Other genes encoded in the MHC may also be important in regulating NK killing; and the BF1 molecule was hypothesized to be inhibitory to NK cell killing (Livant et al. 2004). The HLA-C molecule is an important inhibitory ligand for KIR on human NK cells (Cohen et al. 1999). The structure of the BF1 molecule resembles that of HLA-C in a number of ways. Overall, *BF1* sequences are less polymorphic than *BF2* (Livant et al. 2004); similarly *HLA-C* is less polymorphic than either *HLA-A* or *HLA-B* (Parham et al. 1995). The C terminus of the helix of the α1 domain of the BF1 sequence is also less polymorphic in that it displays high conservation with a number of conserved residues that are highly variable in the BF2. These residues tend to be located in regions that are predicted to interact with either antigenic peptide or the TCR (Livant et al. 2004). Again, similar comparisons can be made between HLA-A, HLA-B and HLA-C with the latter having conserved residues in the α1 helix region (Parham et al. 1995) that interact with inhibitory NK receptors (reviewed in Lanier 2005). Therefore, based on level of expression, available functional data and patterns of polymorphism it has been proposed

that the BF1 molecule may interact with killer inhibitory receptors (KIR) on NK cells to negatively regulate activity (Ewald and Livant 2004). Recent experiments by collaborators (Hunt and Miller) demonstrated that BF1 expression by target cells strongly inhibits killing by chicken NK-like cells (manuscript in preparation).

Additionally, a number of other genes in the chicken MHC have been characterized and have homology with mammalian C-type lectin NK receptors, namely the *B-lec* and *B-NK* genes (Rogers et al. 2005; Shiina et al. 2007). In mammals, C-type lectin proteins have a number of regulatory functions in the immune response (Weis et al. 1998). Some of these proteins form NK surface receptors that bind MHC class I molecules and either activate or inhibit NK killing (reviewed in Lanier 1998). However, in mammals these proteins are not encoded in the MHC, but on a region termed the NK complex (NKC) (Yokoyama and Seaman 1993). Although the ligands in chickens for B-lec and B-NK are not known, it is predicted that B-lec may function as activator of NK cells and B-NK as an inhibitory receptor (Rogers et al. 2005). Because they may function to regulate NK cells, B-lec and B-NK are also candidates for conferring resistance to MDV.

However, it must be pointed out that it is known that other genetic regions that lie outside the MHC are also involved in determining resistance or susceptibility to MDV, and other pathogens, in the chicken (Bumstead 1998; Yonash et al. 1999; Zhu et al. 2003). Genetic mapping studies in lines 6_1 and 7_2, which have the same MHC haplotype but differ in susceptibility to MDV, have revealed that resistance or susceptibility may be linked to a region in the chicken chromosome that is syntenic with that in mammals that contains the NK complex (Bumstead 1998).

NK cells

NK cells function to remove intracellular pathogens from the body without prior sensitization. They are part of the innate immune response and are important in the early stage of infection (0-5 days), prior to the mobilization of adaptive immunity (reviewed in Backstrom et al. 2004; French and Yokoyama 2003). NK cells are lymphoid cells that are derived from the pluripotent hematopoietic stem cells and develop outside the thymus. The effector functions of NK cells are important in defense against intracellular bacteria, parasites and certain viruses. In addition, activated NK cells have recently been implicated as providing a link between the innate and adaptive immune response, as activated cells produce cytokines such as IFNγ, tumour necrosis factor and macrophage inflammatory proteins 1a and 1b, (Backstrom et al. 2004; Shi et al. 2001). A number of inhibitory and activation receptors have been identified on the surface of NK cells, and the interplay of signals from both is important in the activity of NK cells (Byron 1997). Some of the inhibitory receptors are specific for MHC class I molecules on target cells, and those target cells that lack the MHC class I surface molecule, especially virally infected cells, are killed by NK cells (Lanier 1998). The MHC class I molecule is expressed on the surface of most healthy cells, however it is downregulated following viral infection or transformation of the cell (Byron 1997).

Activated NK cells function to lyse infected cells through NK cell-mediated cytotoxicity (Backstrom et al. 2004; Lanier 1998). This requires that the NK cell come into contact with the target cell to facilitate the formation of an immunological synapse (Davis 2002; Kunz and Held 2001). This allows the perforins in the NK cells to generate pores in the target cell through which the NK granzymes can be introduced, leading to

apoptosis in the target cell (Backstrom et al. 2004; Byron 1997). In addition, a further function that has been identified for NK cells is the activation of macrophages to kill phagocytosed microbes and this is achieved by the secretion of IFNγ (Lanier 1998).

NK cell receptors

To date, two categories of receptors that modulate NK cell activity have been identified: the immunoglobin (Ig) superfamily and C-type lectin family. The Ig superfamily contains the killer immunoglobin like receptors (KIR) and the leukocyte immunoglobin like receptors (LILR), both multigene families. In humans, the *KIR* and *LILR* genes are encoded by a region named the leukocyte receptor complex (LRC), located on chromosome 19q13.4 (Wilson et al. 2000). The C-type lectin family includes the human and mouse CD94/NKG2 receptors and mouse Ly49 (Backstrom 2004; Lanier 1998). There is no rodent *KIR* - in mouse, the Ly-49 family is the major family of NK receptors specific for MHC class I molecules. Similar to *KIR*, they are encoded by a small family of polymorphic genes and have inhibitory and activating forms (Backstrom 2004). To date, over 23 *Ly-49* genes have been identified, termed *Ly-49a-w* (Sundbäck et al. 2002).

Common to inhibitory receptors in both groups is a consensus amino acid sequence in the cytoplasmic domain – the Immunoreceptor tyrosine inhibitory motif (ITIM) (Ravetch and Lanier 2000). Binding of these receptors by their ligands results in tyrosine phosphorylation, by an Src kinase and the subsequent recruitment of a cytoplasmic Src homology domain phosphatase. The ITIM can bind SHP-1, SHP-2 or SHIP (Ravetch and Lanier 2000). This causes the dephosphorylation of intracellular

substrates, the net result being the neutralization of activating signals and regulation of NK activity (reviewed in Vivier et al. 2004).

A primary function of NK cells is surveillance for self-MHC class I on cell surfaces, consequently it is necessary that these cells are able to rapidly detect the presence of MHC I on normal cells, dissociate and continue surveying the population (Boreggo et al. 2001). The nature of the KIR-HLA interaction is such that it allows for fast on-off rates and so may facilitate surveillance for MHC (Vilches and Parham 2002). KIR2DL (inhibitory) and KIR2DS (activating) exhibit a high degree of homology in the D1 and D2 domains and both bind HLA-C ligands. However KIR2DS binds HLA-C weakly whereas KIR2DL receptors bind HLA-C avidly (Vilches and Parham 2002). The functional consequences of these interactions are that if both KIR molecules bind MHC class I at the same concentration, the inhibitory binding is stronger and so this signal will predominate (Ravetch and Lanier 2000).

In chickens, candidate genes for KIR-like NK receptors have only recently been identified. Seventy putative functional chicken Ig-like receptors (CHIR) have been described (Denis et al. 2000; Nikolaidis et al. 2005; Viertleboeck et al. 2005). These molecules have been broadly classified into putative activating receptors (CHIR-A) that have short cytoplasmic domains, inhibitory receptors (CHIR-B) that have a long cytoplasmic domain that contains an ITIM and bifunctional receptors that contain both activating and inhibitory motifs (CHIR-AB) (Nikolaidis et al. 2005; Viertlboeck et al. 2005). In 2005, Viertleboeck et al characterized five members of the CHIR family; with the exception of CHIR-AB1 and CHIR-AB2, both of which were shown to have only one Ig domain, the extracellular domains of the remaining CHIR characterized had two Ig

domains. All CHIR-A and CHIR-AB molecules contain positively charged residues in the transmembrane region (Viertlboeck et al. 2005). Similar to mammalian activating NK receptors, this region may interact with adaptor molecules in the target cells. Consistent with inhibitory molecules, CHIR-B1-CHIR-B4 contain 2 cytoplasmic ITIMs. The *CHIR* gene cluster is located on microchromosome 31, a region that is orthologous to the human leukocyte receptor complex (LRC) (Viertlboeck et al. 2005). These genes have features in common with both *KIR* and *LILR*. Like *KIR*, the *CHIR* genes that have been analyzed to date show a high degree of variability while the expression of these *CHIR* on multiple cell types resembles the expression of *LILR*, which are not restricted to NK and T cell subsets but are expressed on B cells, macrophages, monocytes and dendritic cells. But many more *CHIR* genes remain to be characterized.

In chickens, cells with NK-like activity have been described (Chai and Lillehoj 1988; Sharma and Coulson 1979). Additionally, cells that are phenotypically similar to mammalian NK cells in that they are CD8α positive, CD8β negative and CD3 positive have been characterized in the embryonic spleen and among the intraepithelial lymphocyte (IEL) population of the intestine (Göbel et al. 1994, 2001). In addition, the activity of chicken NK-like cells differs depending on whether or not the chickens are genetically susceptible to the disease. In those that are genetically resistant, there is increased NK activity when they are challenged with MDV; conversely, those chickens that are susceptible show a decrease in NK activity (Sharma 1981). To date there has been no demonstration of receptor-ligand interaction involving MHC class I molecules and NK cells in non-mammals. The recently characterized *CHIR* genes include putative inhibitory receptors (CHIR-B), putative activating receptor (CHIR-A) and a novel

bifunctional CHIR-AB molecules that displays both activating and inhibitory characteristics. The similarities to the KIR and LILR suggest that some of these receptors may bind MHC class I molecules and so contribute to the regulation of NK and T cells in chickens (Nikolaidis et al. 2005; Viertlboeck et al. 2005). Recently, one of the receptors, CHIR-AB1, was shown to bind the Fc region of chicken IgY (Viertlboeck et al. 2007).

As previously described, chicken BF1 molecules resemble human HLA-C class I MHC molecules in that they exhibit high conservation in the carboxy-terminus of the α helix of the α1 domain and inhibit NK-like killing. Therefore, it is possible that the BF1 molecule interacts with one or more of the recently described CHIR molecules to modulate NK function and the hypothesis is that genes similar to the putative inhibitory-like *CHIR B1-B4* encode receptors for BFI ligands and function to negatively regulate NK and T cells in chickens, similar to KIR-HLA-C in humans. At least one researcher is actively searching for the inhibitory receptor on chicken NK-like cells that interacts with the BF1 ligand (Marcia Miller, personal communication).

Interferon Induced Immunity

Viruses depend on host cells for their replication and spread. The innate immune response is the first line of defense against pathogens and NK cells are part of the innate response to viruses. Additionally, detection of virus in infected cells results in the activation of a number of signal transduction cascades; these ultimately converge in the nucleus of the cell and regulate the transcription of genes and expression of proteins that result in the antiviral state - an environment antagonistic to viral survival and replication.

The innate immune response is rapid, non specific and of a limited duration (Medzhitov and Janeway 2000). Cells that become infected with viruses initiate a cascade of events leading to an antiviral state. These events fall into three categories. First, virus derived molecular motifs are recognized by cellular pathogen recognition receptors (PRRs). This leads to the production of IFN, which in turn activates expression of interferon stimulated genes (ISGs) that actually mediate the antiviral state. Cells of the innate immune system express receptors that utilize PRRs to detect microbial structures and initiate an antiviral response (Medzhitov and Janeway 1997). PRRs are constitutively expressed on or in all cells of a given type. They are germline encoded and not dependent on immunological memory (reviewed in Akira et al. 2006). The PRRs do not recognize specific pathogens but highly conserved pathogen-derived molecular motifs, termed pathogen associated molecular patterns (PAMPs) present across a wide range of microbes including viruses, bacteria and fungi (reviewed in Kawai and Akira 2006).

Pattern Recognition Receptors

TLRs are type I membrane glycoproteins that are highly conserved from *C. elegans* to humans (Janeway and Medzhitov 2002). These function as PRRs and can be expressed on the cell surface or intracellularly across a number of cell types, including dendritic cells (DCs), macrophages and T cells (reviewed in Akira et al. 2006). They are characterized by having extracellular domains that contain leucine rich repeats, a transmembrane domain and a cytoplasmic Toll-Interleukin 1 (IL-1) receptor (TIR) homology signaling domain (Bowie and O'Neill 2000). Following recognition of

PAMPs, the TLR undergoes a conformational change enabling the recruitment of adaptor molecules to the TIR domain (Dunne and O'Neill 2003). These adaptor molecules are Myeloid Differentiation Factor 88 (MyD88) (Kawai et al. 2001), TIR associated protein (TIRAP)/ MyD88 adaptor like (Mal) (Fitzgerald et al. 2001; Horng et al. 2001), TIR-domain-containing adaptor protein-inducing IFNβ (TRIF)/TIR-domain-containing molecule 1(TICAM1) (Oshiumi et al. 2003; Yamamoto et al. 2002) and TRIF-related adaptor molecule (TRAM) (O'Neill et al. 2003) .

To date, 12 TLRs have been identified in mammals (Akira et al. 2006). These can be grouped according to the type of microbial component that they recognize, for example nucleic acid or protein, with some TLRs capable of recognizing more than one type of structure. However the TLRs that are implicated in viral recognition are TLR2, TLR3, TLR7, TLR8, TLR9 (in Kawai and Akira. 2006).

TLR7, TLR8 and TLR9 are endosomally expressed in plasmacytoid DCs (pDC), a subset of DCs also known as interferon producing DCs due to the high amount of IFN secreted following stimulation by virus (Colonna et al. 2004; Hemmi et al. 2003). TLR 7 and 8 recognize single stranded RNA – IFN secretion has been described in response to synthetic ssRNA derived from human immunodeficiency virus (HIV) and influenza virus, both ssRNA viruses (Heil et al. 2004; Diebold 2004) and by stimulation by RNA homologues such as imiquimod and R848 (Hemmi et al. 2003; Heil et al. 2004).

TLR 9 stimulates secretion of IFN following recognition of unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs; certain viral genomes, for example herpes simplex virus -1 (HSV1) and mouse cytomegalovirus (MCMV) are rich in these motifs (Hochrein et al. 2004; Krug et al. 2004a,b). In addition, synthetic CpGs

are also able to stimulate IFN secretion through TLR9 (Krug et al. 2001; Verthelyi et al. 2001). Following recognition of viral PAMPs by TLR 7, 8 and 9, the adaptor molecule MyD88 is recruited, initiating a biochemical cascade that results in the activation of NFκB and expression of IFN (reviewed in Akira et al. 2006).

TLR 3 is expressed on the surface of epithelial cells and in the endosomes of conventional DCs (Matsumoto et al. 2003). TLR3 recognizes and is activated by double stranded RNA and the synthetic homologue polyinosine-deoxycytidylic acid (poly I:C) (Alexopoulou et al. 2001). Following PAMP recognition, TLR3 signals through the adaptor molecule TRIF, resulting in the activation of IRF-3 and NFκB (Yamamoto et al. 2003).

Viral glycoproteins can also be detected by TLR2 and TLR4. However this leads to the release of pro-inflammatory cytokines and not IFN (in Akira et al. 2006).

PAMPs can also be recognized in a TLR-independent manner. IFNβ production has been reported in TLR3-/- mice following infection with Newcastle disease virus (NDV), vesiclular stomatitis virus (VSV), Sendai virus and treatment with poly I:C (Lopez et al. 2004;Yoneyama et al. 2004). In addition, the expression of IFN in pDCs in response to respiratory syncytial virus (RSV) infection has been shown to be independent of the MyD88 pathway but requires the presence of replicating virus in the cytoplasm (Hornuing et al. 2004). Taken together, this was suggestive of a system that detected actively replicating virus in the cytosol (Kawai and Akira 2006). This alternative pathway of virus recognition and IFN secretion was found to be mediated by the cytoplasmic protein retinoic acid-inducible gene 1 (RIG-1) (Yoneyama et al. 2004). This is an RNA helicase that, in addition to its helicase domains, has 2 caspase recruiting

domain (CARD)-like domains (Yoneyama et al. 2004). The helicase domain interacts directly with the viral dsRNA and the CARD-like domains are responsible for the signaling that leads to the activation of the transcription factors NFκB and IRF3 (Kawai and Akira 2006; Yoneyama et al. 2004). In RIG-1 knockout mice, there is virtually no production of type I IFNs or inflammatory cytokines in response to infection with NDV, VSV or Sendai virus (Kato et al. 2005).

A number of TLRs have been described in chickens. Two forms of chicken TLR2 have been described and shown to recognize lipoproteins and LPS (Boyd et al. 2001 Fukui et al. 2001). Chicken TLR 3 has been identified by DNA sequencing and database searching (Yilmaz et al. 2005) and upregulates IFNβ production in response to poly (I:C) (Karpala et al. 2007: Schwarz et al. 2007). Polymorphisms in the chicken TLR4 gene have been associated with susceptibility to infection with *Salmonella* (Leveque et al. 2003). Chicken TLR5 has been described and it displays conserved sequence and structural similarity to its mammalian counterpart (Yilmaz et al. 2005); in addition, it has been shown to function in the upregulation of IFNβ in response to aflagellar *Salmonella enterica* serovars (Iqbal et al. 2005). Chicken TLR 7 has been described and there is some experimental data to suggest that, like its mammalian counterpart, it is located in the endosome (Philbin et al. 2005). In addition, a TLR unique to chickens –TLR15 – has recently been characterized and is shown to be upregulated in response to bacterial infection (Higgs et al. 2006).

Interferon Expression

The IFN regulatory factor (IRF) family of transcription factors consists of 9 known members and these are important in regulating the IFN response (reviewed in Samuel 2001). IFN expression is transcriptionally regulated by activation of latent transcription factors. These include IRF3, IRF7 and NFκB (Takaoka and Taniguchi 2003). Of these, IRF3 and IRF7 have a major role in regard to IFN production following viral infection but NFκB also functions to interact with these and upregulate IFN production (Takaoka and Taniguchi 2003).

The chicken IFN gene was the first non-mammalian IFN gene to be cloned (Sekellick et al. 1994) and subsequently IFNα, IFNβ and IFNγ have all been described in chickens (Digby and Lowenthal 1995; Sick et al. 1996, 1998; Weining et al. 1996). The type I chicken IFN genes, IFN α and β, have low sequence homology (<30%) with the mammalian counterpart but structurally there is some similarity in the promoter region. Chicken IFNα genes do not have an NFκB response element, whereas chicken IFNβ genes do (Sick et al. 1998). Additionally, the mature protein displays a number of conserved motifs (Sick et al. 1996). By contrast, chicken IFNγ has high sequence homology to mammalian IFNγ and has been shown to upregulate MHC class II genes (Weining et al. 1996).

The induction of IFN Type I genes is best understood using the model of the IFNβ promoter. In response to viral challenge, a number of latent transcription factors are activated and a multi-protein unit, the enhancesome, forms at the IFNβ promoter (Maniatis et al. 1998). This consists of, among others, the transcription factors ATF-2/C-Jun, NFκB and IRF3 (Falvo et al. 2000; Wathelet et al. 1998). Inactive NFκB is

sequestered in the cytoplasm by association with a member of the inhibitory κB (IκB) family of proteins (Silverman and Maniatis 2001). Following viral stimulation of the cell, the IκB protein is phosphorylated by IκB kinase (IKK); it is then directed along the ubiquitination pathway which ultimately leads to degradation in the proteasome and so active NFκB is released and translocates to the nucleus (Silverman and Maniatis 2001). Viral recognition also causes the phosphorylation of IRF3 and IRF7 by the IKK like kinases TBK-I and IKKε (Fitzgerald et al. 2003; Sharma et al. 2003). IRF then translocates to the nucleus. The IRF3 pathway results in the production of IFNβ. This then causes the transcription of IRF7 by autocrine feedback; phosphorylation of IRF7 then stimulates the secretion of IFNα. IRF3 is ubiquitously expressed across all cell types; however IRF7 is constitutively expressed in pDCs (in Honda et al. 2005).

When IFNα and β are produced following virus infection they are capable of binding cell surface IFN receptors in an autocrine and paracrine manner and activating the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (reviewed in Samuels 2001). The STAT proteins are latent transcription factors that are phosphorylated by the JAK enzymes following cytokine stimulation. There are 7 known members of the STAT family and 4 members of the JAK family; of these, the ones that are implicated in mediating Type I IFN responses are JAK1, JAK2, STAT1 and STAT2, as well as the non-STAT transcription factor IRF9 (reviewed in Samuels, 2001).

Following phosphorylation three transcription factors, STAT 1, STAT 2 and IRF9, translocate to the nucleus. The complex they form, the IFN stimulated gene factor 3 (ISGF3), binds the IFN stimulated response element (ISRE) a conserved sequence in

the promoter region of IFN-inducible genes, and activates transcription of these genes (reviewed in Samuels 2001).

Mediators of the Type I IFN-induced antiviral state

Among the IFN-induced proteins implicated in antiviral actions in virus infected cells are protein kinase R (PKR), 2',5' oligoadenylate synthetase (OAS) (which couples with RNase L) and protein GTPase Mx (Mx) (reviewed in Samuel 2001).

PKR is an RNA-dependent protein kinase found mainly in the cytoplasm that, when activated, leads to the phosphorylation of eukaryotic initiation factor 2 (eIF2) and so inhibits translation in the cell (Clemens and Elia 1997). Activation of PKR is mediated by dsRNA, resulting in autophosphorylation (Galabru and Hovanessian 1987). Once activated, PKR then catalyses the phosphorylation of a number of other proteins, including eIF2 (Pathak et al. 1988; Samuel 1979). The phosphorylated eIF2 forms a complex with eIF-02b, impairing the guanine nucleotide exchange reaction and so inhibiting translation in the cell (Clemens and Elia 1997). Chicken *PKR* has been cloned and transfection experiments have demonstrated antiviral activity against VSV (Ko et al. 2004a,b).

Type I IFN also induces the coupled 2',5' oligoadenylate synthetase – RNase L system. The enzyme OAS, which is activated by binding dsRNA, synthesizes adenylate oligomers that then activate the latent enzyme RNase L resulting in the degradation of RNA, both cellular and viral (Rebouillat and Hovanessian 1999). In humans, a number of isomers of OAS and three *OAS* genes, each of which contains an ISRE at the 5' end, have been described – *OAS1, OAS2* and *OAS3* (Rebouillat and Hovansessian 1999).

Differential splicing of *OAS1* m RNA produces the small (40- and 46-kDa) enzymes and these isoforms only differ at the C terminal region (Benech et al. 1985). Differential splicing of *OAS2* mRNA produces the middle-size 69- and 71k-Da enzymes; these proteins contains two repeat domains that are homologous to OAS1 (Marie and Hovanessian 1992). *OAS3* encodes a larger, 100-kDa protein that has three repeat domains, again these are homologous to OAS1 (Rebouillat et al. 1999). These different-sized proteins differ with regard to cellular localization and the concentration of dsRNA required for their activation (reviewed in Samuel, 2001). A number of genes encoding mouse OAS isozymes have also been reported (Ghosh et al. 1991; Rutherford et al. 1991). More recently, OASL has demonstrated antiviral activity against West Nile virus in humans and mice (reviewed in Samuel 2002). Most importantly, OASL1 plays a critical role in mice for resistance to West Nile virus (Mashimo et al. 2002). Chickens have two *OAS* alleles, one of which has a large deletion and encodes an unstable protein (Tatsumi et al. 2000; Yamamoto et al. 1998). Transcription of *OAS* in chickens in response to IFN has also been documented (West and Ball 1982).

Mx proteins are probably the best characterized of the known IFN inducible genes with antiviral activity. They are found in a variety of organisms from yeast to vertebrates. *Mx* was first described in mice, when it was shown that resistance to influenza virus was due to the Mx1 protein (Thimme et al. 1995; Staeheli et al. 1988). Rodents are known to have both nuclear and cytoplasmic forms, where location correlates with function in that nuclear Mx1 proteins are protective against viruses that replicate in the nucleus such as influenza-like viruses and orthomyxoviruses (Krug et al.

1985; Staeheli et al. 1983) while the cytoplasmic Mx 2 protein is protective against viruses that replicate in the cytoplasm (Meier et al. 1990).

In humans MxA and MxB are found in the cytoplasm, but only MxA has been shown to have antiviral activity; however this activity is targeted against a broad spectrum of RNA viruses including paramyxoviruses (measles) (Zhao et al. 1996), bunyaviruses (Frese et al. 1996), orthomyxoviruses (Marshall et al. 2000: Pavlovic et al. 1992) and rhabdoviruses (VSV) (Pavlovic et al. 1990). MxA has also been shown to be protective against Hepatitis B, a DNA virus (Gordien et al. 2001). More recently, antiviral activity has also been shown in transfected cells against the double stranded RNA viruses infectious bursal disease virus (IBDV), which affects poultry, and a human reovirus (Mundt 2007).

The exact mechanism of action of Mx proteins has not been fully elucidated; however, Mx appears to be a multi functional protein. It appears to target the nucleocapsid in both Thogoto virus and La Crosse virus infection; however with VSV infection the transcription of viral RNA was inhibited (Haller and Koch 2002). A model of action has been proposed by Haller and Koch.

In chickens a single *Mx* gene has been described and first reported as having no antiviral activity in transfection experiments (Bernasconi et al. 1995). This was based on cDNA obtained from one line of the White Leghorn breed. However, further analysis of DNA sequences from a number of different breeds revealed extensive polymorphism in the *Mx* gene with 25 nucleotide substitutions over the length of the gene – altogether 19 independent combinations were described (Ko et al. 2002). Further studies indicated that the Mx protein from some of these breeds demonstrated antiviral activity against VSV

and influenza virus in transfected cells, whereas others did not. This antiviral activity was found to correlated to a single nucleotide polymorphism (SNP) that encoded a specific amino acid substitution at position 631, with the presence of serine indicating no antiviral activity while the presence of asparagine resulted in a protein with antiviral activity (Ko et al. 2004b).

Infectious Bursal Disease Virus

Infectious bursal disease virus (IBDV), an immunosuppressive pathogen of chickens, was first described over forty years ago and continues to be a pathogen of economic importance to the commercial chicken industry. The disease caused by IBDV was first described in 1962 as "Gumboro Disease" named after the location of the first recorded outbreak in Gumboro, Delaware (reviewed in Lukert and Saif 2003). This is a disease of considerable economic importance for two reasons – mortality due to the virus can result in losses of up to 20% and the resulting immunosuppression that occurs due to destruction in the bursa can cause exacerbation of other diseases and insufficient response to vaccines (Allen et al. 1972: Jackwood and Saif 1987). Currently the disease is controlled by extensive vaccination programmes (reviewed in Lukert and Saif 2003). IBDV belongs to the family *Birnaviridae;* viruses in this family are non-enveloped capsids and have a genome consisting of two segments of double stranded RNA (Dobos et al. 1979; Kibenge et al. 1988). Other viruses in this family are infectious pancreatic necrosis virus (IPNV), a pathogen of fish and crustaceans, and Drosophila X virus (DXV), which infects insects (Dobos et al. 1979). The two segments of the IBDV genome, A and B, encode five proteins designated VP1, VP2, VP3, VP4 and VP5 (Lukert

and Saif 2003). Segment A is the larger of the two containing approximately 3400 base pairs (bp). It encodes a large, 110-kDa polyprotein in a single open reading frame (ORF) that is auto-processed into VP2, VP3 and VP4 (Azad et al. 1987; Hudson et al. 1986; Kibenge et al. 1997). VP2 and VP3 are the structural proteins with VP2 forming the outer capsid of the virion while VP3 forms the inner capsid (Bottcher et al. 1997; Lomabardo et al; 1999). VP4 is a viral protease that contributes to autoprocessing of the polypeptide (Azad et al. 1987). Segment A also encodes the smaller 17-kDa protein VP5 on a second ORF (Mundt et al. 1997). VP5 is detectable only in IBDV-infected cells and while it is not necessary for replication it is speculated that it may contribute to the release and dissemination of the virus (Lombardo et al. 2000: Mundt et al. 1997). Segment B is the smaller genome segment, having approximately 2800 bp and it encodes the 97-kDa protein VP1, the viral RNA polymerase (Müller and Nitschke, 1987). In addition, VP1 contributes to the viral structure as it forms complexes with VP3 (Lombardo et al. 1999).

There are two distinct serotypes of IBDV, serotype 1 and serotype 2 (reviewed in Lukert and Saif, 2003). Serotype 2 is mainly isolated from turkeys and is avirulent in chickens while serotype 1 strains are pathogenic in chickens. Serotype 1 strains are further classisfied as apathogenic, mild, intermediate, classic variants and very virulent according to theur virulence (reviewed in van den Berg et al. 2000). The structural protein VP2 is the major antigen that induces an immune response. Sequencing of the *VP2* gene of different strains of IBDV has revealed that the antigenic determinants for serotypic specificity are encoded in a region of the gene that has a high rate of nucleotide changes, termed the *VP2* hypervariable region (Bayliss et al. 1990; Öppling et al. 1991;

Schnitzler et al. 1993; Vakharia et al. 1994). Since the mid-1980's antigenic variation among serotype 1 isolates has appeared in the US (Jackwood and Saif 1987; Snyder et al. 1992). This has proved problematic in that antigenic variant strains have emerged in vaccinated flocks, which can lead to immunosuppression (Müller et al. 2003). Very virulent IBDV (vvIBDV) has emerged in some parts of the world. The acute form of IBD caused by vvIBDV first emerged in Europe in the late 1980s and has since spread to Africa, Asia and more recently South America; however, vvIBDV has not yet been reported in the US (reviewed in Lukert and Saif, 2003).

It appears that chickens are infected by IBDV via the oral route or inhalation, and the virus is transported to other tissues by macrophages in the bloodstream (reviewed in Sharma et al. 2000; van den Berg et al 2000). IBDV infects and replicates mainly in dividing immunoglobulin-M bearing B cells in the Bursa of Fabricius, thereby causing their destruction and eventually the destruction of the bursa itself (Becht 1980; Kaufer and Weiss 1980; Rodenberg et al. 1994). Monocytes are also infected and this contributes to the dissemination of the virus (Burkhardt and Müller, 1987). Following infection, virus is detected in most bursal follicles after 13 hours, and by 16 hours post-infection a second viremia occurs leading to virus replication in other organs (Müller et al. 1979). Chickens are most susceptible to mortality at 3-6 weeks of age, when the bursa reaches maximum development, whereas younger chicks are more susceptible to prolonged immunodeficiency (reviewed in Lukert and Saif 2003; van den Berg et al. 2000). The virus is also detected in the spleen and lymphoid tissues but in much smaller amounts (van den Berg et al. 2000). CD8+ and CD4+ T cells infiltrate the following the appearance of viral antigen. T cells are first detected at 1 day post-infection, with the

highest number detected after 7 days and persisting for up to 12 weeks (Kim et al. 2000). Infection with IBDV can result in either acute disease or death (Lukert and Saif 2003; van den Berg et al 2000). The acute stage of the disease caused by IBDV lasts no longer than one week and is characterized by anorexia, depression, ruffled feathers, diarrhea and death (reviewed in Lukert and Saif 2003; Sharma et al. 1989). In surviving birds, the bursal follicles become repopulated and some degree of immunocompetence can return. However recovered chicks generally have some degree of immunosuppression due to the loss of developing B cells in the bursa and a reduced antibody titer (Kim et al. 1999; Withers et al. 2005). Disease caused by vvIBDV is similar but the acute stage is exacerbated (van den Berg et al. 2000).

Infectious Bursal Disease and Mx

Currently some evidence exists for differential genetic resistance to very virulent IBDV, among chicken lines. In 1993 the Bumstead group looked at resistance to IBDV in different chicken lines and results of experiments using F2 and backcross chickens indicated that resistance may be due to a single gene. A second group (Hassan et al. 2004) examined IBD and Newcastle disease and concluded that an innate factor is responsible for resistance as they did not see any correlation between resistance and antibody response or lymphocyte response. Neither of these studies identified any candidate genes.

Innate immune responses that might control IBDV have received little attention. Recently, the effect of recombinant chicken IFNα on IBDV infection has been investigated in a limited study and it was shown to both suppress plaque formation in

infected cells in a dose dependent manner and ameliorate infection in commercial and specific pathogen free (SPF) birds (Mo et al. 2001). Chicken IFNα is known to have Mx-inducing activity (Schultz et al. 1995) and recently the transcription of *Mx* has been shown to be upregulated in chicken embryonic fibroblasts (CEFs) infected with IBDV (Wong et al. 2007). Nonetheless, the antiviral effect of chicken IFNα against IBDV has not been well characterized.

IFN-induced expression of *Mx* has also been reported in fish (Staeheli et al. 1989). An important pathogen of fish, infectious pancreatic necrosis virus (IPNV), like IBDV, is a dsRNA virus belonging to the family *Birnaviridae* (Dobos, 1995). Recently, salmon Mx1 was shown to possess antiviral activity against IPNV in transfected cells by reducing cytopathic effect, reducing virus yield, inhibiting viral protein synthesis and reducing viral transcription (Larsen et al. 2004). As mentioned earlier, human MxA expressed in transfected Vero cells strongly inhibits the replication of IBDV (Mundt 2007). Chicken *Mx*631 alleles have not been evaluated in transfection experiments for antiviral effect against IBDV. However, because chicken Mx proteins resemble human MxA in terms of cytoplasmic location and activity against both influenza and VSV, it seems likely that Mx Asn631 will be similar to MxA in its ability to limit IBDV replication.

Statement of Research Objectives

Because BF1 molecules have been shown to inhibit chicken NK cells, it is important to learn more about their regulation and possible allelic differences in expression to better understand a potential role in disease resistance. Previous reports

that have determined *BF2* as the dominantly expressed MHC class I locus in chickens have been based on conventional RT-PCR. This is not truly quantitative and results may be affected by differences in efficiency. In order to further confirm that *BF2* is the dominantly expressed MHC class I locus in chickens, locus specific quantitative RT-PCR will be used to evaluate relative expression of the *BF1* and *BF2* alleles. In an attempt to give a molecular explanation for the difference in expression among the *BF1* alleles, nucleotide sequences in the 5' region containing promoter elements will be obtained and analysed. Sequence of promoter regions may provide some evolutionary insights and indicate past selective pressure on expression. Additional sequence analysis in the coding region of poorly expressed *BF1* alleles will be performed when promoter region sequences cannot account for unusually low expression.

It is very difficult to determine the functional effects of polymorphism in a single gene of the MHC, because there are many genes that are highly polymorphic. To evaluate the functional consequences of polymorphism in a gene of the innate immune system, we chose the chicken *Mx* gene. The Mx protein is a candidate for determining resistance to the double stranded RNA virus IBDV for two reasons: (1) the human MxA and salmon Mx1 have both shown antiviral activity against dsRNA viruses, including IBDV in the case of MxA; (2) the chicken *Mx* Asn631 allele demonstrates antiviral activity against VSV and influenza virus in transfection experiments. The role of chicken IFNα and Mx Asn631 will be assessed in response to infection with IBDV in chicken embryo fibroblast cultures.

III. THE CHICKEN BF1 (CLASSICAL MHC CLASS I) GENE SHOWS EVIDENCE OF SELECTION IN PROMOTER AND SIGNAL PEPTIDE REGIONS AS WELL AS THE MATURE PROTEIN CODING EXONS

Abstract

The chicken B-MHC contains two classical class I genes, *BF1* and *BF2*, with the exception of two related haplotypes lacking *BF1* due to insertion/rearrangement. BF2 molecules are recognized as antigen-presenting molecules by CTL. Evidence for BF1-restricted antigen presentation to CTL is lacking, but BF1 is an inhibitory ligand for chicken NK-like cells. In light of this functional specialization of BF1 and BF2 molecules, we were interested in evaluating their relative expression at the mRNA level. We evaluated five MHC haplotypes for class I gene expression by RT-quantitative PCR. *BF1* transcript levels were 2- to 5-fold lower than *BF2*, with the exception of one haplotype in which *BF1* expression was very low. To investigate molecular explanations for differences in BF expression, we determined nucleotide sequences of enhancer A and proximal promoter elements of nine different BF1 alleles, as well as their signal peptide sequences. Results showed that all *BF1* alleles (unlike mammalian non-classical class I genes) exhibit conservation of most of the identified promoter elements, but divergence from the Enhancer A sequence identified in the more highly expressed *BF2* locus. Nonetheless, extensive *BF1* allelic polymorphism was found in the promoter region and in the signal peptide, with two strongly separated allelic lineages identified for

both. Patterns of promoter lineages, signal peptide lineages, and exon 2 mature protein coding sequences in individual *BF1* alleles suggest that recombination among these elements has contributed to diversification of *BF1* alleles. Finally, identification of a novel inactivating mutation in one *BF1* allele suggests past selective pressure to eliminate BF1 function.

Introduction

Expression of mammalian class I genes is regulated through *trans*-acting transcription factors binding to *cis*-linked DNA elements located 5' of the transcription initiation site (reviewed in van den Elsen et al. 2004). *HLA-A, -B*, and *–C* classical class I genes in humans are characterized by locus-specific promoter regions (Cereb and Yang 1994), and exhibit cell-type specific differences in constitutive and cytokine-induced expression (Johnson 2003).

In the chicken, classical class I and class II molecules are encoded in the B-MHC region (Miller et al. 2004). The B-MHC is highly polymorphic, and different haplotypes determine strong differences in resistance or susceptibility to infectious diseases such as Marek's disease. The gene(s) responsible for differential disease resistance is not known, in part because the chicken MHC is so compact that no recombinants within the B-MHC have been detected in experimental matings. Obvious candidates include the class I MHC genes.

Most chicken MHC haplotypes contain two classical class I loci, *BF1* and *BF2* (Shaw et al. 2007; Livant et al. 2004; Miller et al. 2004). It has been reported that the *BF2* gene is expressed at the mRNA level in much greater amounts than the *BF1* gene (Shaw et al. 2007), supporting the "minimal essential MHC" hypothesis that a single dominant class I molecule (BF2) determines MHC haplotype-associated differences in disease resistance (Wallny et al. 2006). Among the five haplotypes with transcribed *BF1* genes that were described in detail by Shaw et al. (2007), two promoter lineages were observed, distinguished by divergence or deletion of Enhancer A sequence and differing at numerous single nucleotide polymorphisms (SNPs) downstream from the Enhancer A

region. Nonetheless, most of the promoter elements identified in the *BF2* gene were remarkably conserved in both BF1 promoter lineages. Two haplotypes lack an identifiable *BF1* gene (Shaw et al. 2007).

BF2 molecules have been demonstrated to present antigen to CTL in an MHC-restricted manner (Fulton et al. 1995). The *BF2* locus is more polymorphic than the *BF1*. Nonetheless, multiple *BF1* alleles exist, and they exhibit evidence of positive selection for diversity in the peptide binding region; in the midst of this allelic diversity, BF1 allotypes are characterized by the presence of conserved, locus specific residues concentrated in the alpha-1 helix, resembling a pattern of diversity observed in HLA-C molecules in humans (Livant et al. 2004).

Functionally, HLA-C molecules play an important role in regulating natural killer (NK) cell activity by interacting with inhibitory receptors on NK cells, through interactions involving the conserved locus specific residues in the alpha 1 helix region (reviewed in Vilches and Parham 2002). HLA-C molecules are expressed, at both the transcript level and on the cell surface, in lower amounts than either HLA-A or –B (McCutcheon et al. 1995). It is thought that the reduced expression of HLA-C is related to its specialized function as a ligand for NK regulatory receptors. In part, lower expression of the *HLA-C* locus may be attributed to substitutions in the Enhancer A sequences (Gobin et al. 1998).

Recent studies (Hunt et al., submitted) demonstrated that BF1 molecules expressed on target cells strongly inhibit killing by chicken NK-like cells. This new function raises questions of whether a more extensive evaluation of B haplotypes, and more quantitative assay of *BF1* and *BF2* gene expression, will support the hypothesis of *BF2* as the

dominantly expressed MHC class I gene. A related question is whether allelic differences exist in the expression of the *BF1* gene, indicating that selective pressures during evolution may have diversified this inhibitory NK ligand for expression as well as for structural polymorphism.

The assignment of the *BF2* locus as the major expressed classical class I locus in chickens was based on the number of cDNA clones obtained from *BF1* and *BF2* by conventional RT-PCR and cloning (Shaw et al. 2007). However, conventional PCR is not truly quantitative, and differences in efficiency of amplification between related sequences (for example, *BF1* and *BF2*) may skew the output. Indeed, we found that among cDNA clones from some broiler MHC haplotypes, putative *BF1* sequences (class I sequences that matched or clustered with known *BF1* sequences), represented the predominant sequence, or were equally abundant as sequences that matched or clustered with *BF2* sequences (Li et al. 1999; unpublished observations). Finally, cDNA clone numbers provide no information regarding whether allelic differences in *BF2* or *BF1* expression exist, as is the case for mammalian MHC class II genes (Vincent et al. 1996; Fernandez et al. 2003)

Relatively few *BF1* alleles have been evaluated for expression or for promoter elements. Our group previously characterized 16 MHC haplotypes identified in broiler chickens for *BF1* and *BF2* exon 2 and exon 3 sequences, and identified several new haplotypes, including new *BF1* alleles. Using these novel haplotypes, as well as two haplotypes previously identified in experimental Leghorn lines, we addressed several questions. Will a highly quantitative method to measure *BF2* and *BF1* mRNA expression corroborate the large difference in expression between the two genes that was estimated

from cDNA clone numbers, and is lower expression of *BF1* gene relative to *BF2* gene a general rule? Are there allelic differences in the expression of the *BF2* or *BF1* gene? Are there more than two different promoter lineages of the *BF1* gene, and how much diversity exists within a given lineage? Does linkage disequilibrium exist between *BF1* promoter alleles and coding exons (interpreted as evidence of "drift" by Kaufman)? Is there evidence for deterioration in promoter elements in any of the BF1 alleles? Does the signal peptide vary markedly among *BF1* alleles? The ultimate question is whether allelic diversity in *BF1* promoter or expression can shed light on the newly discovered function of BF1 molecules in regulating NK cell function.

Therefore we (1) evaluated relative expression of *BF2* and *BF1* alleles in several MHC haplotypes by a locus-specific reverse transcription (RT) quantitative PCR method, normalizing with B-actin as a housekeeping gene; (2) evaluated the degree and patterns of polymorphism in the enhancer/promoter regions and signal peptide coding exons of nine *BF1* alleles, of which eight have not been described in the literature; (3) sequenced the entire gene of one *BF1* allele to determine the molecular explanation for its very low expression. Results are discussed in light of implications for the evolutionary history of the *BF1* gene.

Materials and methods

Chickens

Five birds of each of five MHC haplotypes (broiler haplotypes C5, A12, C7, A9 and A1) were used to compare expression of the *BF1* and *BF2* genes by RT-quantitative PCR methods. The C5 haplotype has *BF1, BF2, BLB1* and *BLB2* alleles identical to the B21 standard haplotype; however, C5 differs from B21 in the B-G region. The *BF* and *BL*

alleles of the remaining haplotypes do not match any standard haplotypes, and were first described in commercial meat-type chickens (Li et al. 1999; Livant et al. 2001; Livant et al. 2004).

For determination of promoter regions, the five MHC haplotypes listed above were used, as well as four others in broiler lines: A8 (matches standard B6 for both BF and BL sequences), C1, C2v and J3 (Livant et al., 2004). Genomic DNA isolated from two different birds of each haplotype was used for long distance PCR amplification and sequence analysis (below).

Preparation of constructs as standards for quantitative PCR

For quantification of expression of *BF1* and *BF2* alleles in lymphocytes, exons 2-4 of each haplotype, encoding the α1 and α2 domains of the *BF1* and *BF2* genes were PCR amplified from cDNA, cloned into pBluescript2 by standard methods and used as a standard control in order to generate a standard curve. The plasmids were linearized by digestion with HindIII and purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA, USA) according to manufacturer's instructions and resuspended in 10 mM Tris HCl, pH 8.5, 0.1 mM EDTA ($T_{10}E_{0.1}$).

The PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, Oregon) was used to determine the DNA concentration; standard diluent consisting of $T_{10}E_{0.1}$ containing 20 ug/ml of sheared salmon sperm DNA was used to dilute the DNA to contain 10^5, 10^4, 10^3 or 10^2 molecules of DNA per 5ul. Diluent containing no plasmid DNA was used as a negative control.

Preparation of cDNA for Quantitative PCR

Each of the broiler B haplotypes A12, C7, A1and A9 was compared with the C5 haplotype (B21 haplotype in the *BF/BL* region). Collection of blood, extraction of RNA and preparation of cDNA was conducted simultaneously with a C5 bird and one of the second haplotype to which comparison would be made. All samples were processed individually from blood collection through quantitative PCR. A two-step reverse transcription (RT) and PCR reaction protocol was used.

Blood was collected from the wing vein of each bird in heparin. Lymphocytes were isolated from the peripheral blood using slow speed centrifugation (50 x g for 15 min) and resuspended in phosphate buffered saline to a concentration of 5×10^6 cells/ml. Total RNA was extracted from lymphocytes using the High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) as per manufacturer's directions, and cDNA was synthesized using Thermoscript RTPCR system (Invitrogen Life Technologies, Carlsbad, CA, USA). Reverse transcription was performed according to manufacturer's instructions in a final volume of 20 µl using 1 µl of random hexamers per reaction. The RT conditions were 25^{o}C for 10 min, 55 ^{o}C for 50 min, 85 ^{o}C for 5 min followed by a 20 min incubation at 37^{o}C after the addition of 2 U of RNase H per reaction. Each cDNA preparation was stored at –80 ^{o}C until use.

Table 1. Primers for Amplifying *BF1, BF2* and *B-actin* from cDNA for Quantitative PCR

Primers Sequences	Direction	Sequence	Haplotype
BF1			
Bfup83-a	Forward	GTGGTACGTGGACGTGGGGTATGT	B21,A12,BC7
BFI100NUP81	Forward	CCGTGGTTCGTGGACGTGGGGTAC	A1, A9
BFIdn259d	Reverse	ACCGTGTGAGACCCGCCGGT	B21,BA12,BC7
Bfdn259p	Reverse	CAGCGTGTGAGACCCGCCAGT	BA1,BA9
BF2			
Bfup81e	Forward	CGTGGTTCGTGGACGTGGGGTAC	B21
BfIup120e	Forward	CGTGCACTACAACAGCACCGCG	BA12
Bfup81b	Forward	CCGTGGTTCGTGACTGTGGGGTAC	BC7
bfIV100Nup81	Forward	CCGTGGTTCGTGATTGTGGGGTAC	BA1,BA9
bfIVdn259d	Reverse	CACTGTGTGAGACCCGCCGGT	B21,BA12,BC7
BFIdn259d	Reverse	ACCGTGTGAGACCCGCCGGT	BA1
BFdn259m	Reverse	CCGTGTGAGACCCGCCGGT	BA9
B-actin			
acup861	Forward	CCTTCAACTCCATCATGAAGTGTGATGTG	All
acdn975b	Reverse	CAGGTGGGGCAATGATCTTGATTTTC	All

Table 2. Probes for Detecting *BF1*, *BF2* and *B-actin* Amplicons in Quantitative PC

Probe Sequences	Label	Sequence	Haplotype
BF1			
BFI121rp3	3'-FAM	GACGCAGATCGGACAGCGC	B21, BA12,BC7
BFIV2nrp2	3'-FAM	GACAGACGCAGATCGGACAGCTC	BA1,BA9
BFI121an3	5'-Bodipy	ATGAGCGGAGTGTGAAAGTGAGC	B21,BA12,BC7,B A1, BA9
BF2			
FIV21rp2	3'-FAM	AGAGACGCAGATCGTACAGGGC	B21
FIV6Nrp3	3'-FAM	ACAGACGCAGATCGTACAGGGCA	BA12
FIV10Nrp3	3'-FAM	AGACGCAGCTGGGCCAGGG	BA9
BFIV7Mrp3	3'-FAM	GACGCAGATCGCACAGGGCA	BC7
BFIV0Nrp1	3'-FAM	GAGAGACGCAGATCGAACAGCACA	BA1
FIV21an2	5'-Bodipy	GTGAGCAGATTAACCGCGAGAAC	B21
FIV6Nan2	5'-Bodipy	TGAGCAGATTGACCGCGAGAACCT	BA12,BC7,BA1
FIV10Nan1	5'-Bodipy	CATGAGCAGGTTAACAGCGAGGACC	BA9
B-actin			
acreprt2	3'-FAM	CACAATGTACCCTGGCATTGCTG	All
acanchr1	5'-Bodipy	CAGGATGCAGAAGGAGATCACAGC	All

Amplification and detection of specific products

Primers and probes for FRET- qPCR were designed using DNAstar software and obtained from Qiagen. One primer was designed to cross intron-exon boundaries to prevent amplification of genomic DNA (specifically, the downstream primers for BF1 and BF2 spanned exons 2 and 3). *BF2* locus specificity for the primers and probes of each haplotype was confirmed by testing primer-probe mixes designed for one locus with standard prepared from the second locus in that haplotype. In all cases, there was no cross-reaction between *BF1* and *BF2* detection systems (data not shown).

Aliquots of cDNA prepared from individual birds were evaluated for expression of *BF1* and *BF2* transcripts. Additionally, *β-actin* transcripts were quantified in each cDNA preparation, so that BF1 and BF2 expression could be normalized. One primer for *β-actin* was designed to cross the exon 4 and exon 5 boundary. The other primer was located in exon 4. Probes hybridized within exon 4 sequences (Tables 1 and 2).

Table 3. LightCycler Protocol for Quantitative PCR Analysis of *BF1* and *BF2* Alleles

		Temperature (^{o}C)	Time (sec)
Denaturation		95	120
Stringency 1	Denaturation	95	0
5 cycles	Annealing	68	12
	Extension	72	8
Stringency 1	Denaturation	95	0
7 cycles	Annealing	66	12
	Extension	72	8
Stringency 1	Denaturation	95	0
3 cycles	Annealing	64	12
	Extension	72	8
Hybridization	Denaturation	95	0
20 cycles	Annealing	53	6
	Extension	72	10

Real time PCR reactions were carried out in glass capillaries in the LightCycler (Roche) in a final volume of 20µl. Each reaction contained 5µl of cDNA sample or standard DNA and 15µl of PCR mastermix as previously described (Huang et al. 2001). Primers were used at a concentration of 1µM, FRET probes at a concentration of 0.5µM. Each 20µl reaction contained 1U of hot start Platinum *Taq* DNA polymerase and 0.2U Uracil-N-Glycosylase (Invitrogen Life Technologies). Standard controls ranged from 0-10^5 template molecules of cDNA as described above. Samples were run in duplicate with sample that was not reverse transcribed used as a negative control. The thermal cycling procedure (Table 3) consisted of 15 high-stringency step-down cycles and 20 relaxed stringency fluorescence acquisition cycles (Wang et al 2004). The stringency cycles increased specificity of the reaction.

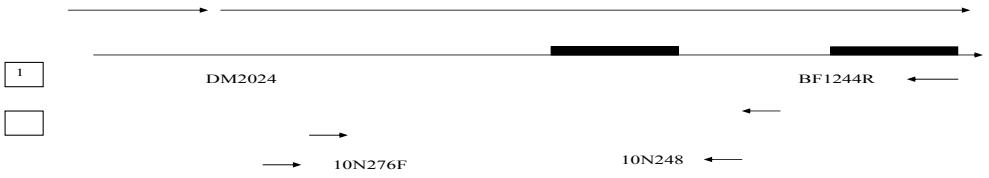


Figure 2. Location of (1) amplification and (2) sequencing primers for determining nucleotide sequence of the Enhancer A, promoter and signal peptide regions of *BF1* alleles

Sequence Analysis of Promoter, Enhancer A and signal peptide regions

DNA used was isolated from chicken erythrocytes using standard methods. Primers for long-distance amplification of the *BF1* locus Enhancer A/promoter region from genomic DNA are listed in Table 4. The upstream *BF1* primer (DM2024) was designed against the sequence of the DMB2 gene of the B12 MHC (Gen Bank AL023516) using Vector NTI software (Version 9). The downstream primer (BFI244R) was located in *BF1* exon 2 and was designed to be specific for the *BF1* locus (Livant et al 2004). Locations of primers used for initial amplification and for subsequent sequence determination are shown in Fig. 2.

Table 4. Primers for Amplifying and Sequencing Promoter/Enhancer A Region of *BF1* Alleles

Name	Direction	Sequence	Haplotypes
PCR Primers			
DM2024	Forward	ATTGCCATCAATGAGGGCTG	All
BFI244R	Reverse	GCCGGTCTGGTTGTATCGTTC	All
Sequencing			
DM2024	Forward	ATTGCCATCAATGAGGGCTG	All
BFI244R	Reverse	GCCGGTCTGGTTGTATCGTTC	All
5M208F	Forward	TGACGGTGATTGACATTGTGC	**B21**, BC7, BA12
5M751R	Reverse	TGAGCCGCGGGTGGGGGTCT	**B21**, BC7, BA12, BC1, BC2v, **B6**
5M88F	Forward	ATGAGTCATCCTAATTAAGGAG	**B21**,B C7, BA12
10N276F	Forward	CAGTTCTGCTCAGTGCCTCCA	BA1,BA9, BJ3
10N248R	Reverse	TGAGGATCCCACGGGCACAG	BA1,BA9, BJ3
B1N297F	Forward	AAGTGATCTGGATAGGTCG	BC1, BC2v, **B6**

Sequence analysis of *BF1* gene downstream of exon 2.

PCR amplification of *BF1* from the promoter region to Exon 2, containing the promoter region was performed using primers DM2024 (5') and BF1224R (3'), the latter being

specific for *BF1*. The upstream primer BFI244 (5') (reverse of the downstream primer used to amplify promoter region) and downstream primer TAP11035 (3') were used to amplify the *BF1* gene in C5 and C7 haplotypes from Exon 2 to the promoter region of *TAP 1*, so allowing for sequencing of BF1 to the 3'UTR. All primers were synthesized by Operon Biotechnologies, Huntsville, AL, USA.

The Expand Long Template PCR system (Roche) was used and the PCR reaction mix contained 5 µl of Buffer number 3, 10 mM of each dNTP, 30 µM of each primer, 200 ng of genomic DNA and 2.5U (for haplotypes C7, A1, A9, C1, A8, C2v, and J3, or 3.75U (for haplotypes B21, A12) of Expand Long Template Enzyme mix in a final volume of 50 µl. The PCR was performed on an Applied Biosystems Thermal Cycler; conditions were an initial 2 minute hold at 94^{o}C, 10 cycles for 94^{o}C for 10 s, 60^{o}C for 30 s and 68^{o}C for 4 min, 25 cycles of 94^{o}C for 15 s, 60^{o}C for 30 s and 68^{o}C for 4 min plus 20 s each additional cycle and a final elongation step of 68^{o}C for 7 min.

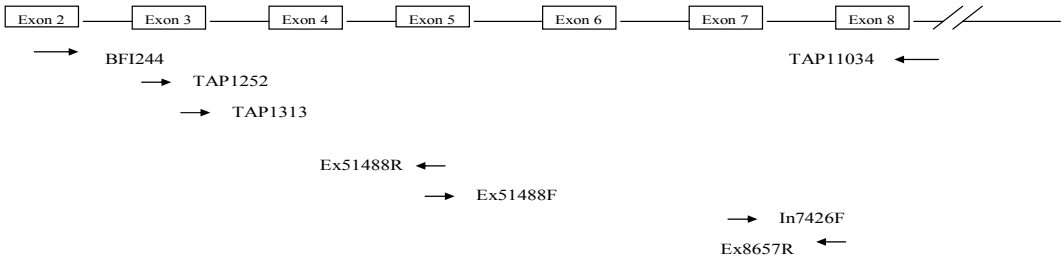


Figure 3. Location of amplification and sequencing primers used for determining nucleotide sequence of *BF1* alleles from exon 2 to the 3' untranslated region.

Table 5. Primers for Amplifying and Sequencing Exon 2 to 3'UTR of *BF1* Alleles

Name	Direction	Sequence	Haplotype
PCR Primers			
BFI244	Forward	GAACGATACAACCAGACCGGC	**B21**, BC7
TAP11034	Reverse	CCCTCGTGTCCCGCCTGC	BC7
3UTR922R	Reverse	AAGCATAACAGTCAGCATAGGAAGC	**B21**
Sequencing			
TAP1252	Forward	ATGTTCGGCTGTGACATCC	BC7
TAP1313	Forward	ATGGGAAAGACTTCATTGC	BC7
TAP51488R	Reverse	ATGGCCACAATGGCGACG	BC7
TAP51488F	Forward	CGTCGCCATTGTGGCCAT	**B21**, BC7
TAPIn7426F	Forward	ATGTGCTCTCTGTGGTACTGC	**B21**, BC7
TAPEx8657R	Reverse	ATGTGAGAGGTCAAGAGGATGG	**B21**, BC7

PCR products of the appropriate size were isolated using 1% Nusieve gel electrophoresis and stained with ethidium bromide. The fragments were excised and purified using the QIAquick gel extraction kit (Qiagen). Sequences were obtained from two MHC homozygous animals of each haplotype. The product was then sequenced in both directions by automated sequence analysis (Genomic Sequencing Laboratory, Auburn University, Auburn, AL, USA). For the PCR product from 5'UTR to Exon 2, initial sequencing was performed in both directions, using the DM2024 upstream primer and the BF1224R downstream primer. Further sequences were then obtained from primers designed from this initial sequence (Table 5, Fig. 2). The PCR product that contained the region from Exon 2 to the 5'UTR of the TAP gene was initially sequenced only in the forward direction, using the upstream PCR primer BF1224; subsequent primers were then obtained from this sequence to allow for sequencing in both directions (Table 5, Fig. 3). Promoter elements were identified by reference to Kroemer et al. (1990), van den Elsen

(2004) and the program SIGNALSCAN (http://bimas.dcrt.nih.gov/molbio/signal/) by Prestridge (1991). The dN to dS rate of the signal peptide (omitting the 15 bp insertion) was estimated by Mega 3.1 program. Exon 1 sequences were evaluated for predicted function as signal peptides by PSORTII (http://psort.ims.u-tokyo.ac.jp/).

Table 6. Efficiencies of *BF1* and *BF2* Reactions. Numbers in parenthesis are range of efficiency values obtained from three separate PCR runs for each haplotype.

Haplotype	**BF1 Reaction**	**BF2 Reaction**
C5 (BF/BL = B21)	1.92 (1.76 – 2.05)	1.97 (1.90 – 2.01)
A9	1.91 (1.84 – 1.98)	1.97 (1.90 – 2.03)
A1	1.95 (1.87 – 2.10)	2.01 (1.95 – 2.09)
C7	1.83 (1.78 – 1.91)	1.87 (1.84 – 1.90)
A12	1.90 (1.78 – 1.99)	1.94 (1.90 – 1.99)

Results

The *BF2* Gene is Consistently Expressed in Higher Amounts than the BF1 Gene

To quantify transcripts from the *BF1* and *BF2* alleles of five different haplotypes, RT- real time quantitative PCR was performed. For each bird, the amounts of BF1 and BF2 were compared to β-actin transcripts from the same cells in order to normalize the data and comparisons were made between each of the A12, C7, A9, and A10 haplotypes with C5 (BF/BL = standard B21) samples collected and processed at the same time. A B21 haplotype was used for comparison with each of the others, because it matches a standard haplotype that was characterized by another laboratory for relative BF2 and BF1 transcript amounts by determining the number of cDNA clones (Shaw et al.

2007). To ensure that each PCR reaction was carried out with equivalent efficiency, PCR reactions using only standard controls were performed and efficiency compared (Table 6).

In all five haplotypes, *BF1* was expressed at a lower level than the *BF2* locus. As shown in Figure 4A, the ratio of *BF1* to *β-actin*, *BF2* to *β-actin* and *BF1* to *BF2* mRNA was similar between BC5 and A12 haplotypes. In both haplotypes, the *BF2* message was expressed at approximately 3 times the level of *BF1*. In a comparison of A9

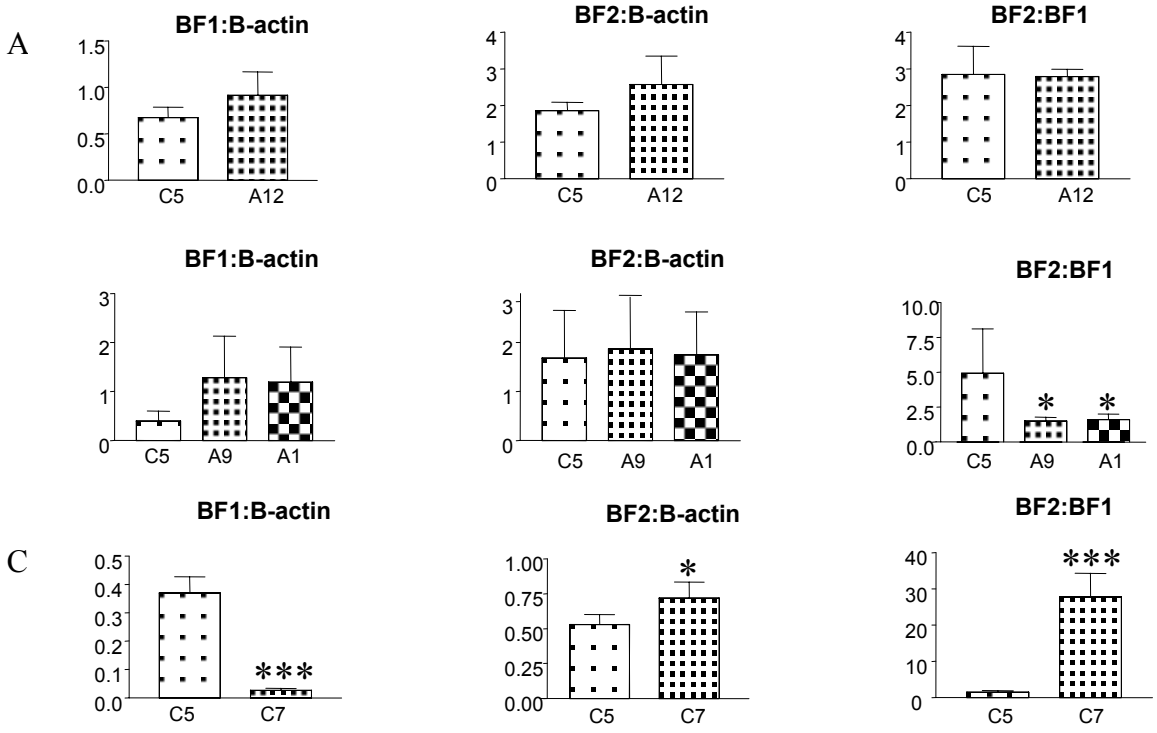
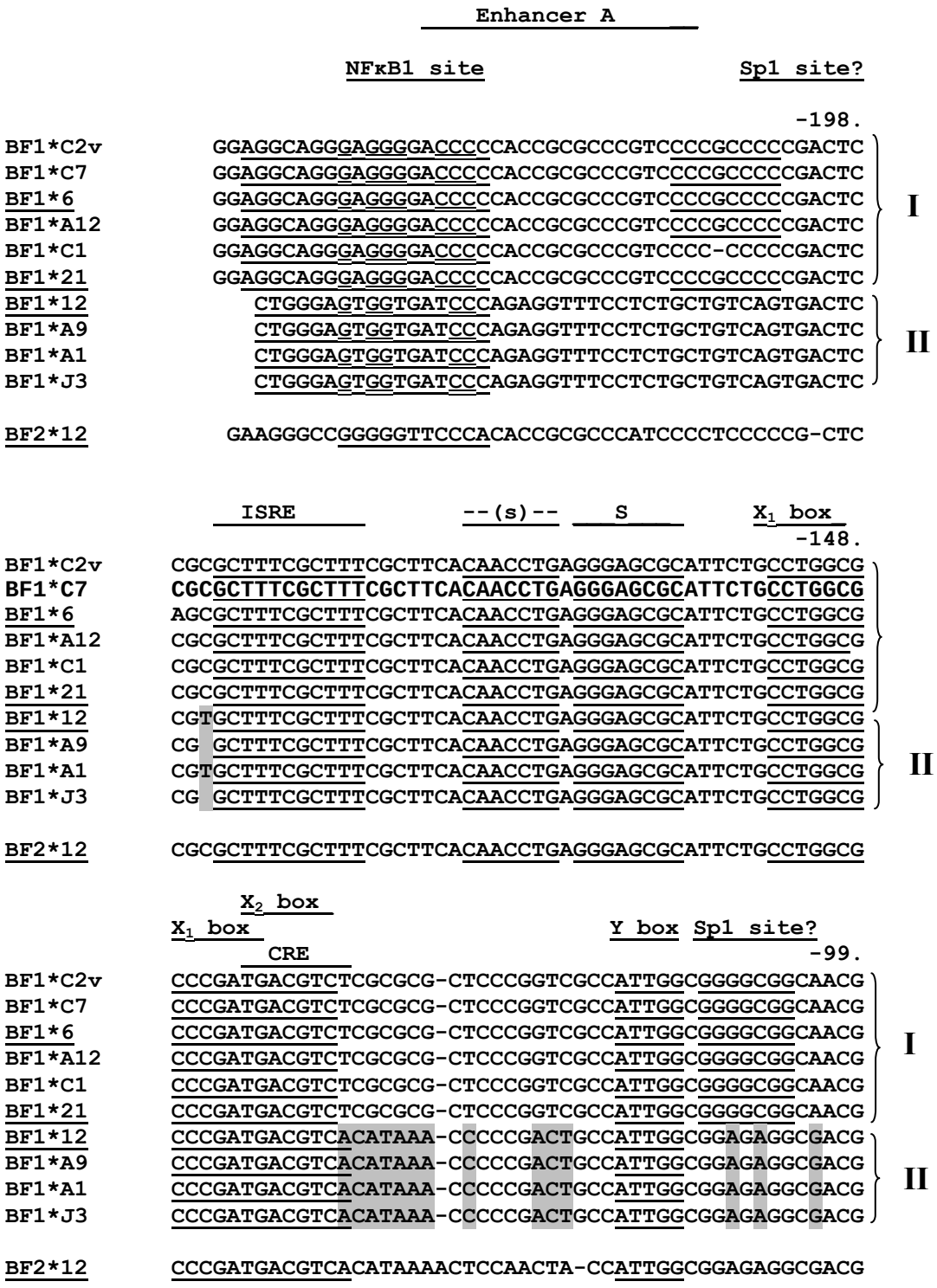


Figure 4. RT Quantitative PCR of RNA from peripheral blood lymphocytes of various MHC haplotypes. Mean values were derived from five independent experiments for each haplotype with duplicates of each sample in each PCR run. Error bars indicate standard deviation for each haplotype; * = $p < 0.05$; *** = $p < 0.001$


Enhancer A
NFκB1 site
Sp1 site?
-198.
BF1*C2v GGAGGCAGGGAGGGGACCCCCACCGCGCCCGTCCCCGCCCCCGACTC
BF1*C7 GGAGGCAGGGAGGGGACCCCCACCGCGCCCGTCCCCGCCCCCGACTC
BF1*6 GGAGGCAGGGAGGGGACCCCCACCGCGCCCGTCCCCGCCCCCGACTC
BF1*A12 GGAGGCAGGGAGGGGACCCCCACCGCGCCCGTCCCCGCCCCCGACTC
BF1*C1 GGAGGCAGGGAGGGGACCCCCACCGCGCCCGTCCCC-CCCCCGACTC
BF1*21 GGAGGCAGGGAGGGGACCCCCACCGCGCCCGTCCCCGCCCCCGACTC
I
BF1*12 CTGGGAGTGGTGATCCCAGAGGTTTCCTCTGCTGTCAGTGACTC
BF1*A9 CTGGGAGTGGTGATCCCAGAGGTTTCCTCTGCTGTCAGTGACTC
BF1*A1 CTGGGAGTGGTGATCCCAGAGGTTTCCTCTGCTGTCAGTGACTC
BF1*J3 CTGGGAGTGGTGATCCCAGAGGTTTCCTCTGCTGTCAGTGACTC
II
BF2*12 GAAGGGCCGGGGGTTCCCACACCGCGCCCATCCCCTCCCCCG-CTC
ISRE
--(s)--
S
X1 box
-148.
BF1*C2v CGCGCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
BF1*C7 CGCGCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
BF1*6 AGCGCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
BF1*A12 CGCGCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
BF1*C1 CGCGCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
BF1*21 CGCGCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
I
BF1*12 CGTGCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
BF1*A9 CG GCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
BF1*A1 CGTGCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
BF1*J3 CG GCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
II
BF2*12 CGCGCTTTCGCTTTCGCTTCACAACCTGAGGGAGCGCATTCTGCCTGGCG
X2 box
X1 box
CRE
Y box
Sp1 site?
-99.
BF1*C2v CCCGATGACGTCTCGCGCG-CTCCCGGTCGCCATTGGCGGGGCGGCAACG
BF1*C7 CCCGATGACGTCTCGCGCG-CTCCCGGTCGCCATTGGCGGGGCGGCAACG
BF1*6 CCCGATGACGTCTCGCGCG-CTCCCGGTCGCCATTGGCGGGGCGGCAACG
BF1*A12 CCCGATGACGTCTCGCGCG-CTCCCGGTCGCCATTGGCGGGGCGGCAACG
BF1*C1 CCCGATGACGTCTCGCGCG-CTCCCGGTCGCCATTGGCGGGGCGGCAACG
BF1*21 CCCGATGACGTCTCGCGCG-CTCCCGGTCGCCATTGGCGGGGCGGCAACG
I
BF1*12 CCCGATGACGTCACATAAA-CCCCCGACTGCCATTGGCGGAGAGGCGACG
BF1*A9 CCCGATGACGTCACATAAA-CCCCCGACTGCCATTGGCGGAGAGGCGACG
BF1*A1 CCCGATGACGTCACATAAA-CCCCCGACTGCCATTGGCGGAGAGGCGACG
BF1*J3 CCCGATGACGTCACATAAA-CCCCCGACTGCCATTGGCGGAGAGGCGACG
II
BF2*12 CCCGATGACGTCACATAAAACTCCAACTA-CCATTGGCGGAGAGGCGACG

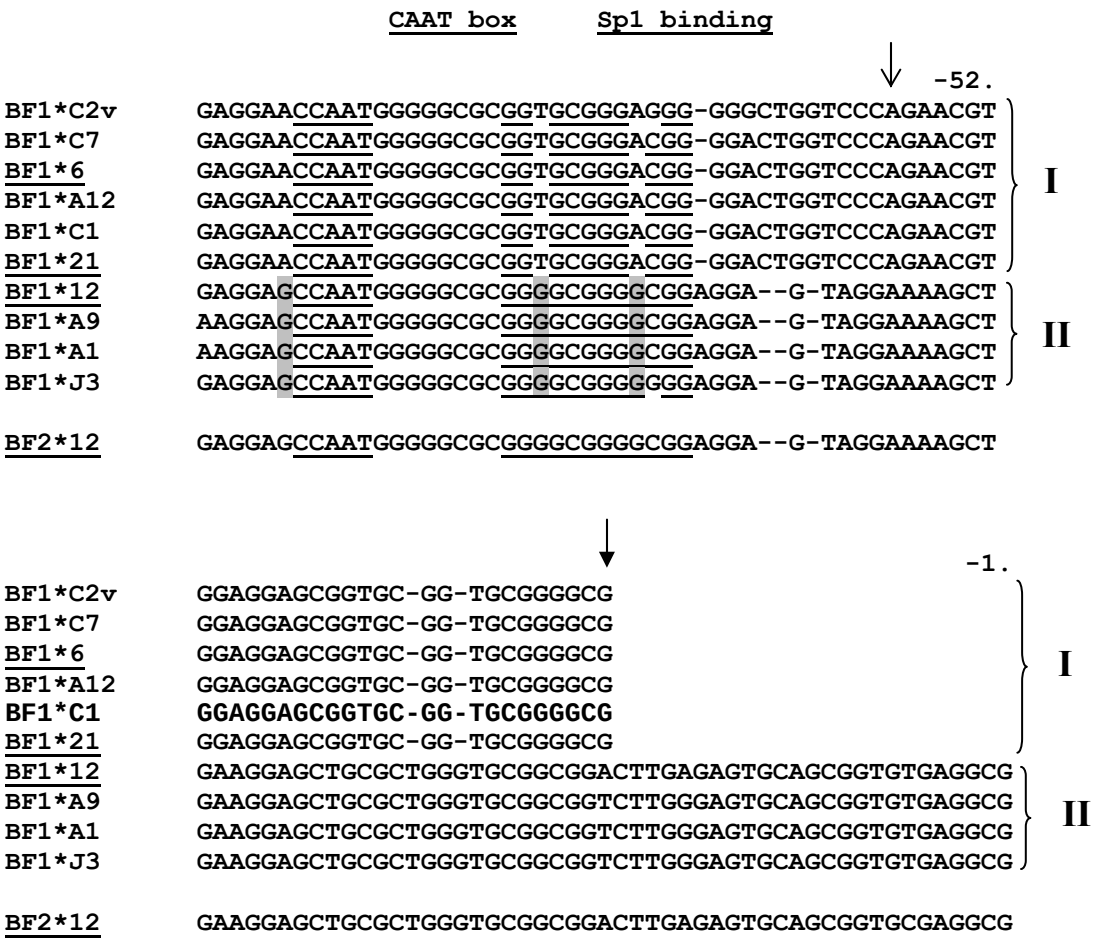


Figure 5. Alignment of promoter region sequences. Numbering is based on the sequence of BF1*12. Dashes indicate gaps introduced to maximize sequence identity. Position +1 is A of start codon. Arrows indicate transcription start points identified for BF2*12 (Kroemer et al. 1990). Shading indicates lineage specific polymorphisms delineating Type I or Type II within the region that is homologous among all alleles. Promoter elements based on van den Elsen et al. 2004 and Moon et al. 2005. Note that distance between S box and X1 box is 15 bp, which is the same as human. Distance between X2 box and Y box is 19 bp (human is 18 bp).

and A1 haplotypes with B21 (Fig. 4B), the ratios of *BF1* to *β-actin*, *BF2* to *β-actin* and *BF2* to *BF1* expression between A9 and A1 were similar. The ratio of *BF2* to *BF1* in B21, however, was approximately 2 to 3 times higher than that seen in A9 and A1. In both A9 and A1, *BF2* message was only approximately 1.2 times higher than that of *BF1*. However, the most marked difference in level of expression by comparison with the B21 haplotype occurred in the C7 haplotype. Mean *BF2* expression (relative to *β-actin*) was similar for the two haplotypes, though they differed statistically ($p < 0.05$) (Figure 4C). However, *BF1* expression relative to *β-actin* was dramatically lower in C7 than in B21 haplotype. As a result, the *BF2*:*BF1* ratio in the C7 haplotype was almost 30:1.

Putative *BF1* Genes Are Located Adjacent to *DMB2* Gene

In standard haplotypes characterized by long distance PCR from flanking genes, the *BF1* gene is adjacent to the *DMB2* gene (Shaw et al 2007). Earlier, we tentatively assigned *BF* sequences from novel MHC haplotypes to *BF1* or *BF2* locus based on their phylogenetic clustering with known *BF2* or *BF1* sequences, in which *BF1* sequences separated into a strongly defined clade (Livant et al 2004). To localize putative *BF1* sequences and to examine whether differences in the promoter regions may be responsible for the differential expression of the *BF* alleles, the 5' upstream region (from the flanking *DMβ2* gene to exon 2) of several *BF1* alleles was amplified by long range PCR from genomic DNA from 9 different haplotypes. The upstream primers were located in the 3'UTR of the *DMβ2* gene and the downstream primer was the putative *BF1* locus specific nucleotide sequence encoding the alpha 1 helix. Sequence determination

of the exon 2 region confirmed that it contained the sequence tentatively assigned as a *BF1* gene on the basis of phylogenetic clustering, so formally localizing it to this area.

Only Two, Highly Diversified, BF1 5' Upstream Region Lineages Exist

Direct sequencing through the promoter region was performed on the DNA fragments amplified from *BF1* alleles by long distance PCR from the flanking *DMβ2* gene. The sequences obtained here together with *BF1*12* (Kaufman, Genbank Accession AL023516) were examined for conserved regulatory elements and putative transcription regulatory elements that have been reported for the B12 *BF2* allele (Fig. 5) (Kroemer et al. 1990; Zoeller et al. 1992). Analysis of these sequences showed two differing sequences for Enhancer A, which is bound by NFκB family members, and other polymorphisms in downstream promoter sequences between haplotypes.

All nine *BF1* alleles fall into one of two bi-allelic lineages (here designated as Type I and Type II) with respect to Enhancer A and promoter sequences. The positive regulatory element, Enhancer A, was identified in *BF2**12 on the basis of homology with mammalian class I MHC Enhancer A sequences (Kroemer et al. 1990). In the alignment shown in Fig. 5, Type I and Type II sequences differ from each other by 9 nucleotides in the region containing Enhancer A. In fact, the *BF1*12* allele (and its regulatory region relatives A1, A9, and J3) appear to have undergone a deletion of 272 bp in the region between the *DMB2* gene and the *BF1* gene including the region containing Enhancer A, as noted by Shaw et al. (2007). However, the putative Enhancer A region identified in the *BF2* gene has not been evaluated functionally, nor does it have the palindromic sequence (GGGGATTCCCC) creating dyad symmetry described as important for κB1

site in mammalian MHC class I molecules (Mansky et al. 1994). Thus, the actual effect of differences in the two *BF1* lineages from *BF2* enhancer A region cannot be addressed.

Between the distal site of deletion involving Enhancer A in Type II sequences (-202) and the proximal site of divergence involving loss of transcription sites in Type I sequences (-69), there are 18 lineage specific SNPs (boxed nucleotides in Fig. 5). Complete linkage disequilibrium exists between the two Enhancer A types, and polymorphisms in the proximal promoter region (Fig. 5).

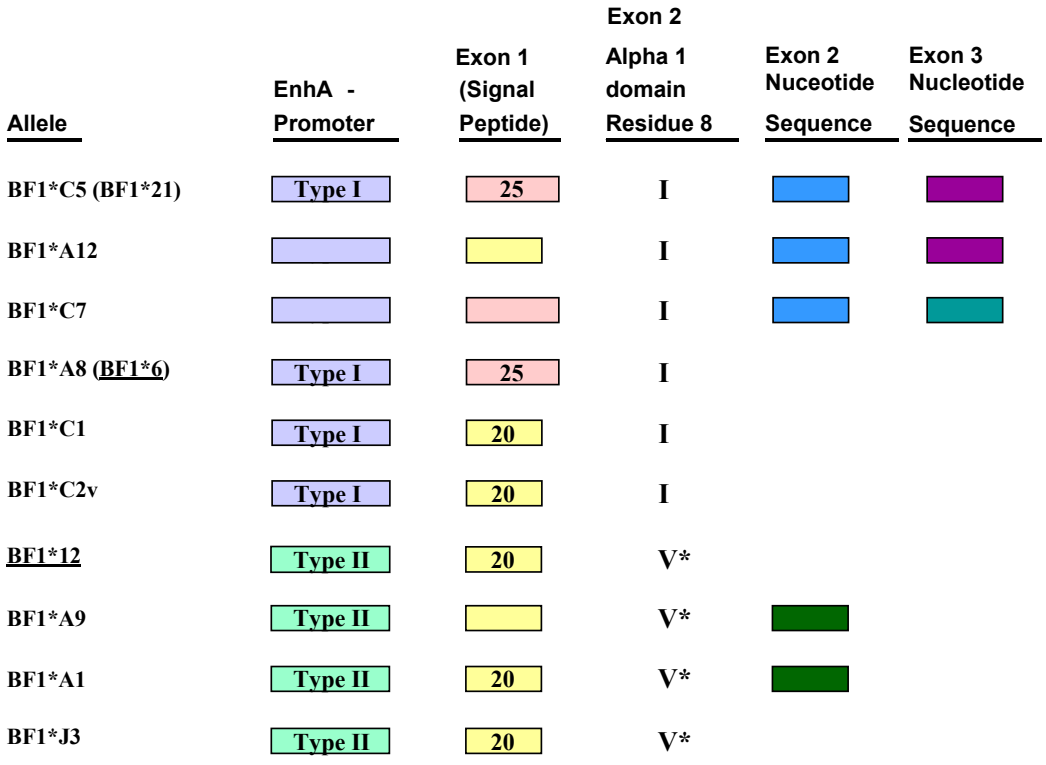


Figure 6. Diagrammatic representation of BF1 "haplotypes" consisting of various promoter lineages, signal peptide alleles, and α1 domain sequences

The only apparent linkage between promoter types and exon 2, encoding the α1 domain (that contributes to the peptide binding site), is with residue 8 of the mature protein (Ile in all Type I, Val in all Type II promoters, p = 0.0048 by Fisher's exact test) (Fig. 6). Except for the residue 8 association, promoter lineages do not necessarily correspond to exon 2 coding sequence relationships. Among the Type II lineages, BA1 and BA9 exon 2 sequences are identical with each other, and closely related to B12. But *BF1*J3* allele exon 2, which also has a Type II promoter, is on a separate and strongly supported branch from the BA1/BA9/B12 alleles, clustering with B21 (Type I lineage promoter) (Fig. 7).

BF1 Alleles Contain Highly Conserved Promoter Elements, with Polymorphism Concentrated Near the CRE

The sequence downstream from the Enhancer A contains a putative interferon stimulated response element (ISRE). Transfection experiments demonstrated that this motif acts as a strong IFN responsive element in the BF2 gene (Zoeller et al. 1992). To date, this evaluation of the ISRE motif is the only functional study of any of the putative regulatory motifs in the chicken *BF* promoter region. The sequence of the putative ISRE in the nine *BF1* alleles sequenced here is identical to that in *BF2* and includes the interferon consensus sequence (G/C)TTTCNNTTC present in many mammalian genes that are induced by interferon (Zöller et al. 1992). This sequence is intact across all haplotypes, showing significant homology with that of mouse and man. In addition, the *BF1* promoter region, like the *BF2*, contains 2 tandem repeats of the sequence motif

CTTTCG which binds nuclear trans-acting factors in a number of mammalian IFN responsive genes (Zöller et al. 1992).

The S box, X boxes and Y box are elements that are typically found in the 5' flanking region of mammalian MHC class II genes, although they are now recognized in class I genes as well. The *BF1* gene contains sequences that are similar to these; across all haplotypes the sequences were identical and corresponded to those previously described in the *BF2*12* gene.

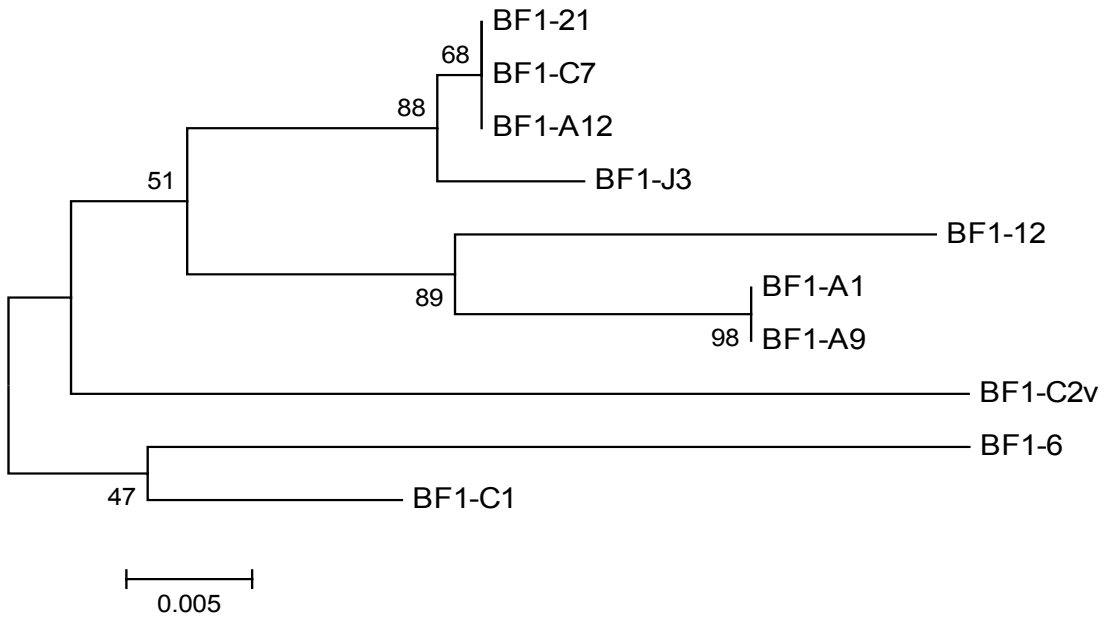


Figure 7. Phylogenetic analysis of *BF1* exon 2 sequences. Neighbour-joining tree analysis was conducted using MEGA version 3.1 (Kumar et al. 2004). The numbers at nodes indicate bootstrap values obtained from 500 replicates. The scale indicates the proportion of nucleotide sites at which two sequences being compared are different.

Two further regulatory DNA sequence elements have been described in MHC class I genes – the TATA box and the CAAT box (reviewed in Singer and Maguire 1990). All of the haplotypes sequenced here had a CAAT box; there is no TATA box sequence in either chicken *BF2* or *BF1* genes, but an Sp1 binding site is located where a TATA box would be expected (Kroemer et al. 1990).

The *BF2* gene in the B12 haplotype has a tandem repeat of the Sp1 Binding Site (GGGCGG) (Kroemer et al. 1990). The sequences of the *BF1*12, BF1*A1*, and *BF1*A9* alleles (all Type II) are identical to this, also having two tandem repeats. The *BF1*J3* allele (also Type II), however, has a polymorphism that disrupts the second site. The *BF1*C2v* allele has three nucleotide differences, whereas the other *BF1* alleles (A8, A12, C1 C5, and C7) differ by two nucleotides in this region; in all these Type I promoters, both tandem repeats are disrupted. On the other hand, two additional potential Sp1 sites, between –211 to –205 and between -104 and –110, were identified by SIGNAL SCAN (Prestridge 1991) in all the Type 1 alleles but not in Type II (Fig. 5).

In the *BF1*A1*, *BF1*A9* and *BF1*J3* alleles (Type II promoter), the cAMP Response Element (CRE) is identical to that of *BF1*12*. In all the other alleles (Type I), there is a one nucleotide difference (T for A at the 3' end of the CRE). Almost half of the SNPs distinguishing Type I and II promoter lineages (7/16) are in either the CRE or the "CRE zone" consisting of 10 nucleotides to either side of the CRE octamer (Mitchison and Roes, 2002).

The Two BF1 Signal Peptide Lineages Exhibit Evidence of Recombination with Exon 2 Coding Alleles

In addition to the polymorphisms that are present in the 5' flanking region of the various haplotypes, sequencing through exon 1 allowed identification of differences among signal peptides (SP). Two distinct signal peptide lengths were identified, with three of the alleles–*BF1*C7*, *BF1*21* (C5 haplotype) and *BF1*B6*– having a 15 bp in-frame insertion in the middle of the signal peptide sequence (Fig. 8). The result is an SP of either 21 or 26 amino acids. The extra 15 bp appear to be the result of a duplication of the sequence encoding "LGLLL" (positions 9-13), followed by a single point mutation to encode "LGLLR" in the inserted sequence. [Interestingly, one of the *BF2* alleles (BF2*1401) has a similar but shorter insertion encoding "GL", which appears to be the result of a duplication of sequence encoding positions 10-11 (GenBank AM282694).]

The signal peptide length polymorphism is restricted to the Type I promoter lineage; however it is not present in all haplotypes with Type I promoters. Within that lineage, the two SP lengths appear to be randomly associated with the polymorphic exon 2 (encoding the alpha 1 domain) (Fig. 6), which together with the alpha 2 domain (encoded by exon 3) forms the peptide binding site. Some *BF1* alleles share identical exon 2 sequences, including BF1*21, BF1*A12 and BF1*C7 (Livant et al. 2004). Among these alleles, two (*BF1*21* and *BF1*C7*) have a SP region encoding 26 amino acids, whereas BF1*A12 SP is predicted to be 21 amino acids in length. *BF1*6* and *BF1*C1* exon 2 sequences are more closely related to each other than to any other alleles (Fig. 7), yet they differ in SP length. Thus, these alleles have a patchwork pattern of linked polymorphisms involving exons 1 and 2. This pattern in coding regions of MHC class I

molecules has traditionally been interpreted to indicate allelic exchange by recombination.

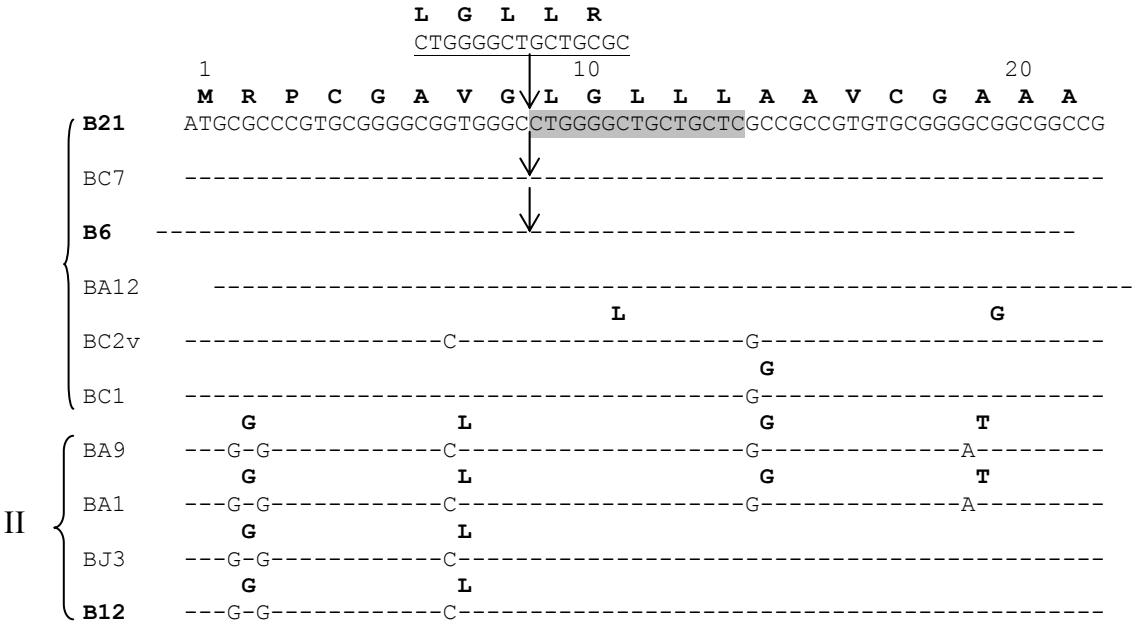


Figure 8. Alignment illustrating predicted signal peptide-coding sequences of *BF1* alleles. Bold indicates standard haplotype.

In addition to the insertion encoding an extra five amino acids, other SNPs exist in the signal peptide coding region. Residues 2 and 7 are linked polymorphisms, occurring in a pattern that typifies either Type I or Type II promoter lineage (amino acids R2, V7 for Type I; G2, L7 for Type II). An exception to this pattern is the *BF1*C2v* allele, which is Type I promoter lineage and has R2 with L7. Other SNPs (A14G, A19T) are seen in BF2 signal peptides, although R2 is thus far observed in only *BF1* (Type I promoter lineage) alleles. The rate of nonsynonymous to synonymous substitution (dN to dS ratio) is 1.76 for BF1 signal peptide coding regions, suggesting positive selection has

exerted an influence on this region. All the *BF1* allelic exon 1 sequences, including the ones with 15 bp insertion, are predicted to function as signal peptides by PSORTII analysis.

BF1*C7 has Two Potential Inactivating Polymorphisms in Intron 7 and Exon 8

Because the promoter sequence of the *BF1*C7* allele did not explain its very low expression, the *BF1* alleles of C7 and C5 (B21) haplotypes were also sequenced from exon 2 through to the 3'UTR and comparisons made to examine whether differential expression may be explained by polymorphism in this region of the gene.

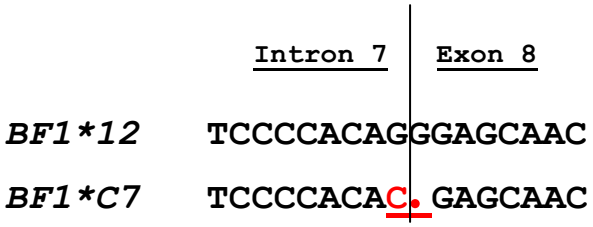


Figure 9. Location of putative inactivating mutations in *BF1*C7* nucleotide sequence

A 5' splice site sequence that differs from the mammalian consensus sequence by one bp has been identified in the *BF2*B12* sequence at the beginning of Intron 7 (Kroemer et al. 1990). This identical sequence, in the same location, is present in both the *BF1*C7* and *BF1*21* (from the C5 broiler haplotype) alleles. The canonical 3' splice site noted by Kroemer et al is also present approximately 155 bp from the 5' splice site in both the *BF1*B12* and *BF1*21* (C5 haplotype); however in *BF1*C7* there is a mutation in the putative 3' splice site (Figure 9). If this is indeed a 3' splice site in *BF1*B21*, then

this mutation could account for the markedly reduced mRNA levels of BF1*C7. Additionally, Exon 8 encoding a cytoplasmic exon, has a one bp deletion in the *BF1*C7* allele when compared with nucleotide sequences of *BF1*21* in the C5 haplotype (our data) and the sequences of *BF1* alleles from standard haplotypes in the GenBank database (Shaw et al. 2007). This complex deletion/substitution mutation is predicted to abolish intron 7 acceptor site for RNA splicing.

Two canonical polyadenylation sites have been described in the *BF1*B12*. A polymorphism in the more 5' poly A site was seen in *BF1*C7* allele. However, the same polymorphism was described in *BF2*14* and *BF2*15* alleles, which are well-expressed (Shaw et al. 2007).

Discussion

This study reports difference in expression of the *BF2* and *BF1* loci, with all but one of the haplotypes evaluated here exhibiting a BF2 to BF1 ratio between 1.2:1 and 5:1. This BF2 to BF1 ratio is is somewhat lower than the approximately 10:1 ratio reported in cDNA clones of most haplotypes reported by Shaw et al. (2007), although the B21 haplotype in their studies had a BF2 to BF1 ratio of 1.5 to 1. The approximately 2-fold difference between B21 and A1/A9 in our experiments may not be real, because the quantitative PCR is not sensitive enough to reliably distinguish differences less than about 2-fold. On the other hand, the remarkably low BF1 expression in the C7 haplotype, with a resulting high BF2 to BF1 ratio, is certainly real. The finding that *BF1*C7* has a

polymorphism that results in loss of a 3' splice site (intron 7) could explain the reduced expression of this allele (Magor et a1.1997).

We also report extensive polymorphism in the 5' upstream region of the various *BF1* alleles, containing regulatory elements for expression, but conservation of most identified promoter elements including ISRE, S boxes, X1 and X2 (or CRE) boxes, Y box, and CAAT box. Almost all of the individual polymorphisms (SNPs), however, can be assigned to one of two lineages. Finally, the fact that all the novel *BF* sequences that were previously assigned to the *BF1* locus (on the basis of locus-specific sequence elements and phylogenetic analysis) could be amplified by long distance PCR from the flanking *DMβ2* gene in effect localizes these sequences to the *BF1* locus. One broiler haplotype evaluated in the present study (A8) has a *BF/BL* region that matches standard B6 haplotype based on identical *BF1* and *BF2* exon 2 and 3 sequences, as well as *BLB1* and *BLB2* exon 2 sequences (Li et al. 1999; Livant et al. 2004; unpublished data). In this haplotype, the BF6 sequence designated "major" (Shaw et al. 2007) localized by long distance PCR to the *BF1* region, in agreement with phylogenetic cluster analysis placing this sequence in the *BF1* group (Livant et al. 2004). These results further reinforce the importance of evaluating expression of BF genes by quantitative PCR methods. However, we did not evaluate BF1 and BF2 expression in this haplotype.

The concentration of SNPs in the CRE and CRE zone is reminiscent of the picture seen in three mouse MHC class II gene (Aα, Aβ, and Eβ) promoters, which interestingly also belong to bi-allelic lineages defined by numerous SNPs in linkage disequilibrium (Mitchison and Roes 2002). As these authors point out, the CRE is responsive to external signals. Functional consequences of the two promoter lineages are not known. In

lymphocytes from peripheral blood lymphocytes (mostly T cells), constitutive expression of BF1 transcripts did not differ markedly between different promoter lineages (comparing B21 with BA9 or BA1). In the case of mouse MHC class II promoter polymorphism, however, some of the expression differences were tissue-specific (Janitz et al. 1997).

Type I or II promoters are in linkage disequilibrium with one nucleotide substitution in exon 2 encoding either valine or isoleucine at residue 8 in the α1 domain of the mature protein (Type I is associated with Ile, Type II with Val). Linkage disequilibrium between regulatory sequences and coding sequences has been suggested to indicate co-evolution between the two, with selection acting on expression (Mitchison and Roes 2002). On the other hand, except for residue 8 in the α1 domain of the mature protein, other exon 2 polymorphisms appear not to be in linkage disequilibrium with promoter lineages, based on phylogenetic analysis. The only proposed contact for MHC I residue 8 is with β2-microglobulin (Kaufman 1992). Residue 8 is invariant (Ile) in all known BF2 alleles, and is an invariant position in the 1746 known human MHC classical class I molecules as well, with the sole exception of the *B5525* allele (IMG/HLA database; http://www.ebi.ac.uk/imgt/hla/). Type I and II promoters are also in linkage disequilibrium with exon 1 SNPs encoding residues 2 and 7 of the signal peptide.

The various combinations of signal peptide length polymorphism (exon 1) with exon 2 alleles described herein could most easily be explained as a result of recombination between the two exons. How could signal peptides of differing length be selected? In general, polymorphism in the SP may affect efficiency of transport into the endoplasmic reticulum, so selective pressure could operate to regulate expression at post-

translational steps. Beyond the common function of transporting nascent protein into the endoplasmic reticulum, in mice and humans, MHC I signal peptide has the unusual function of providing peptides that bind to and stabilize cell surface expression of an MHC I nonclassical protein (HLA-E in humans, Qa-1 in mice) both of which are recognized by an inhibitory receptor on natural killer cells (CD94/NKG2A); reduced MHC I peptide amounts signal infection by viruses that downregulate MHC I expression (reviewed in Lanier, 2005). MHC alleles differ in signal peptide sequences, with some sequences having superior binding affinity (HLA-E) to others (Vales-Gomez et al. 1998). Whether a similar system exists in chickens is not known, although a gene with homology to both *CD94* and *NKG2* was identified in the chicken genome (Chiang et al. 2007).

In humans, Enhancer A of *HLA-A* and *–B* are identical to each other and to mouse Enhancer A. However, the less well expressed *HLA-C* gene differs from the 11-bp sequence "GGGGATTCCCC" NFκB1 site by 2 nucleotides in the majority of *HLA-C* alleles (Cereb et al. 1994). As a result, the dyad symmetry believed to be important for interaction with nuclear binding factors is disrupted; the NFκB1 and NKκB2 sites of *HLA-C* Enhancer A failed to bind any proteins of the NFκB/Rel family. Furthermore, the *HLA-C* enhancer A did not have any detectable NFκB induced transactivating activity in a reporter assay (Gobin et al. 1998). Despite the lack of NFκB transactivating activity associated with the *HLA-C* Enhancer A, the amount of message for HLA-C measured by McCutcheon et al. (1995) was 10% to 50% the level of HLA-B message. This figure is remarkably close to the amount of BF1 relative to BF2 transcripts in the present report. Strong conservation of most promoter elements in *BF1* alleles examined indicates that

BF1 are classical class I molecules, as the nonclassical class I genes demonstrate highly diverged (and sometimes unrecognizable) promoter elements (Howcroft and Singer 2003).

At least two independent events have resulted in inactivation of *BF1*. Extremely low expression of BF1 locus in C7 haplotype is reported here, and is probably explained by a mutation in a splice acceptor site. A similar mutation in an *HLA-A* allele is associated with reduction of transcript amounts to about 10% of the normal allele (Magor et al. 1997). The substitution and deletion mutation we observed in *BF1*C7* may be of the sort described in KIR3DS1 (Martin et al. 2007), which was thought to result from the misalignment of DNA strands during DNA replication. B14 and B15 haplotypes lack *BF1* transcripts due to apparent insertion and rearrangement (Shaw et al 2007). In humans, *HLA-A24* has been inactivated in 3 independent events, which the authors suggest may be the result of selective pressure to eliminate expression of that allele (Magor et al. 1997). Null or poorly expressed alleles of *KIR2DL* (van den Bussche et al. 2006) and *KIR3DL* (Pando et al. 2003), inhibitory receptors on NK cells recognizing HLA-C or HLA-B molecules as ligands, have been described; the argument has been made that selective pressure to reduce or eliminate these inhibitory receptors exists because the presence of the KIR on immune cells inhibits responses directed toward eliminating infections. An ortholog of *HLA-C* has been found in some but not all orangutan MHC haplotypes, and the presence of an apparently co-evolving KIR ortholog suggests that the orangutan *HLA-C* orthologue is functional (Guethlein et al. 2007). Thus, the fact that some chicken MHC haplotypes lack BF1 expression has a precedent,

and would be consistent with a model in which pathogen-driven selective pressures may have acted to adjust the degree of immune regulation.

Previously, we argued that the *BF1* gene appears to be under selection for diversification in coding sequences, because sites in the peptide binding region have a dN to dS rate of substitution greater than 1.0 (Livant et al. 2004). Based on analysis of seven MHC haplotypes (only five of which encode an identifiable *BF1* gene), Shaw et al. (2007) argue that *BF1* genes are not under selection but subject only to drift from more ancestral alleles. With the addition of eight different haplotypes here, it becomes apparent that the *BF1* gene has hallmarks of a gene under selection for variation in transctipt abundance, and for varied promoters, or combinations of signal peptides with coding sequences of the mature protein.

IV. INTERFERON-α INDUCED INHIBITION OF INFECTIOUS BURSAL DISEASE VIRUS IN CHICKEN EMBRYO FIBROBLAST CULTURES DIFFERING IN MX GENOTYPE

Abstract

Interferon-induced antiviral activity in cells forms an important early line of defense against viral pathogens. IFN-induced mediators are well documented in mammals, with one of the best characterized antiviral proteins being Mx. In chickens, many alleles of *Mx* have been described, but functionally only the polymorphism at a site encoding residue 631 in the protein determines differential antiviral activity against vesicular stomatitis virus and influenza virus in transfection experiments. The role of chicken Mx has not been assessed with regard to infectious bursal disease virus (IBDV), an important pathogen of chickens. To examine the role of chicken IFNα and *Mx*631 SNP against IBDV, chicken embryo fibroblast cultures that differed in *Mx* genotype (antivirally positive *Mx* Asn631 or antivirally negative *Mx* Ser631) were treated with IFNα and cell death following IBDV infection and viral yield assessed. IFNα was shown to have strong antiviral activity in this system in terms of reduced cell death and virus yield. Furthermore the reduction in viral yield did not differ significantly among *Mx* genotypes, indicating that *Mx* Asn631 is not a pivotal determinant of resistance to IBDV.

Introduction

Infectious bursal disease virus (IBDV), a member of the birnavirus family, is a small bisegmented dsRNA virus that encodes 5 proteins (Dobos et al. 1979). It was first described in chickens in 1962 as the causative agent of Gumboro disease (Cosgrove 1962). The virus infects and replicates mainly in lymphoid cells in the Bursa of Fabricius, thereby causing their destruction and eventually the destruction of the bursa itself (Becht 1980; Kaufer and Weiss 1980). Chickens aged 3-6 weeks are most susceptible to mortality, whereas younger chicks are more susceptible to prolonged immunodeficiency (Lukert and Saif 2003). This is a disease of considerable economic importance for two reasons – mortality due to the virus can result in losses of up to 20% and the resulting immunosuppression that occurs due to destruction in the bursa can cause exacerbation of other diseases and insufficient response to vaccines (Allen et al. 1972; Jackwood and Saif 1987). Currently the disease is controlled by extensive vaccination programs (Lukert and Saif 2003). However, some evidence exists for differential genetic resistance to mortality from highly virulent IBDV among chicken lines (Bumstead et al. 1993; Hassan et al. 2004). The involvement of a single gene in IBDV resistance was indicated in one of the Compton (Institute for Animal Health, England) Leghorn lines by the results of experiments using F2 and backcross chickens (Bumstead et al. 1993). However, the identity of this gene is not known.

Innate immune response genes are likely candidates for genes that may influence resistance to IBDV. Type I IFNs are a major component of the innate response to viruses but only limited studies have been conducted to evaluate the effect of chicken IFNα in IBDV infection. Mo et al (2001) evaluated a narrow dose range of a recombinant

chicken IFNα preparation of unspecified titer and demonstrated a modest reduction of IBDV plaque formation. They also demonstrated that their recombinant IFNα protein could ameliorate IBD in specific-pathogen free (SPF) chickens.

The antiviral state is established in cells by the effect of interferons. The production of the type I IFNs α and β is induced following viral infection and subsequently a number of IFN-responsive genes are induced (reviewed in Samuel 2001). Among the type I IFN-induced proteins that have antiviral activity in infected cells are protein kinase R (PKR), 2',5' oligoadenylate synthetase (OAS) (which couples with constitutively expressed RNase L) and Mx (reviewed in Samuel 2001). Of these, Mx proteins in humans and mice are probably the best characterized of the known IFN-inducible genes with antiviral activity against a wide range of viruses (reviewed in Haller and Kochs 2002). Mx is a high molecular weight GTPase that blocks viral replication by directly associating with the virus; however, the exact mechanism of action is unknown (in Haller and Kochs 2002). Mx was first described in mice (Horisberger et al. 1983) and the antiviral properties of this protein have been well established. In rodents these properties appear to be related to the cellular location of Mx. The nuclear Mx1 protein blocks influenza virus replication (Krug et al. 1985; Staeheli et al. 1983); however, antiviral activity against vesicular stomatitis virus (VSV), but not influenza, is conferred by the cytoplasmic Mx2 protein (Meier et al. 1990).

The human homologue, MxA, has been shown to have activity against a wide range of RNA viruses including orthomyxoviruses (Marshall et al. 2000; Pavlovic et al. 1992), paramyxoviruses (Zhao et al. 1996), the rhabdovirus VSV (Pavlovic et al. 1990) and bunyaviruses (Frese et al. 1996). In addition, MxA also confers resistance to

hepatitis B, a DNA virus (Gordien et al. 2001) and has recently been shown to be effective against two double-stranded RNA viruses - infectious bursal disease virus (IBDV) and a human reovirus - in transfected cells (Mundt 2007). Chicken IFNα is known to have *Mx*-inducing activity (Schultz et al. 1995) and recently the transcription of *Mx* has been shown to be upregulated in chicken embryonic fibroblasts (CEFs) infected with IBDV (Wong et al. 2007).

When chicken *Mx* cDNA was first cloned and sequenced it was reported that it was devoid of antiviral activity against a number of viruses including influenza virus, Thogoto virus, Sendai virus and VSV (Bernasconi et al. 1995). However, this work was carried out in one line of White Leghorn breed and subsequent sequencing of *Mx* cDNAs from many breeds revealed extensive polymorphism in the *Mx* Gene (Ko et al. 2002). Cell transfection experiments indicated that some of the *Mx* genes conferred resistance to highly pathogenic avian influenza virus and VSV. A single SNP at position 2,032 (G to A) that encoded an amino acid substitution (Ser to Asn) at position 631 was demonstrated to determine differential antiviral activity. Asn at position 631 conferred positive antiviral activity and Serine at position 631 made the Mx protein negative for antiviral activity (Ko et al. 2002; 2004b). In addition, preliminary data from *in vivo* studies carried out by collaborators has indicated that chicks that are homozygous for the *Mx* Asn631 allele survive longer following infection with highly pathogenic avian influenza H5N2 than those that are homozygous for the *Mx* Ser631 allele (Suarez and Ewald unpublished).

Mx proteins have also been described in fish (Staeheli et al. 1989). An important pathogen of fish, infectious pancreatic necrosis virus (IPNV), like IBDV, is also a dsRNA virus belonging to the family *Birnaviridae* (Dobos 1995). Recently, salmon Mx1 was

shown to possess antiviral activity against IPNV in transfected cells by reducing cytopathic effect, reducing virus yield, inhibiting viral protein synthesis and reducing viral transcription (Larsen et al. 2004).

Chicken *Mx*631 alleles have not been evaluated in transfection experiments for antiviral effect against IBDV. More important than the ability of ectopically expressed Mx proteins to limit viral replication is the question of whether Mx proteins that are expressed endogenously after infection or IFNα treatment play a pivotal role in resistance to virus. Because chicken Mx proteins resemble human MxA in terms of cytoplasmic location and activity against both influenza and VSV it seems likely that chicken Mx Asn631 will be similar to human MxA in its ability to limit IBDV replication. There are reasons to hypothesize that cells endogenously expressing Mx Asn631 or Ser631 after IFNα treatment will differ in antiviral effect against IBDV. In normal cells endogenously expressing IFN stimulated genes (ISGs) in response to IFNα, many different antiviral proteins are expressed. Nonetheless in some viral infections a single ISG (eg *Mx* or and *OAS* isotype) is the predominant determinant of resistance to the disease (Arhneiter et al. 1990; Mashimo et al. 2002; Scherbik et al. 2007). Additionally *MxA*, *OAS* or *PKR* polymorphisms in individual members of the human species are associated with differences in outcome to infection with SARS virus (Hamano et al. 2005; He et al. 2006), hepatitis C virus (Knapp et al. 2003) or West Nile virus (Yakub et al. 2006). An *in vitro* model of virus infection is appropriate to evaluate *Mx* influence on viral replication: embryo fibroblasts from genetic variants typically reflect the same difference as *in vivo* susceptibility or resistance to infection, as has previously been demonstrated with mouse *Mx1* and influenza infection (Arnheiter et al. 1990; Engelhardt et al. 2004;

Staeheli et al. 1988) and mouse *OASL1* and West Nile virus infection (Mashimo et al. 2002).

The aim of the present work was to investigate whether the chicken *Mx* allele having antiviral activity against avian influenza virus (AIV) and VSV is a major determinant of IFN-induced antiviral activity against IBDV. This was achieved by establishing chicken embryo fibroblast cultures from embryos differing in *Mx*631 SNP genotype, treating with recombinant chicken IFNα to induce the antiviral state and infecting with IBDV. We found that IBDV replication was adversely affected by IFNα in this system, and that reduction in virus yield did not differ significantly among Mx genotypes.

Materials and Methods

Experimental Design

A specific pathogen free (SPF) broiler flock segregating for high frequency of both *Mx* Asn631 and *Mx* Ser631 was maintained at the Auburn University Poultry Science Farm in an SPF facility. Chicken embryo fibroblast (CEF) cultures were established from individual embryos which were then typed for *Mx*631 SNP and individually infected.

Typing of parents and embryos

To type parents, blood was collected in EDTA from the wing vein of each bird. To type embryos, approximately 5 ml of allantoic fluid were collected from each egg at the time of CEF preparation and erythrocytes obtained from this. DNA was isolated

from erythrocytes using the Gen Elute Blood genomic DNA kit (Sigma Aldrich, St Louis, MO) as per manufacturer's instructions and PCR amplification of a 450-bp region in exon 13 performed using the primers Mx14up1 (GAATAGCAACTCCATACCGTG) and Mx14dn2 (GTCTACCAGGTATTGGTAGGCTTTG). PCR was performed in a final volume of 50 µl, each reaction containing 200-400 ng of DNA, 2.4 U of AmpliTaq Gold, 5µl 10x Buffer I, 100 mM of $MgCl_2$, 4µl of dNTP mixture (2.5 mM each) and 50 µM of each primer (Operon, Huntsville, AL). The PCR was performed on an Applied Biosystems Thermal Cycler; conditions were an initial 11 minute hold at 94^{o}C, 40 cycles of 94^{o}C for 1 minute, 50^{o}C for 30 seconds and 72^{o}C for 1 minute plus a final extension at 72^{o}C for 10 minutes.

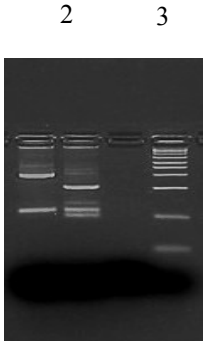
For restriction endonuclease digestion of the amplicons, 25 µl of PCR product was digested with 2.5 U *Hpy*8I following manufacturer's instructions (Fermentas Inc, Glen Burnie, MD). Following digestion, RFLP bands were visualized on 2.5% agarose gel stained with Sybr Green under UV illumination. Cells/birds were then described as *MxAA* (homozygous for the antivirally positive Mx Asn631)*; MxGG* (homozygous for antivirally negative Mx Ser 31); or as *Mx GA*(heterozygous) (See Figure 10).

Sires and dams were also typed for *OAS*A* and *B* alleles by PCR using the method of Yamamoto et al (1998).

Cell cultures and viruses

Individual primary CEF cultures were prepared from 10-12 day old SPF embryos from the flock segregating for *Mx* 631 SNP. Fifteen ml of cell suspension at a density of 1 x 10^5 /ml cells was seeded into 75 cm^2 flasks and grown in MEM containing 80 µg of

tylosin and 100 μg of gentamycin per ml with 4% fetal bovine serum (FBS) (Gibco), and 10% Tryptose phosphate broth (Sigma Aldrich) in 5% CO_2 at 38^{o}C. CEF cells were harvested one day after culturing. To harvest, the cells were rinsed 1x in phosphate buffered saline (PBS) and trypsinised with TrypLE (Gibco) for 5-10 minutes and 2 ml of growth medium was added to stop trypsinization. The cells were then collected by centrifugation for 5 minutes, and cell pellets were either (1) resuspended in medium as described but with 2% FBS, seeded into 96 well plates, 180 μl per well, at a density of 3.4 x10^5 per ml and used for IFNα treatment and infection with IBDV or (2) resuspended in freeze media and stored in liquid nitrogen until needed or (3) seeded into 75cm^2 flasks at 1x10^5 ml for harvesting and 3rd passage.



a

Mx Asn631/Asn631	Mx Ser631/Ser631	Mx Asn631/Ser631
307		307
	206	206
112	112	112
	101	101
31	31	31

b

Figure 10. Mx PCR RFLP bands visualized on 2.5% agarose gel. (a) Lane 1 shows *Mx Asn631/Asn631* genotype; Lane 2 shows *Mx Ser631/Ser631* genotype; Lane 3 is 50-2000 kb marker. (b) The size of RFLP bands in each genotype.

Preparation of recombinant chicken interferon alpha (IFNα)

COS 7 cells were grown to 80% confluency and transfected with the chicken IFNα expression plasmid, CHIFNα-pcDNAI, a kind gift from Peter Staeheli (University of Freiburg, Germany). Cells were transfected using PolyFect Transfection reagent (Qiagen, Valencia, CA) as per manufacturer's instructions. The cultures were incubated at 37^{o}C for 15-20 hours and medium changed; the medium containing IFNα was harvested 72 hrs after transfection and centrifuged to remove cell debris. This crude recombinant IFNα preparation was then stored at 4^{o}C until evaluation to determine titer, then aliquoted and stored at -20^{o}C. The determination of the IFNα titer was performed on CEF cultures infected with vesicular stomatitis virus (VSV), Indiana strain. The antiviral activity of the IFN was measured by a cytopathic effect inhibition assay as described (Schultz et al. 1995) and the titer was determined to be 200,000 U/ml.

Determining Virus Dose and Time-Point for Evaluating IFNα Effects

The IBDV vaccine strain D-78 (Intervet Inc, Millsboro, DE) was propagated in primary CEF cells. In order to harvest the virus, the cells and medium were frozen and thawed 3 times in the culture flask; cells and medium were removed from the flask and centrifuged to remove cell debris. To determine virus titers, 3rd passage *Mx Asn631/Ser631* heterozygous CEFs seeded in 96 well plates grown to confluency were infected with serial 10-fold dilutions of virus stock. MTT viability assay was performed after 3, 4, 5 and 6 days (Figure 11). At 6 days post-infection, the cell cultures showed maximum death from viral replication while control cells were still viable. The $TCID_{50}$ was determined using the method of Reed and Muench to be $10^{7.4}$/ml. A virus stock dilution resulting in 50 $TCID_{50}$/20 µl was chosen for evaluating IFNα and Mx effects.

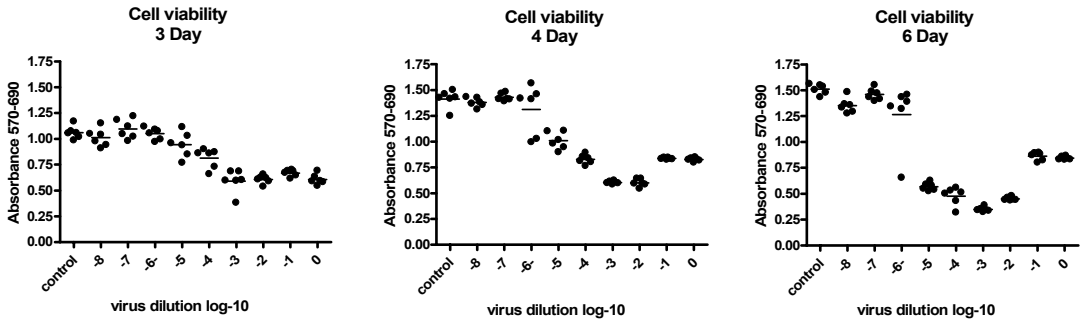


Figure 11. Time course of cell viability for determination of IBDV stock titer. Absorbance 570-690 nm measures cell viability, where loss of absorbance indicates a reduction in cell viability.

IFNα treatment and virus infection

CEFs were grown to 80-100% confluency at 38^{o}C in 5% CO_2, washed once with PBS and treated with eight 4-fold serial dilutions of IFNα, beginning at 10,000 U/ml for 16-20 hours. The medium was removed, cells washed in PBS and infected with 50 $TCID_{50}$ IBDV D78 in 20µl of MEM with gentamycin and tylosin but without FBS and Tryptose phosphate broth. After 1 hour of incubation at 38^{o}C, 160 µl of medium containing 2% FBS and 10% Tryptose phosphate broth was added. In some experiments, IFNα was added back in the same concentration as pretreatment. At 4 days post-infection, a sample of medium was removed from each well and pooled within each treatment replicate and stored at -80^{o}C to evaluate virus yield by $TCID_{50}$.

In order to determine virus yields, ten-fold serial dilutions of the collected supernatants were used to infect confluent 3rd passage CEFs in 96 well plates and cell viability assays (MTT) performed 6 days post infection. The Cell Proliferation Kit I

(MTT) (Roche Applied Science, Mannheim, Germany) was used, following manufacturer's instructions, to assess cell viability. UV absorbance was measured at 570 -690 nm on an ELx800 Universal Microplate Reader (Bio-Tek Instruments, Winooski, VT).

Results

IFNα has strong antiviral activity against IBDV

In preliminary experiments we investigated the effect of IFNα and *Mx*631 SNP on virus replication. Individual 3rd passage *Mx Asn631/Ser631* heterozygous and *MxSer631/Ser631* homozygous cell cultures were treated with 4-fold dilutions of crude recombinant IFNα expressed from a eukaryotic expression vector. IFNα concentrations ranged from 10,000 U/ml to 0.6 U/ml; medium without IFNα was used as a control. Cell death was assessed at 4, 5 and 6 days post infection by MTT staining. Following treatment with IFNα, the amount of cell death was shown to decrease in relation to the amount of IFNα added to the cells; this effect was apparent in both *Mx* genotypic groups with no marked difference observed in cell death between the two. Results indicated that IFNα had strong antiviral activity against IBDV infection in this culture system (Figure 12).

Cell death was compared in cultures that were pretreated with IFNα versus replicate cultures that were pretreated in the same way but also treated with the same IFNα concentration immediately after infection, when it was added back in maintenance media and remained for the duration of the experiment. The addition of IFNα post

infection did not have a marked or reproducible effect on cell death as measured by MTT (Figure 12 and data not shown).

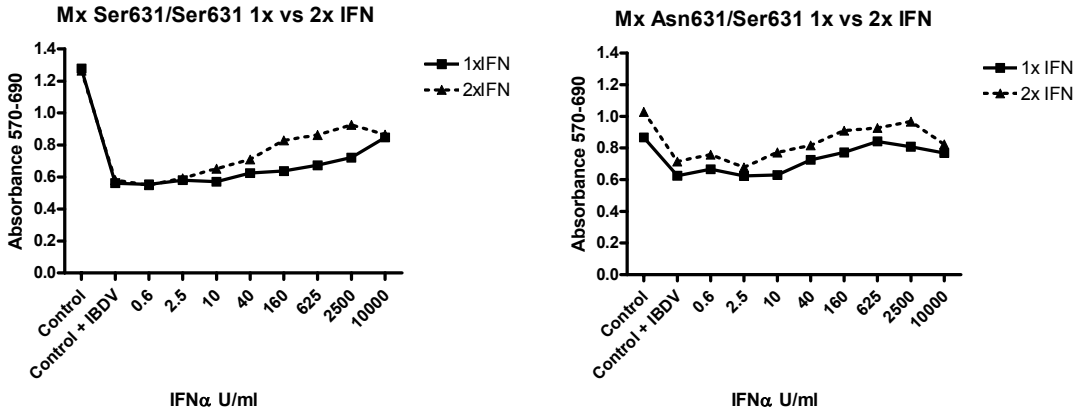


Figure 12. A representative example of comparison of cell death between 1x and 2x IFN treated cells infected with IBDV. 1x IFN indicates pretreatment with IFNα only whereas 2x IFN indicates pretreatment and post-infection treatment with IFNα. Absorbance 570-690 nm measures cell viability, where loss of absorbance indicates a reduction in cell viability 4 days post infection.

The antiviral activity of IFNα was further demonstrated by reduction in viral yield. Individual 2nd passage CEF cultures were treated with IFNα, as described, and infected with 50 $TCID_{50}$ IBDV. Following infection maintenance medium without IFNα, or with the same dilution of IFNα as original treatment, was added. After 4 days, a sample of medium was collected from each well, pooled and used in virus yield determinations. For virus yield determination, 3rd passage CEFs were used. These were grown to confluency and infected with 10-fold serial dilutions of medium from cultures

that had been infected with virus in the absence of IFNα or 625 U/ml or 40 U/ml IFNα. After 6 days culture, an MTT assay was performed to determine tissue culture infectious dose (virus yield) (Figure 13). Overall, the amount of viral replication was reduced in all cells that had been treated with IFNα, but the addition of IFNα in maintenance medium reduced virus yield more. In the cultures treated once with IFNα, virus yield decreased 10 fold at the highest IFN concentration (625 U/ml). In contrast, in cultures where IFNα was added back post infection at this concentration, virus yield decreased by between 100- and 1000-fold. This was seen in both *Mx* genotypes that were available for comparison (Figure 13).

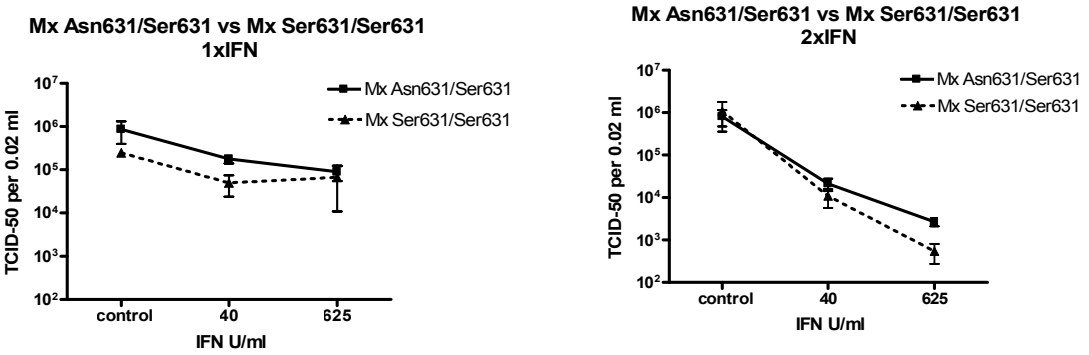


Figure 13: Comparison of virus yields from supernatants collected from IFNα treated *Mx Asn631/Ser631* or *Mx Ser631/Ser631* CEFs four days post-infection with IBDV. 1xIFN indicates pretreatment with IFNα only, whereas 2xIFN indicates pretreatment and post-infection treatment with IFNα. CEF cultures established from 3 individual embryos of each genotype were compared, (n=3 independent experiments).

Influence of *Mx* genotype on IFN-induced antiviral activity

The effect of IFNα on cells derived from embryos with different *Mx*631 genotypes was assessed in greater depth. Briefly, on any given day, CEFs were prepared from 2 to 4 embryos and individual cell cultures established. Allantoic fluid was collected from the embryonated egg at the time of CEF preparation and DNA from cells was used to type the embryos. Because IFNα pre-treatment alone had minimal effect on virus yield (Figure 13), the cultures were treated with IFNα pre- and post-infection, as described, infected with IBDV, and medium collected after 4 days and $TCID_{50}$ determined as described.

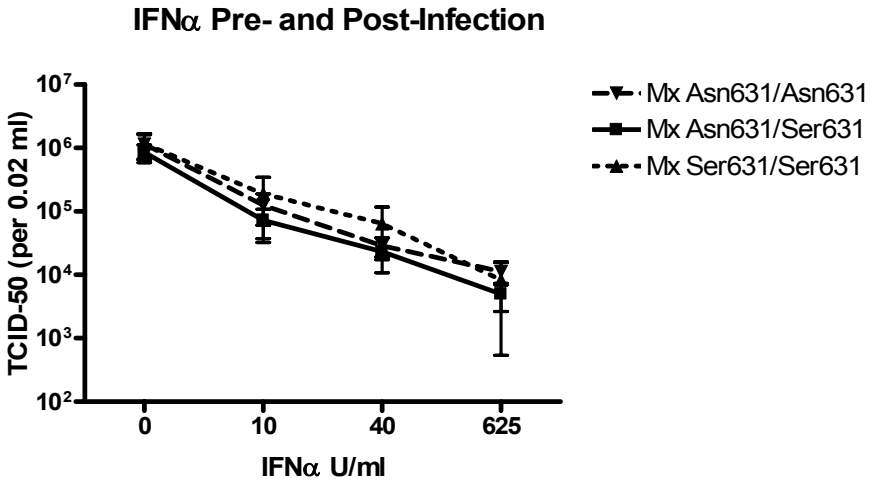


Figure 14. Mx genotype effect on reduction in IBDV yield following IFNα treatment. CEF cultures were prepared individually from four Mx Asn631 homozygotes, four Mx Ser631 homozygotes and five Mx Asn631/Ser631 heterozygotes. Virus yield differs from control (no IFNα treatment) at all IFNα concentrations evaluated ($p<0.001$ by ANOVA).

Virus yield was shown to decrease in response to IFNα treatment across all genotypes. However there was no significant difference among *Mx* genotypes at the IFN dilutions described here (Figure 14).

Discussion

There is little information in the literature describing the antiviral properties of chicken IFNα against IBDV infection. Results of the present study provide evidence to suggest that chicken IFNα has strong, dose dependent antiviral activity against IBDV, even at a dose as low as 10 U/ml when exogenously added IFN is present before and after virus infection. In comparing cells that had been treated once or twice with IFNα, there did not appear to be any reproducible reduction in cell death in those cells that were treated twice. However when virus yield was assessed on selected IFNα dilutions, cells treated before and throughout viral infection with IFNα showed a 100- to 1000-fold decrease in viral yield at the highest IFN concentration when compared to the control without IFNα, compared with a 10-fold decrease in cultures treated once with IFNα.

This leads to the question of which interferon stimulated gene or genes is responsible for activity against IBDV? Mx is an obvious candidate. A recent report described that human MxA has been shown to strongly reduce to replication of the birnavirus IBDV (Mundt 2007). Salmon Mx1 demonstrates potent antiviral activity against another birnavirus IPNV, a fish pathogen (Larsen et al. 2004). In addition, in CEFs that were infected with IBDV the transcription of *Mx* was upregulated in comparison to uninfected cells (Wong et al. 2007). This data is suggestive of an antiviral role for Mx proteins against double stranded RNA viruses. However, to date the antiviral

role of chicken Mx against IBDV has not been assessed either in transfection experiments or in cells endogenously expressing Mx in response to IFNα. In cells endogenously expressing Mx in response to IFNα, we were able to compare cultures with antivirally positive *Mx* Asn631 and antivirally negative *Mx* Ser631 alleles for IBDV antiviral activity following IFNα treatment to induce ISGs. Results indicated that when cell survival was evaluated 4 days post infection following IFNα treatment, there was little difference between genotypes. Medium samples from these cultures were then analyzed to assess virus yield, and it was shown to decrease across all genotypes with increasing concentrations of IFNα. There was no significant difference in reduction in virus yield among *Mx* genotypes (Figure 14). In all *Mx* genotypes, the virus yield was lowest in cells treated twice with IFNα.

The similarity in virus reduction induced by IFNα across all *Mx* genotypes may have several explanations. Firstly, the Mx protein in chickens may be a redundant ISG for IBDV: the antivirally positive Mx Asn631 may have activity against IBDV but other ISGs may compensate for the absence of antiviral activity against IBDV by Mx Ser631. Chicken *PKR* has been cloned and transfection experiments have demonstrated antiviral activity against VSV (Ko et al. 2004a). Chicken *OAS* is also a candidate for IFNα induced antiviral activity – upregulation of this protein has been reported in chicken embryo cells treated with IFN (West and Ball 1982) and in chicken CEFs infected with IBDV (Li et al. 2007). Two alleles of *OAS* have been described in the chicken, *OAS-A* and *B*, with OAS-B exhibiting only 10-15% activity of OAS-A (Yamamoto et al. 1998). However, preliminary data from our laboratory suggests that different OAS genotypes did not influence IBDV replication. Secondly, Mx Asn631 may not have antiviral

activity against IBDV despite its differential activity against VSV and influenza virus. Finally, it is possible that both Mx Asn 631 and Ser631 have antiviral activity against IBDV and that the Mx 631 polymorphism affects only vesicular stomatitis and influenzaviruses.

Reverse transcription quantitative PCR in our lab demonstrated that *Mx* was well expressed, relative to *β-actin*, in CEFs treated with the crude recombinant IFNα preparation in a dose dependent manner (Livant unpublished). This demonstrated that the IFN preparation was active and the CEF in this culture system responded well in terms of upregulation of Mx expression.

This study demonstrates the antiviral properties of IFNα on IBDV in CEFs. Further studies are required to evaluate the role of the various ISGs in IFNα-induced anti-IBDV activity.

V. CONCLUSION

The innate immune response is one of the first lines of defense against viral pathogens and is characterized by rapid, non-specific responses of limited duration. A key component of the innate immune response is the NK cell, which destroys virally infected cells. The Type I IFN pathway, which ultimately establishes an antiviral state in cells, is another critical component of the innate immune response to viruses. Numerous studies in mammals have shown that polymorphism of genes in both these pathways is linked with differences in disease resistance. However, similar studies in chickens are limited. The first study presented here focused on polymorphism in a gene of the chicken MHC complex (*BF1*), encoding a class I molecule that was recently shown to regulate NK-like cells. In the second study, the role of the Type I IFN pathway, and polymorphism in a gene involved in the IFN-induced antiviral state (*Mx*), was evaluated against IBDV, a commercially important pathogen of chickens.

Although the association of the chicken MHC with resistance to infectious diseases was first identified about 40 years ago, and the list has been growing, in no case is the identity of the MHC gene or genes responsible for differential resistance known. Similarly, the role of individual MHC proteins in immune mechanisms (CTL or NK cell killing, etc.) is incompletely understood. However, the molecule encoded by one of the MHC class I genes (*BF2*) presents antigen peptide to CTL. And recent studies by collaborators demonstrated that expression of the BF1 molecule (encoded by the other

MHC class I gene) on target cells strongly blocks NK-like killing. These results indicate that the BF1 molecule is a ligand for an as yet unidentified killer inhibitory receptor on NK cells. Mammalian cells tightly regulate expression of MHC class I molecules (such as HLA-C) that may be functionally homologous to BF1, and the NK-inhibitory class I molecules are less well expressed at both the mRNA and protein levels than the class I molecules that present antigen to CTL. In mammals, allelic variation in expression or recognition of both the NK inhibitory receptors and their MHC class I ligands has been noted, and is thought to be driven by pathogen pressure. This study attempted to further characterize the expression and promoter polymorphism of the chicken MHC class I molecule, BF1, in light of its NK-like cell inhibitory activity.

Previous studies to quantify expression of the *BF1* and *BF2* loci relied on counting relative numbers of cDNA clones obtained from conventional PCR, which is not truly quantitative. This study reports difference in levels of expression of the *BF2* and *BF1* loci, using quantitative RT-PCR, with all but one of the haplotypes evaluated here exhibiting a *BF2* to *BF1* ratio on the order of 1.2 to 5. The exception, the BF1*C7 allele, was expressed in very low amounts, resulting in a *BF2* to *BF1* ratio of 27:1. To further evaluate mRNA expression of *BF1* genes, nucleotide sequence analysis of the 5' regulatory region containing promoter elements of nine *BF1* alleles was performed. Results showed extensive polymorphism in the 5' upstream region of the various *BF1* alleles but conservation of most identified promoter elements; additionally, all the *BF1* alleles showed divergence from the Enhancer A sequence identified in the more highly expressed *BF2* gene. The fact that all the novel *BF* sequences that were previously assigned to the *BF1* locus (on the basis of locus-specific sequence elements and

phylogenetic analysis) could be amplified by long distance PCR from the flanking *DMβ2* gene in effect localizes these sequences to the *BF1* locus. Finally, the extremely low expression reported of the BF1 allele in one haplotype, C7, may be explained by a mutation in the 3' splice site of intron 7. In light of the demonstration that BF1 molecules inhibit NK cell killing, the polymorphism causing low expression of the *BF1*C7* allele may have been selected by pressure from a specific pathogen that was better controlled by host NK cells having a lower threshold of activation conferred by absence of the BF1 inhibitory ligand on infected cells.

In the second study presented here, the effect of the Type I IFN (IFNα) on IBDV was evaluated. Futhermore, we evaluated the influence of polymorphism in the *M*x gene, which is induced by Type I IFNs and contributes to the antiviral response, on infection *in vitro* with IBDV.

IFN induced mediators have been well documented in mammals; in particular, the antiviral role of Mx proteins in humans and mice has been well established. The antiviral role of this protein has also been demonstrated against dsRNA viruses, in fish and transfected human cells. Furthermore, artificial mutants of chicken Mx cDNA confirmed that positive antiviral was conferred by the presence of Asn at position 631, with the presence of Ser indicating an antivirally negative protein. However to date the role of chicken Mx has not been assessed with regard to IBDV, an important pathogen of chickens. In this study, CEF cultures differing in Mx631 genotype were established. In this system, we were able to compare cultures from antivirally positive Mx Asn 631 and antivirally negative Mx Ser 631 embryos for activity following IFNα treatment to induce ISGs.

IFNα clearly has a strong antiviral effect against IBDV in this culture system; in cultures of all Mx genotypes, there was a reduction in cell death in infected cells that had previously been treated with IFNα. Virus yields were also compared among genotypes and it was shown to decrease across all genotypes in response to IFN in a dose dependent manner, with no significant difference among Mx genotypes.

This study demonstrates the antiviral properties of IFNα on IBDV in CEFs. The reduced virus yield in cell cultures of all Mx genotypes indicates that the Asn/Ser dimorphism at position 631 is not a critical determinant of IFN-induced anti-IBDV activity. Clearly, one or more ISGs control IBDV infection in chicken CEF cells. The IFN-induced antiviral state may potentially be mediated by both Mx Asn631 and Ser631 alleles possessing antiviral activity against IBDV, or by one or more other ISG compensating for the lack of Mx Ser631 activity against IBDV.

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