The Type I IFN of *Bos taurus*

A Dissertation

presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

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MAY 2008

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THE TYPE I IFN OF BOS TAURUS

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My Dedications….

I would like to dedicate the first page and the last page to my brother because he is always at the beginning of every exciting adventure and always at the end of the best journeys. Plus he is just the bestest brother in the whole wide world….

I would like to dedicate the Literature Review to my mom for giving me a strong foundation

and Chapter II to my two dads for both being there for me in their own way.

Chapter III I is dedicated to Grandma for a long and incredible life with lots of "hugs around the neck."

The appendix goes to Knickkack because she still has a lot of story left to write,

and finally Gypsy gets the last period on the last page because she just wants to know I'm done so she can go play in the sun again.

Acknowledgements

I have spent many years in Dr. Roberts laboratory from my undergraduate through veterinary school and then through my doctoral training, and I have learned a great deal from him. I would like to thank him for supporting me through all of my steps and mis-steps. He has an incredible mind, which together with his passion for science, is fascinating and inspiring to witness.

I would also like to thank my committee members, Dr. Gary Johnson, Dr. Gayle Johnson, Dr. Venkataseshu Ganjam, and Dr. Jonathan Green, for their time and input in my doctoral work. As well, Dr. Gayle Johnson together with Dr. O'Brien provided me the first opportunity to work in a laboratory as an undergraduate and I am grateful I had that experience. Dr. Gary Johnson gave me the most direction for my genome work and provided the most useful tips in tackling the project. Dr. Ganjam always has a kind word of encouragement when I see him and Dr. Green has had the pleasure of walking me through a number of computer programs and concepts that I just couldn't figure out on my own. I have a great committee.

I would like to give a special thanks to Koji Kimura for culturing the day 8 bovine blastocysts used in the Chapter II. This dissertation would not have been possible without his assistance. Dr. Alan Ealy taught me all the important stuff from PCR to protein purification to tissue culture. Dr. Anindita Chakrabarty always gave me good advice (even though I failed to take it). Dr. Laura Schulz has served as a sounding board whenever a project has not worked. Finally, I would like to acknowledge the rest all the past and present members of Dr. Roberts' laboratory. I love you all.

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THE TYPE I IFN OF BOS TAURUS

ABSTRACT

The Type I interferons (IFN) have major roles in the innate immune response to viruses, a function that is believed to have led to rapid expansion in the number and complexity of their genes. IFNT, which is a unique Type I IFN restricted to pecoran ruminants, also has a specialized role in maternal recognition of pregnancy in cattle. This work has two main aims 1) determine whether male and female blastocysts differ in the kind and number of *IFNT* they express and whether this pattern changes over development and 2) provide the first comprehensive annotation of the Type I IFN locus in *Bos taurus*, thereby providing an insight into the functional evolution of the Type IFN in ruminants. Data collected for the first aim indicate that female blastocysts do not transcribe a different set of *IFNT*s than males ($p=0.54$). However, significant differences ($p < 0.001$) were evident among conceptuses of different age, indicating that additional genes may be transcribed as IFNT production increases during development. The data collected for the second aim revealed the Type I IFN locus has undergone significant rearrangement and expansion in bovine compared to mouse and human. The *IFNW* subfamily is greatly expanded compared to other species, comprising 24 potentially functional genes. Selective pressure analysis found the regulatory regions of the *IFNW* are diverging faster than the coding regions. The identification of a new Type I IFN subfamily that is expressed from virally challenged bovine kidney cells is the most striking finding of the second aim.

Chapter I

Type I Interferon and Their Extended Family

Cytokines

An Overview

Cytokines are secreted regulatory proteins acting in either a paracrine or autocrine fashion through specific cell surface receptor interactions. The term was coined in 1974 to define a factor secreted by a cell ("cyto") that acts on a target cell (Cohen et al., 1974). Cytokines are usually small, 6-30 kDa (kilodalton), extremely potent polypeptides, often functioning at nanomolar or picomolar concentrations (Geoffrey R, 1994) . These factors are typically produced only for brief periods of time and show restricted capacity for entry into the circulation and generating systemic effects. The temporally and geographically confined actions are further limited by the classically short half-life of most cytokines (Leonard, 2003b). Specific exceptions for the above characteristics exist, however, making the definition of cytokines imprecise. For example, erythropoietin and transforming growth factor β (TGFβ) both enter the systemic circulation (Oppenheim, 1994). There have been many attempts to differentiate cytokines from hormones, which classically act on sites far from their release location, and growth hormones, which are generally constitutively rather than transiently expressed, but these distinctions are becoming increasingly blurred as cytokines become better understood. Consequently, the nomenclature defining these factors will undoubtedly continue to evolve.

Individual cytokines have pleiotropic actions that are often either antagonistic or synergistic with the actions of other cytokines. Thus, cytokines typically operate within a cytokine network to bring forth diverse biological activities for both normal and pathological events. These activities may include cell proliferation, differentiation, apoptosis, chemotaxis, and cytolytic activity involved in immune responses, inflammatory reactions, tissue remodeling, angiogenesis, and neoplastic transformation (Geoffrey et al., 1994). The appropriate balance of cytokines within the network is critical to maintaining homeostasis in these pivotal biological processes.

Classification

The receptors that cytokines bind for initiation of signal transduction have been the most defining element in cytokine classification. Currently five unique cytokine receptor superfamilies have been identified: immunoglobulin receptors, class I receptors, class II receptors, tumor necrosis factor (TNF) receptors, and chemokine receptors (Figure 1) (Kuby, 1997). The cytokine receptor superfamilies are classified based on their tertiary structure rather than amino acid homology, which often is only 20-30% among superfamily members (Renauld, 2003). The ligands are classified into the same superfamilies as the receptors they bind. Structural and functional similarities among cytokines that share receptors reinforce the aptness of this classification system. However, as with the receptor superfamilies, the amino acid identity between cytokines in the same superfamily is often low. For example, interleukin-10 (IL-10), a member of the class II cytokine superfamily, only has 15-25% amino acid identity with other members of its superfamily, but it shares their six α-helical structure (Renauld, 2003).

While not technically accurate, many immunology and cytokine reviews only divide cytokines and their receptors into class I and class II superfamilies (Leonard, 2003a; Langer et al., 2004) because some receptor superfarmilies had previously been placed within other superfamilies (chemokine receptors) (Cohen et al., 1999) or because historically a superfamily was not classified as a cytokine receptor family (immunoglobulin receptors). The class I and class II receptor superfamilies are defined by their highly homologous extracellular domains (Bazan, 1990). The extracellular domains of both superfamilies contain two tandem fibronectin type III domains (Figure 2A). However, the position of four conserved cysteines linked by disulfide bonds, which are responsible for the alpha helix arrangement of the receptors, differs between the superfamilies (Kuby, 1997). The class I receptor superfamily, sometimes referred to as the hematopoietin receptor family, also contains a conserved tryptophan-serine-arbitrary amino acid-tryptophan-serine (WSXWS) motif that is absent in the class II superfamily (Figure 1) (Bazan, 1990; Leonard, 2003b). The class I cytokine family is the largest of the two families and contains most of the receptors associated with immune and hematopoietic systems, including IL-2 related cytokines, IL-3 related cytokines, IL-6 related cytokines, prolactin, leukemia inhibitory factor (LIF), erythropoietin, thrombopoietin, leptin, and growth hormone (Figure 1) (O'Sullivan et al., 2007). The class II cytokine receptor family currently includes the receptors for tissue factor VIIa (FVIIa), IL-10 related cytokines, Type I interferon (IFN), Type II IFN, and Type III IFN (Figure 1 and 2A) (Kotenko and Langer, 2004).

Class II Cytokines

Class II Cytokine Receptors

Class II cytokines activate common signaling pathways by interacting with their specific receptors. All class II cytokine receptors except the tissue factor receptor, a homodimer, and the IL-22 decoy receptor are formed by two subunits: a long intracellular ligand binding receptor (R1) and a short intracellular ligand binding receptor (R2) (Figure 2A) (O'Sullivan et al., 2007). The R1 subunit generally defines the specificity of signaling and the R2 subunit initiates signal transduction (Kotenko and Langer, 2004). Twelve class II receptor subunit chains have been identified in human and are believed to be conserved in other mammalian species. Dimerization of the receptor subunits creates ten known receptor complexes (Table 1) (O'Sullivan et al., 2007). These complexes are unique; however, the high degree of structural homology within the cytokine superfamily allows receptor complexes to bind multiple cytokines (Figure 2A and 3) (Renauld, 2003). The shared cytokine receptors help explain the redundant actions initiated by cytokines.

The Type I IFN receptor is formed by two subunits: IFNAR1 and IFNAR2. IFNAR2 has a higher affinity for IFN than does IFNAR1. Indeed, IFNAR2 binds Type I IFN in IFNAR1 knockout mice, but IFNAR1 alone cannot. However, the complex of IFNAR1 with IFNAR2 has a tenfold higher affinity for IFN than IFNAR2 alone (Cleary et al., 1994; Cohen et al., 1995). In addition, IFNAR1 is necessary for signal transduction to proceed and could be involved in differential ligand specificity (Cohen et al., 1995; Cutrone and Langer, 2001). The IL-10 receptor follows a similar paradigm (Langer et al., 2004). Small variations in this theme occur, however. For example, the IFNGR1 and R2 subunits have reversed roles in initiation of signal transduction and ligand specificity

compared to the other class II receptor subunits. In addition, subunits can be mixed and matched to form receptor complexes. Type II IFN receptor consists of a unique receptor subunit, IFNLR1, and IL-10R2, suggesting Type II IFN is more closely related to IL-10 than to either Type I or Type II IFN.

The best characterized signal transduction pathway for the class II cytokine superfamily involves Janus kinases (Jaks) and signal transducers and activators of transcription (STATs) (Kotenko and Pestka, 2000; O'Sullivan et al., 2007). Jak1 is associated with the intracellular domain of R1 at the Jak Association Site (JAS) (Figure 2B). R1 also contains a Src homology 2 (SH2) domain Recruitment Site (SRS) that usually contains a STAT. R2 is an Accessory Chain (AC) to bring an additional tyrosine kinase to the complex for activation of the Jak-STAT pathway (Kotenko and Pestka, 2000). Binding of the appropriate cytokine to the receptor subunits facilitates phosphorylation of Jak1 through its tyrosine residues (Kotenko and Langer, 2004). The Jak phosphorylation creates docking sites for the STAT or another SH2 domain containing protein. The Jaks then phosphorylate the transcription factors for nuclear transport (Renauld, 2003).

It is interesting to note that, despite the R1 chain possessing both the SRS and JAS, R1 homodimerization does not allow cross-phosphorylation of the Jaks and initiation of the signaling process, although the tissue factor receptor provides an exception to this rule. Activation of the Jak-Stat pathway is possible through R1 homodimerization within the class I cytokine family, but the geometry of the class II R1 subunit only allows the receptors to be within 27Å at their closest points, preventing the Jaks from interacting (Walter et al., 1995; Kotenko and Pestka, 2000). Some speculate

that the necessity of the R2 subunit allows greater signaling control for the ligand and permits additional cellular component interactions for activation of multiple signaling pathways (Kotenko and Pestka, 2000).

Several cytokines including interferon-gamma (IFNG), IFN-beta (IFNB), and IL-10 exist as homodimers in their native state. Cytokine homodimerization alters the standard class II receptor complex. Two copies of both the R1 and R2 subunits oligomerize in the presence of a class II cytokine homodimer (Figure 2A) (Kotenko and Langer, 2004). It is currently unclear how this oligomerization affects signal transduction, but it may help explain how cytokines acting through the same receptor complex can induce different biological processes.

Jak-STAT

Only one Jak, *Hopscotch,* is present in Drosophila (Binari and Perrimon, 1994). The Jak family expanded to four members, Jak1, Jak2, Jak3, and Tyk2, concurrent with the evolution of innate and adaptive immune response in higher animals (Table 1). Jaks are large proteins, ranging from 120 to 140 kDa, which contain seven Janus homology (JH) domains (Figure 3). A classical kinase domain is present at the carboxyl-terminus (JH1) immediately adjacent to a regulatory pseudokinase domain (Yamaoka et al., 2004). The two-faced aspect of these kinase-pseudokinase domains prompted early researchers to name it after the two-faced Roman god Janus. Jaks interact with their receptor through a conserved Four-point-one/Ezrin/Radixin/Moesin (FERM) domain close to their N-termini in JH3-JH7 (Chen et al., 1997). The FERM domain is responsible for receptor selectivity of the Jak family members, and it is ultimately responsible for Jak1/Tyk2

associating with Type I IFN, Type III IFN, and IL-10-related cytokine receptors, as well as Jak1/Jak2 binding to the Type II IFN receptor (Table 1) (Haan et al., 2001; Hilkens et al., 2001). Interestingly, Jaks are not only responsible for signal transduction, but they also regulate the stability and trafficking of the cytokine receptors. Tyk2 reduces endocytosis of IFNAR1 and promotes re-localization of IL-10R2 to the plasma membrane (Ragimbeau et al., 2003). In this way, Jaks regulate class II cytokine activity through multiple mechanisms.

Seven STATs can be activated by Jaks in mammalian species: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (Ivashkiv and Hu, 2004). The affinity varies between each STAT and the class II cytokine receptors based on interactions between the STAT SH2 domains and phosphotyrosine motifs on the receptor (Strobl et al., 2001). Type I IFN can activate all seven STAT transcription factors, but preferentially activates STAT1 and STAT2. The activated STAT1/STAT2 dimer complexes with interferon regulatory factor 9 (IRF9) to form the IFN-stimulated gene factor 3 (ISGF3), also known as p48 or ISGF3γ (Brierley and Fish, 2005). ISGF3 then binds to interferon-stimulated response element (ISRE) to regulate gene transcription (Langer et al., 2004). Despite acting through a different receptor complex, Type III IFN has the same STAT activation pattern as Type I IFN. Both IFNG and IL-10 preferentially activate STAT1 and STAT3, which is even more surprising, because, as described in the next section, these cytokines induce very different biological downstream events despite activating the same signaling pathway.

Biological Functions of Class II Cytokines

It is impossible to pigeon-hole the function of the class II cytokine superfamily because its members are highly divergent and often antagonistic in function (Table 1). For example, IFNG is commonly classified as a pro-inflammatory molecule known for activation of macrophages and endothelial cells (Dalton et al., 1993; Goodbourn et al., 2000). On the other hand, IL-10 is an anti-inflammatory protein that inhibits production of pro-inflammatory molecules and activation of co-stimulatory molecules by macrophages, monocytes, and T cells (Moore et al., 2001). Class II cytokines have been repeatedly observed to play critical roles in the broad functional categories of viral defense, inflammatory modulation, and cancer prevention (Renauld, 2003; Langer et al., 2004). These roles will be discussed in more detail below. It should be noted that tissue factor VIIa is a structural and functional outlier from the other class II cytokines and is not included in this general functional categorization (Langer et al., 2004). Accordingly it is not discussed further in this chapter.

Viral Defense

Both Type I and Type III IFN have dominant roles in viral defense, a topic discussed in depth later in the chapter. Briefly, Type I IFN is rapidly induced by most viruses, a process that subsequently upregulates other antiviral genes and provides a barrier to viral replication (Takaoka and Yanai, 2006). Type III IFN is less well characterized, but appears to have an analogous role in viral protection (Renauld, 2003). Type II IFN has a less dominant role in viral defense and does not upregulate the same antiviral genes as Type I or Type II IFN. However, Type II IFN is released by T cells and natural killer (NK) cells during viral infection, and both Type II IFN and its receptor

are pivotal in the defense against specific viral agents such as mouse hepatitis virus (Mello et al., 1993; Schijns et al., 1996). IL-10, as alluded to earlier, is antagonistic to Type II IFN (and Type I IFN) and, actually, may indirectly aid viral invasion. Homologues to IL-10 and IL-10 related cytokines are produced by a variety of viruses including Epstein-Barr virus, baboon herpes virus papio, equine herpes virus Type 2, and ovine Orf parapoxirus, presumably to neutralize the host response (Hsu et al., 1990; Rode et al., 1993; Fleming et al., 1997)

Inflammatory Modulation

Type II IFN (IFNG) and IL-10 are at either end of a see-saw controlling inflammatory response. IL-10 was first identified as a soluble product from $CD4^+$ T helper-type 2 (Th2) cells that inhibited IFNG and IL-1 from Th1 cells (Fiorentino, 1989). IL-10 downregulates major histocompatibility (MHC) class II molecules and B7 on antigen presenting cells, thereby, preventing optimal T cell activation (de Waal Malefyt et al., 1991). IFNG pushes the see-saw in the opposite direction through upregulation of MHC class II molecules on B cells, dendritic cells, and professional antigen presenting cells (Kuby, 1997). It inhibits population growth of Th2 populations and promotes activation of Th1 populations skewing the immune response to a Th1 phenotype (Schroder et al., 2004). These opposing actions are clearly demonstrated through the administration of lipopolysaccharide (LPS), which provides a classic experimental model to characterize inflammatory regulators. LPS induces the expression of IFNG and its pro-inflammatory actions. Furthermore, blocking IFNG increases resistance to LPS-induced shock (Heremans et al., 1990). On the other hand, IL-10 deficient mice are

highly susceptible to LPS-induced shock, and the administration of IL-10 to LPS treated animals reduces endotoxin-induced death (Ishida et al., 1993).

The role of Type I IFN and IL-10 related cytokines are more elusive. Type I IFN has pro-inflammatory effects on T and B cells *in vitro*, but often suppresses the immune system *in vivo*. Two Type I IFN, IFN-alpha (IFNA) and IFNB, are even clinically approved for the treatment of autoimmune diseases such as multiple sclerosis (Hall et al., 1997; Brod et al., 2001; Brod et al., 2006). IL-10 related cytokines, which include IL-19, IL-20, IL-22, IL-24, and IL-26, are not well characterized at this point in time. Many (IL-19, IL-20, IL-22, and IL-24) are assumed to be pro-inflammatory because they are induced by LPS (Renauld, 2003). IL-22 also induces acute phase proteins from hepatocytes and pancreatitis-associated protein in pancreatic acinar cells (Kotenko and Langer, 2004). However, the receptors for these cytokines have not been found on any immune cells examined thus far, including monocytes, NK cells, B and T cells, and bone marrow progenitors (Wolk et al., 2002).

Cancer Prevention

Type I, Type II, and Type III IFN are negative growth regulators through both direct modulation of the cell cycle and induction of pro-apoptotic genes (Goodbourn et al., 2000; Renauld, 2003). For example, IFN upregulate cyclin-dependent kinase inhibitor p21 in human prostate cancer cells and, thereby slow their proliferation (Hobeika et al., 1997). IFN modulation of the cell cycle typically results in arrest at the G1/S checkpoint. However, the arrest at any check point is not guaranteed because the antiproliferative effects of IFN are not ubiquitous across cell types (Goodbourn et al., 2000; Schroder et al., 2004). For example, human IFNA and IFNB inhibit growth of

Daudi cells at relatively low doses (~1unit/ml), but most other cell lines are unaffected at any dose. Similarly, Type II IFN induce apoptosis in certain cell type, e.g. murine pre-B cells, but inhibits apoptosis in chronic lymphocytic leukemia (Grawunder et al., 1993). The pro-apoptotic effect of Type I IFN are limited to virally infected or neoplastic cells suggesting a mechanism is in place to differentiate "healthy cells" from "sick cells" (Tanaka et al., 1998). Despite these incongruities, Type I IFN (IFNA2b and 2a) treatment has been shown beneficial for a variety of human neoplasias, including chronic myelogenous leukemia, B- and T-cell lymphomas, melanoma, multiple myelomas and renal-cell carcinoma (Kirkwood, 2002). In some cases, the growth inhibitory actions of IFN also rely on their close cousins, IL-10 related cytokines. Specifically, a combination treatment of IFNB and a protein kinase c activator, mizerein, upregulates IL-24 in human melanoma cells (Sauane et al., 2003). Consequently, IL-24 has become a promising new candidate for an anticancer therapeutic agent. Over-expression of IL-24 through an adenovirus expression system in a breast cancer cell line induces apoptosis, at least partially through alterations in the BAX to BCL2 ratio (Su et al., 1998). These results have been mimicked in a variety of other cancer types, but overexpression of IL-24 has no growth inhibitory effects on any non-neoplastic cell lines. The tumor suppression was confirmed in animal xenografts, providing rationale for phase I and phase II clinical trials for IL-24 (Sauane et al., 2003).

Interferons

Overview

Class II cytokines, as a group, protect or minimize the damage to the host from infectious or non-infectious agents. IFN were the first discovered cytokines, and information acquired about these factors provided the cornerstone to understanding function, pathways, and evolution of all other class II cytokines. IFN are best known for their role in host defense. Indeed, it was the pursuit of their antiviral activity that led to the discovery of IFN by Isaacs and Lindenman in 1957. The factors interfered with influenza viral replication in the avian chorioallantoic membrane previously treated with heat inactivated influenza virus, hence the name "interfer"ons. (Isaacs and Lindenmann, 1957). IFN are still defined by their ability to induce an antiviral state in cells; however, they now have a number of other recognized effects. Cell proliferation, apoptosis, and immune response are strongly influenced by IFN (Stark et al., 1998). Influencing innate and adaptive immunity is obviously the central role played by IFN, but their actions can also be associated with rapid tissue change and restructuring.

IFN are grouped into three families (Type I, Type II, and Type IIII) based on gene structure, protein structure, and biological properties (Pestka et al., 2004a). At least eight subfamilies of Type I IFN exist in mammals, and they are IFN-alpha (IFNA), IFN-beta (IFNB), IFN-delta (IFND), IFN-epsilon (IFNE), IFN-kappa (IFNK), IFN-tau (IFNT), IFN-omega (IFNW), and IFN-zeta (IFNZ). All Type I IFN evolved from the same ancestral gene, share a high level of structural homology, and induce similar biologic responses (Flores et al., 1991; Uze et al., 1995). The intronless gene structure and action through the same Type I IFN receptor are the most unambiguous criteria for classifying

the Type I family members. Type II IFN is comprised of a single IFN-gamma (IFNG) gene with three introns that bears no apparent sequence or evolutionary relationship to Type I IFN (De Maeyer and De Maeyer-Guignard, 1994; Igawa et al., 2006) and binds to a distinct cell-membrane receptor (Stark et al., 1998). Type III IFN, which is commonly called either IFN-lambda (IFNL) or IL28-29, is a recently discovered cytokine family resembling both Type I IFN and the IL-10 families (Yanai et al., 2001). Most Type III IFN genes have either four (IFNL1/IL-29) or five introns (IFNL2/IL-28a and IFNL3/IL-28b) and all act through a receptor composed of an IL-10 receptor subunit (IL-10R2) and a specific IFNL receptor subunit (IFNLR1) (Langer et al., 2004).

The Type III IFN genes were discovered at virtually the same time by two different groups: Sergei Kotenko from the University of Medicine and Dentistry, Piscataway, New Jersey and Wayne Kindsvogel from ZymoGenetics Inc., Seattle, Washington. Both groups first presented their findings at the same "Cytokines and Interferon" meeting in Italy and published their findings in the same issue of Nature Immunology (Kotenko et al., 2003; Sheppard et al., 2003). Kotenko termed members of this new family IFNL based on their mechanisms of induction, biological actions, and signal transduction pathways. Zymogenetics, on the other hand, termed the new cytokines IL-28 and IL-29 based on their gene structure in humans and interaction with the IL-10R2 subunit. Recent studies in fish have reinforced the appropriateness of the IFNL nomenclature and their inclusion in the IFN family, but some sources still refer to these cytokines as IL-28 and IL-29 (Uze and Monneron, 2007).

Evolution

Primitive cytokine receptors have been identified in sea squirts indicating cytokines were present in early chordate lineages approximately 525 million years ago (MYA) (Liongue and Ward, 2007), but, to the best of current knowledge, IFN did not appear until the advent of bony fish, Osteichthyes, approximately 450 MYA (Krause and Pestka, 2005). IFN-like proteins were found to be inducible by viruses in fish in the early 1970s (de Kinkelin and Dorson, 1973); however, characterizing the responsible genes has only become possible recently through advances in fish genomics. These virally induced genes have a five exon structure that most closely resembles that of IL-10 family members; however, their induction pattern and biological actions more closely resemble those of Type I IFN (Uze and Monneron, 2007). Consequently, the classification of these proteins has been unclear. Some authors have referred to these proteins simply as "IFN" (Altmann et al., 2003; Phelan et al., 2005) while others have referred to them as "Type I IFN" (Robertsen et al., 2003; Long et al., 2006; Novoa et al., 2006; Robertsen, 2006; Wang et al., 2006). Knockdown experiments in zebrafish utilizing specific morpholino oligonucleotides ascertained the piscine IFN receptor is comprised of an IL-10 receptor subunit and a unique receptor subunit analogous to the IFNL receptor in mammals. These results led Levraud to classify piscine virus inducible IFN as Type III IFN.

Genomic evidence from mouse and human suggest all members of the Type I IFN family originated from a common ancestor that existed prior to the divergence of fish and tetrapods (Pestka et al., 2004b). Recent advances in piscine genomics in combination with studies of virally inducible teleost genes have provided strong evidence that an ancient Type III IFN was the predecessor for all Type I IFN (Levraud et al., 2007). A

retroposition event of this ancestral gene is the best hypothesis for the conversion of the five exon piscine gene to the single exon gene present in modern mammals (Figure 4A) (Lutfalla et al., 2003). Interestingly, while the gene structure of mammalian IFNL/IL28-29 corresponds to IL-10 in most cases, one of the IFNL/IL28-29 genes in dogs is intronless (Pestka et al., 2004b). It is tempting to speculate Type III IFN can be preferentially targeted for retrotransposition.

Type I IFN has been identified in reptiles and avian species (Isaacs and Lindenmann, 1957; Galabov and Velichikova, 1975). Unfortunately little sequence information is currently available about reptilian IFN and no reptilian genome has been sequenced. More information is available about avian Type I IFN, and recent genome sequencing projects have further increased our understanding of the IFN family in these species. The original ten Type I IFN genes identified in chickens can be grouped into two families termed IFN1 and IFN2. Type I IFN identified in other birds, particularly geese and ducks, are homologous to one of these two avian IFN families. All of these genes have less than 25% amino acid identity with their closest related mammalian counterpart (Kaiser et al., 2005), and the genes encoding them are present on sex chromosomes rather than on autosomes (Nanda et al., 1998). The relationship between these avian and mammalian Type I IFN is still debatable. Early phylogenetic analysis, which included only mammalian IFN, and structural examination of the IFN proteins and their gene control regions suggested these avian IFN are homologues of mammalian IFNA and IFNB (De Maeyer and De Maeyer-Guignard, 1994; Sick et al., 1998). These findings led Lowenthal to propose avian IFN1 and IFN2 to be termed IFNA and IFNB respectively and this nomenclature is still currently used by some (Lowenthal et al.,

2001). Later phylogenetic analysis including both avian and mammalian Type I IFN found that both IFN1 and IFN2 fell within a distinct avian IFN clade with strong bootstrapping support. These results indicate the duplication event giving rise to IFN1 and IFN2 occurred after the divergence of mammals and birds and IFN1 and IFN2 are not homologues to mammalian Type I IFN (Figure 4B) (Hughes, 1995; Roberts et al., 1998). Interestingly, a third chicken IFN subtype, IFN3, has been discovered through close examination of the chicken genome. It is unclear if IFN3 is expressed in the chicken, but it does group with mammalian Type I IFN in phylogenetic analysis (Figure 4B) and may be a true avian homologue to mammalian Type I IFN (Krause and Pestka, 2005).

IFNK is the most evolutionarily ancient mammalian Type I IFN currently identified (Figure 4B) and is separated from the main Type I IFN locus. It is likely represented in most mammalian species based on its early divergence (Pestka et al., 2004b), and evidence of its existence has been found in the genomes of opossum (NW_001581976), pig (CU463237), cat (AANG01430981), horse (NW_001799690), dog (NW_876253), chimpanzee (NW_001240439), rhesus monkey (NW_001101662), human (AF384048), and mouse (NM_199157) (LaFleur et al., 2001; Vassileva et al., 2003). All mammalian species listed have a single copy of IFNK, and, except for mouse, these genes share at least 60% amino acid identity with each other. Murine IFNK, on the other hand, only has 35% amino acid identity to the first IFNK discovered (human), and EST evidence suggests its expression pattern differs from that of human, where it has been best studied (Vassileva et al., 2003; Buontempo et al., 2006). Murine IFNK is slightly separated from the Type I IFN locus in mouse, as it is in human, but the distance

between IFNK and the main Type I IFN locus has not been conserved (Krause and Pestka, 2005). These findings suggest that IFNK has evolved to fill a unique role in rodents that differs from the one it plays in other mammalian species.

IFNB is predicted to have diverged next from the remaining Type I IFN early in mammalian radiation (Roberts et al., 1998). Genomic evidence for IFNB is present in both marsupials (NW_001581976) and monotremes (NW_001794408) supporting its early divergence. *IFNE* is believed to have first appeared close to the same time as *IFNB* based on phylogenetic analysis, and in some analyses it is even predicted to have diverged prior to *IFNB* (Krause and Pestka, 2005). *IFNE* has been found in all eutherian mammals examined; however, no evidence of *IFNE* has been found in marsupials or monotremes to date making it uncertain if it was present prior to eutherian mammalian radiation. The early divergence of *IFNB* and *IFNE* is reflected in the chromosomal arrangement of the genes in humans and mouse (Hardy et al., 2004). *IFNB* and *IFNE* define the limits of the main Type I IFN locus in all mammalian species so far described, with the more recently evolved subfamilies distributed between the two ancient IFN.

IFND and *IFNZ,* also known as limitin, are the most perplexing of the mammalian Type I IFN in regard to their evolutionary history. Phylogenetic analyses predict that they emerged together around 180 MYA (Roberts et al., 1998; Krause and Pestka, 2005). If this prediction is correct, both of these subfamilies might be expected to be present as genes or pseudogenes in most mammalian species, because they emerged long before the appearance of mammals. However, each of these subfamilies has only been identified in a single species- pig for *IFND* and mouse for *IFNZ* (Lefevre et al., 1998; Oritani et al., 2000). *IFND* and *IFNZ* might be homologues to one another, based on the similar

divergence times predicted from open reading frame (ORF) phylogenetic analyses (Krause and Pestka, 2005), but their highly divergent induction patterns do not support this theory. *IFND* has a unique non-virally inducible promoter that is only known to be activated in trophoblast cells from swine from day 14 to 20 of pregnancy (Lefevre et al., 1998). *IFNZ*, on the other hand, is activated by the same classic viral induction pathways as all other Type I IFN (Oritani et al., 2000). The two genes possess obviously different gene control regions that do not appear to have arisen from the same original gene (Oritani et al., 2001).

The three remaining Type I IFN, *IFNA*, *IFNW*, and *IFNT*, were predicted to have diverged after eutherian mammalian radiation in phylogenetic analysis and have not been identified outside mammals. *IFNA* is the oldest of these three and is present in multiple copies in all mammalian species examined. Surprisingly, each species has developed their own *IFNA* subtypes and these subtypes are not orthologs of *IFNA* subtypes in closely related species (Woelk et al., 2007). This relationship suggests the Type I locus is highly active and its architecture under constant change through gene duplication and gene conversion events (Hughes, 1995; Woelk et al., 2007). *IFNW* is predicted to have emerged from an *IFNA* subtype approximately 129 MYA (Roberts et al., 1998).

IFNW is the most variable family when comparisons are made across species, and its evolution does not correspond closely with mammalian speciation. For example, a single functional *IFNW* and two pseudogenes are present in humans, but only a single pseudogene can be identified in mice (Hardy et al., 2004). Even more bewildering, the family appears to have expanded in cats, which, on the basis of cDNA evidence, possess

at least 10 variants (Yang et al., 2007a), but not even a relic of the open reading frame can be found in the dog (Roberts et al., 2003).

IFNT is the youngest Type I IFN emerging from *IFNW* only 36.5 MYA during the evolution of the pecoran ruminants (Roberts et al., 2003). *IFNT* fill a unique position in the maintenance of early pregnancy in ruminants that is discussed in depth later in this section. It is not virally inducible and instead prevents the return of ovarian cyclicity by extending corpus luteum lifespan in the pregnant animal (Leaman et al., 1994).

The sole member of the Type II IFN family, *IFNG*, is not located close to the Type I IFN locus (on chromosome 9 in humans), suggesting that it is not closely related to the Type I IFN. *IFNG* is instead located on chromosome 12 in humans (Gray and Goeddel, 1983; Naylor et al., 1983) and, indeed, it appears to have evolved from an ancient IL-10 related cytokine (IL22-26-like gene) rather than Type III IFN (Figure 4A) (Igawa et al., 2006). As discussed previously, the Type I IFN family also operates through a different Jak-STAT signaling pathway than Type II IFN and causes different biological effects. Since the focus of this work is on Type I IFN and because IFNG is evolutionarily and functionally distinct from Type I IFN (Stark et al., 1998), it will not be discussed in any greater depth.

Type I IFN Structure

Type I IFN subtypes have a high level of structural similarity. For example, IFNT shares 30%, 50%, and 75% amino acid identity with IFNB, IFNA, and IFNW, respectively (Imakawa et al., 1987). This shared identity allows competitive binding to the same receptor and induction of the same responses in host cells. Therefore, Type I

IFN subtypes and subtype variants can be directly compared through functional assays. This is particularly valuable in regard to IFNT because it allows activity comparisons between variants to be made without animal use. However, these results should be examined with caution. Slight structural differences among subtype variants can alter interactions with receptor components and activate different signaling pathways (Stark et al., 1998). Moreover, it is never possible to confirm whether a recombinant protein has folded efficiently. Thus potency ratios between functional assays can differ between variants but may not be meaningful and may not accurately reflect the ability of IFNT to function in its role in maternal recognition of pregnancy.

A three-dimensional model of Type I IFN based on the crystal structure of murine IFNB found five tightly associated helices (A-E) are present (Jarpe et al., 1994; Senda et al., 1995). Crystallography was later repeated on ovine IFNT and confirmed the predicted tertiary structure found (Figure 5) (Radhakrishnan et al., 1999). Helices A, B, C, and E are antiparallel and helix D is approximately parallel with B. The helices are joined by three short loops (BC, CD, and DE) and one long loop (AB). Disulfide bonds are present between Cys29 and Cys139 joining the AB loop to helix E and between Cys1 and Cys99 between the N-terminal of helix A and helix C (Senda et al., 1995). IFNT and IFNW also have a 6 amino acid extension at the carboxyl terminus compared to IFNB and IFNA (165 for IFNA2) (Imakawa et al., 1987), but the "loose" nature of this tail has not allowed its precise location on the IFNT structure to be defined.

Mutational mapping studies on both IFNA and IFNB have provided significant advancements in understanding IFN-receptor interactions. Binding to both subunits appears to be driven by hydrophobic forces while association prior to full binding

involves electrostatic interactions (Piehler et al., 2000; Cutrone and Langer, 2001). Helix E lies at the center of the IFNAR2 binding site and it is flanked by regions of helix A and the AB loop (Piehler et al., 2000; Runkel et al., 2000). Thus binding affinity is likely controlled by residues in these regions. These studies are supported by experiments on IFNA showing mutationsI within residues 16-28 of helix A can result in 50-fold differences in activity on human cells (Weber et al., 1987).

The role of the carboxyl terminus in receptor binding and activation has still not been defined. Piehler removed the last five amino acids from IFNA2 and found that it retained all of its activity. On the other hand, removing eleven amino acids from the carboxyl terminus of IFNT almost completely eliminated antiviral activity (Li and Roberts, 1994), while removal of the six amino acid tail of IFNT not present in IFNA did not affect the anti-luteolytic or antiviral activity (Li and Roberts, 1994; Ealy et al., 1998).

Portions of helices B, C, and D and loops BC and DE are predicted to be involved in IFNAR1 binding (Runkel 2000; Piehler 2000). Since IFNAR1 is suspected of altering signaling pathways, sequence differences between subtypes and variants in these regions could be responsible for their specificity in biological activity. Different residues in these regions could induce different structural changes in the receptor and thereby cause ligand specific responses in the host.

Type I IFN Induction by Viruses

Type I IFN can be produced by virtually all cell types in mammals, but most cells constitutively express little, if any, Type I IFN. However, Type I IFN production can be increased through a variety of factors, from other cytokines (IL-1 and IFNG) to bacteria.

Viral particles, such as envelope proteins, CpG DNA, and dsRNA, are the most common way to induce Type I IFN (Goodbourn et al., 2000). The viral particles are detected first by cellular pattern-recognition receptors (PRRs) (Malmgaard, 2004). Toll-like receptors (TLR) and retinoic acid inducible gene-1 (RIG-1) RNA helicases (RLH) are the best characterized PRRs and guard both the membrane bound extracellular and endosomal compartments (TLR) and the cytoplasm (RLH).

Thirteen TLRs have been identified in humans and mouse; but, like the Type I IFN themselves, not all TLRs are expressed in all species (Thompson and Locarnini, 2007). It is a reasonable assumption that TLRs are co-evolving with *IFN* to optimize viral defense in a given species. After binding viral factors, most TLR homo-dimerize, thereby allowing interaction with one or more of the downstream adaptor molecules, MyD88, Mal, TRIF, and SARM. The TLR interaction with the adaptor molecule initiates either MyD88-dependent (through MyD88 and Mal) or MyD88-independent (through TRIF and SARM) signaling pathways (Figure 6A) (Akira and Takeda, 2004; O'Neill and Bowie, 2007). MyD88-dependent signaling is associated primarily with pro-inflammatory cytokine induction. MyD88 can also complex with interferon regulatory factor 7 (IRF7) and IRF5 allowing them to be phosphorylated and then translocated to the nucleus for IFNA induction (Uematsu et al., 2005; Kawai and Akira, 2006). MyD88-independent signaling activates IRF3 and is the primary TRL pathway for Type I IFN production (Akira and Takeda, 2004) (Schoenemeyer et al., 2005). Both MyD88-dependent and independent pathways activate nuclear factorκB (NFκB), a major transcription factor required to induce IFNB. However, the MyD88-dependent pathway

promotes a rapid NFκB release from the cytoplasm, while the MyD88-independent pathway promotes a delayed NFκB release.

While TLR detect extracellular or endosomal viral factors, they cannot detect cytosolic factors. This function is filled by the RLHs- RIG-1 and melanoma differentiation associated gene 5 (MDA5) (Yoneyama et al., 2004; Thompson and Locarnini, 2007). Both RIG-1 and MDA5 are caspase activation and recruitment domain (CARD) helicases that can detect dsRNA through a DExD/H box helicase domain. Binding dsRNA causes a conformational shift in the RLHs allowing the CARDs to associate with a recently discovered adaptor protein, *IFNB* promoter stimulator 1 (IPS1) (Kawai et al., 2005). IPS1 initiates a signal cascade resulting in the activation of IRF7, IRF3, and NFκB and subsequent induction of Type I IFN. Despite similarities, RIG-1 and MDA5 are not redundant detection systems. They respond to dsRNA differently from one another *in vivo* based on MDA5$^{-/-}$ and RIG-1$^{-/-}$ mouse models, indicating the two proteins detect different viruses (Kato et al., 2006). In fact, RIG-1 is required for detection of RNA viruses such as paramyxoviruses, influenza virus and Japanese encephalitis, while MAD5 is necessary for the detection of other RNA viruses such as picornavirus.

Viral Evasion of PRRs

The best indicator of the importance of PRRs is, perhaps, the numerous viral mechanisms that have evolved to avoid their detection (Figure 6B). Hepatitis B directly downregulates TLR2 in CD14^{+}, hepatocytes, and Kupffer cells (Visvanathan et al., 2007) and this downregulation is believed to contribute to long-term infection of hepatitis B.

The TLR adaptor molecules have been targeted by other viruses. Three different pox viruses genes (A52R, A46R, and N1L) target either MyD88, MAL, TRAM, and TRIF or their downstream signaling factors to prevent IRF3 and NFκB activation (Harte et al., 2003; DiPerna et al., 2004; Stack et al., 2005). The activities of all three of these genes are directly correlated to viral virulence *in vivo*. Hepatitis C encodes a protein, NS3/4a, to deflect both TLR3 detection and RIG-1 detection. NS3/4a is a serine proteinase that cleaves both the TLR3-adaptor protein, TRIF, and the RIG-1 adaptor protein, IPS-1. NS3/4a may also inhibit IRF3 activation at later stages in the RIG-1 signaling pathway (Thimme et al., 2006). GBF-V, the closest viral relative to hepatitis C, encodes a very similar protein to NS3/4a. While it does not appear to have all the same properties as NS3/4a, the latter also blocks RIG-1 detection through IPS-1 cleavage (Chen et al., 2007b). Hepatitis A virus, a picornavirus, has evolved a mechanism to evade MDA5 detection by blocking its adaptor molecule, analogous to the manner whereby hepatitis c avoids RIG-1 detection (Yang et al., 2007b).

Antiviral Activity

Type I IFN activate the expression of hundreds of interferon-stimulated genes and their gene products (ISGs) through the Jak-STAT pathway (Haller et al., 2006), including three, (2'-5')oligo-adenylate synthetase, dsRNA-dependent protein kinase (PKR), and myxovirus resistance gene (Mx), which are among the best characterized proteins with established function in viral elimination. PKR inhibits both transcription and protein synthesis. It phosphorylates the elongation initiation factor eIF2, thereby, preventing eIF2 from being recycled and slowing translation of viral genes (Ramaiah et al., 1994). PKR

may also cause apoptosis of infected cells, at least partially, through a BCL2 and caspase-dependent mechanism (Goodbourn et al., 2000). Finally, PKR is a significant regulator of Type I IFN and other cytokines through modulation of their transcription factors and signaling pathway components. For instance, PKR upregulates NFκB and suppresses STAT1, thereby shifting the signaling pathway in the infected cell towards IFNB expression and more rapid viral clearance. (2'-5')oligo-adenylate synthetase also targets viral transcription. It activates endoribonuclease L (RNase L) and subsequent viral mRNA cleavage (Stark et al., 1998). RNase L also cleaves 28S ribosomal RNA resulting in total ribosomal inactivation. If unchecked, the lack of active translational machinery can result in irreparable damage to the cell and apoptosis, but simultaneously stops viral replication dead. Mx proteins interfere with transcription of viral genes by sequestering viral ribonucleoproteins, including the elongation initiation factor eIF2α, to specific subcellular compartments (Stranden et al., 1993). However, many other ISGs undoubtedly function as antiviral agents but by less established mechanisms.

IFNT: A Reproduction Hormone

Maternal Recognition of Pregnancy

Progesterone is essential for the maintenance of pregnancy in mammalian species as it is required for keeping the uterine endometrium in a receptive state. In a normal estrous cycle, the follicular cells transform into progesterone secreting luteal cells following ovulation. The body of luteal cells, the corpus luteum (CL), will eventually regress in nonpregnant animals to allow the next ovulation. In most pregnant animals, a signal from the conceptus must, directly or indirectly, block luteal regression.

A variety of mechanisms have evolved to accomplish this task in different species. In primates, the signal for luteal regression is intra-ovarian, and possibly PGF2α (Wiltbank and Ottobre, 2003). Thus the CL will regress unless influenced by a contradictory signal from the implanted embryo. The primate conceptus implants into the uterine wall immediately after hatching and secretes chorionic gonadotroph (CG), which reaches the maternal bloodstream to eventually bind to LH receptors on luteal cells and provide luteotrophic support to a CL teetering on the brink of regression, but before progesterone declines. In ruminants, the signal for luteal regression, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), comes from the uterus (McCracken et al., 1999). Consequently, the ruminant conceptus can implant later in pregnancy compared to humans because it does not need to manipulate maternal physiology by releasing protein hormones into the maternal bloodstream. Instead, the ruminant conceptus signal, IFNT, acts by a paracrine mechanism to indirectly prevent a decline in progesterone synthesis (Roberts et al., 1992).

Luteolysis in Ruminants

Regression of the corpus luteum, or luteolysis, is caused by the pulsatile release of endometrial $PGF_{2\alpha}$. These pulses travel to the CL via cross-current exchange between the uterine vein and ovarian artery (Bazer et al., 1997). Neurohypophysial oxytocin interaction with uterine oxytocin receptors initiates an arachidonic acid cascade producing a $PGF_{2\alpha}$ pulse. When $PGF_{2\alpha}$ binds to its luteal receptors, oxytocin, present in intracellular granules, is released. The luteal oxytocin generated by this positive feedback loop may help amplify the pulses of neurohypophysial oxytocin. However, the

necessity of luteal oxytocin for luteolysis has not been confirmed (McCracken et al., 1999).

The ability of oxytocin to initiate a pulse of $PGF_{2\alpha}$ occurs through regulation of oxytocin receptor synthesis by progesterone and estrogen. Progesterone inhibits estrogen receptor synthesis in the uterus and hypothalamus during the luteal phase (Norman, 1997). Towards the end of the estrous cycle, progesterone receptors are auto-downregulated and estrogen levels rise. The estrogen then binds to endometrial estrogen receptors and upregulates oxytocin receptor synthesis. Estrogen also binds hypothalamic receptors and increases the frequency of neurohypophysial oxytocin release (McCracken et al., 1999).

Anti-luteolytic and Luteotrophic IFNT Activity

IFNT is secreted at large quantities by the mononuclear trophoblast cells from day 13 to 17 in sheep and 16 to 21 of pregnancy in cattle, corresponding to the time in which the corpus luteum would normally regress in a non-fertile cycle (Farin et al., 1990). IFNT, which is required to prevent luteal regression, appears to act locally on the endometrium rather than on the CL directly. The most widely accepted theory to explain how $PGF_{2\alpha}$ release is modulated predicts that IFNT inhibits estrogen receptor synthesis. The decreased estrogen receptor density prevents upregulation of oxytocin receptor and subsequent pulsatile release of $PGF_{2\alpha}$ (Bazer et al., 1997). Experimental results support this theory in sheep. Bazer and his colleagues showed that bilaterally ovariectimized ewes treated with recombinant ovine IFNT had decreased estrogen receptor and oxytocin

receptor density compared to controls (Spencer et al., 1995; Bazer et al., 1997). The evidence for a similar mechanism in cattle is less clear (Robinson et al., 1999).

IFNT may also act by altering prostaglandin synthesis. *In vitro* studies on bovine endometrial cells found PGE_2 production increased while $PGF_{2\alpha}$ production fell after IFNT treatment (Asselin 1997; Xiao 1999). While $PGF_{2\alpha}$ is an antiluteolytic hormone, PGE_2 may have luteotrophic activity. Thus IFNT could be both blocking the antiluteolytic activity of $PGF_{2\alpha}$ while providing support for the CL maintenance during early pregnancy. There is considerable disagreement on this subject, however. Our laboratory found that IFNT down-regulated expression of *PTGS2,* the gene encoding cyclooxygenase-2, a rate limiting enzyme in prostaglandin synthesis, as well as $PGF_{2\alpha}$ synthase (*AKR1C1*), in ovine luminal epithelial cells, while it up-regulated PGE_2 synthase (*PGES*) (Chen et al., 2007a). Similar results were obtained when day 14 pregnant and nonpregnant ewes were compared and after ewes on the 14^{th} day of their estrous cycles were treated with IFNT (Chen et al., 2006). These findings are not in agreement with those reported by Gray et. al (Gray et al., 2006), and it is difficult to explain the differences in outcomes. Overall, the most logical, current assumption is that IFNT favors PGE_2 production over $PGF_{2\alpha}$ and blocks an increase in the transcription of the oxytocin receptor gene in the endometrium.

Final Comments

In conclusion, Type I IFN constitute a large and diverse protein family conserved in all species from fish to modern mammals. The family is primarily associated with viral defense, but its members have roles ranging from immune modulation to pregnancy

maintenance. The variety of tasks performed by Type I IFN subfamilies is initially disquieting, but becomes more logical when examined in a larger evolutionary context. The family has retained the signaling pathways employed by other class II cytokines and, consequently, is able to modulate many of the same cellular functions as other class II cytokines. The purpose of this literature review has been to provide a framework for the evolutionary and biological significance of the Type I IFN subfamilies and of the IFNT in particular. This provides important background information to understand my work. Chapter II of this thesis concentrates primarily on the diversity encountered within the a single Type I IFN subfamily, *IFNT*, and in Chapter III, I explore the evolutionary origins of the entire Type I IFN gene locus of *Bos taurus*.

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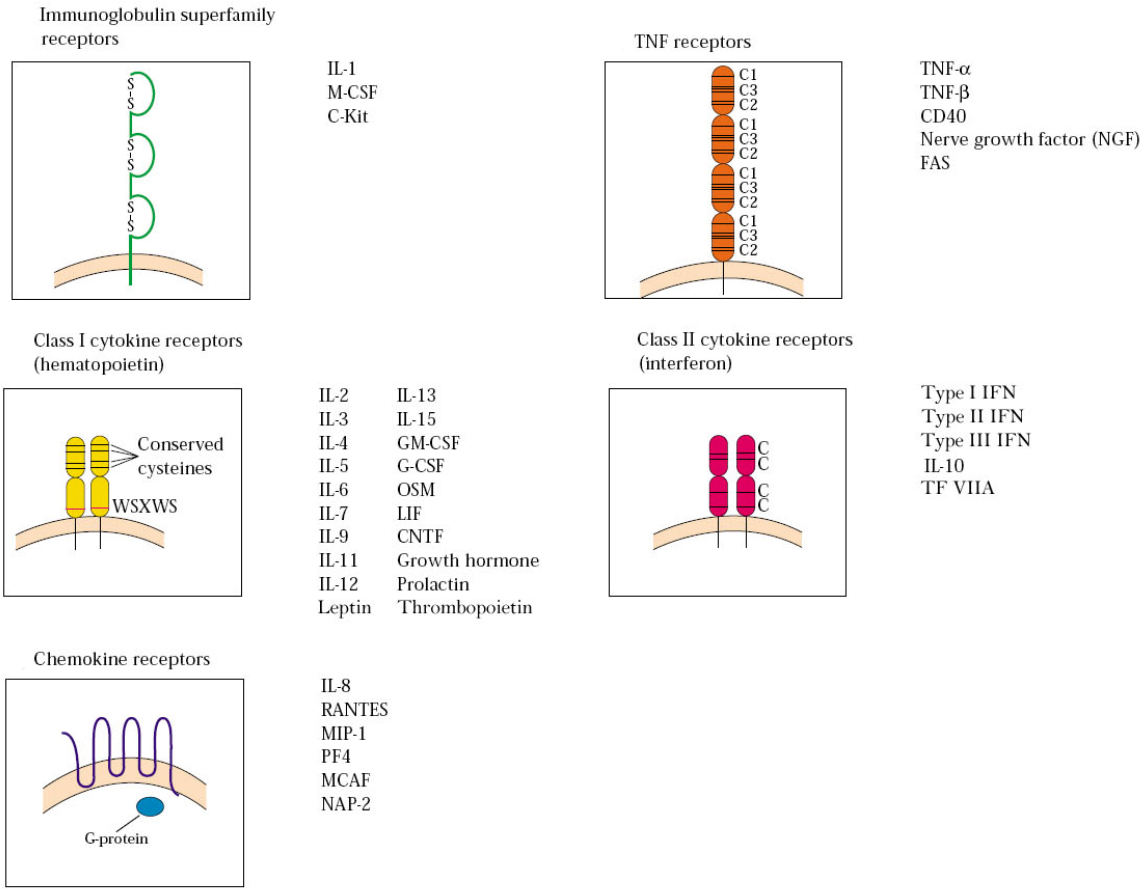
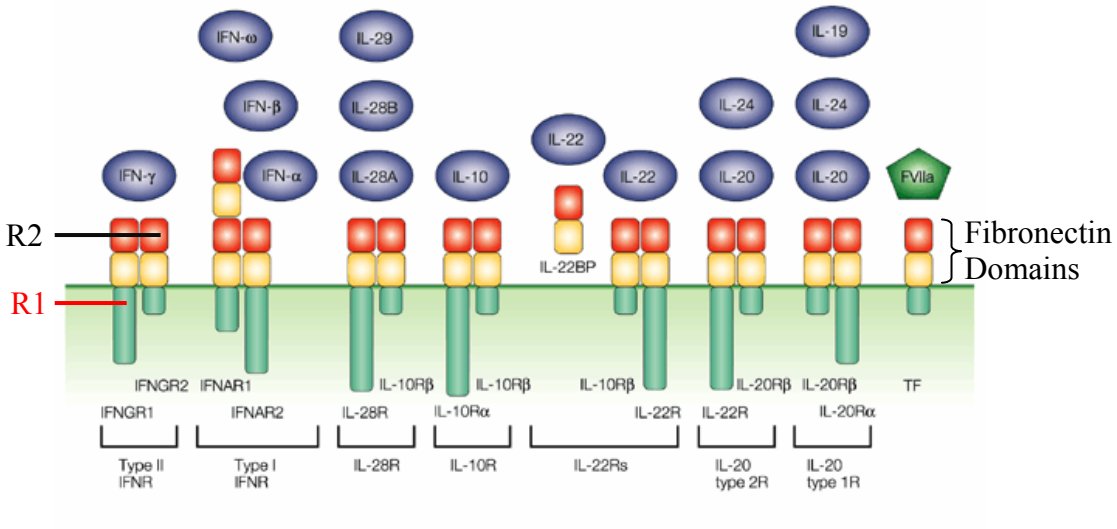


Figure 1. *Cytokine Superfamilies.* Cytokine receptors are divided into five superfamilies based on their structure- immunoglobulin receptors, TNF receptors, class I cytokine receptors, class II cytokine receptors, and chemokine receptors. Common ligands for a receptor superfamily are listed to the right of its cartoon. (Adapted from Immunology by Kuby, J., 5th ed., p: 285)

A



B

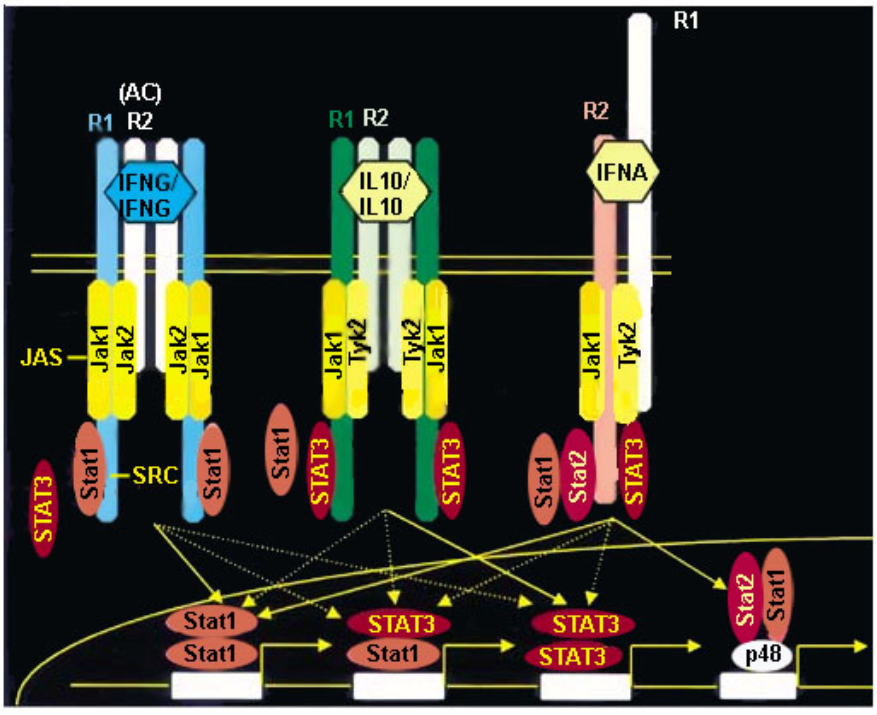


Figure 2. *Class II Cytokine Receptor.* A. Twelve cytokine receptor subunits have been identified in humans and are believed to be present in most other mammalian species. A long R1 chain and a shorter R2 chain dimerize for ligand binding and signal transduction. Multiple cytokines bind many of the receptor complexes. (Adapted from Renauld, 2003) B. The most common signal transduction pathway involves Jak-Stat involving the class II receptor. (Adapted from Kotenko and Pestka, 2000)

Type	Ligand	Complex	Receptors		Downstream Pathways			Functions
Antiviral	IFNA	Type I IFNR	IFNAR1	IFNAR2	Jak1, Tyk2	Stat1,2,3,5	Socs1, Socs3	Antiviral
	IFNB							
	IFNK							
	IFNW							
	IFNE							
	IFNG	Type II IFNR	IFNGR2	IFNGR1	Jak1, Jak2	Stat1,3,5	Socs1, Socs3	Antivirial, pro-inflammatory
	IFNL1	Type III IFNR	IFNLR1	IL10R2		Stat1,2,3,5		Antiviral
	IFNL2							
	IFNL3							
Non-antiviral	IL-10	IL-10R	IL-10R1	IL-10R2				Anti-inflammatory
	IL-26	Type III IL-20R	IL-20R1	IL10R2				Immune response
	IL-22	IL-22R	IL-22R1	IL10R2	Jak1, Tyk2			Pro-inflammatory
	IL-19	Type I IL-20R	IL-20R1	IL-20R2		Stat1,3,5		
	IL-20							Keratinocyte proliferation
	IL-24							Anti-tumour
	IL-20	Type II IL-20R	IL-22R1	IL-20R2				Keratinocyte proliferation
	IL-24							Anti-tumour
	IL-22	IL-22 Decoy	IL-22BP					IL-22 antagonist
	FVIIa	TF	TF					Clotting cascade

Structure
Ligand Binding
Accessory

Table 1. *Class II cytokine receptors and their signal transduction pathways*. Twelve receptor subunits have been identified in humans that complex to form 10 class II receptors. The Jak-Stat signaling pathway is activated by ligand-receptor binding. (Adopted from O'Sullivan et al., 2007)

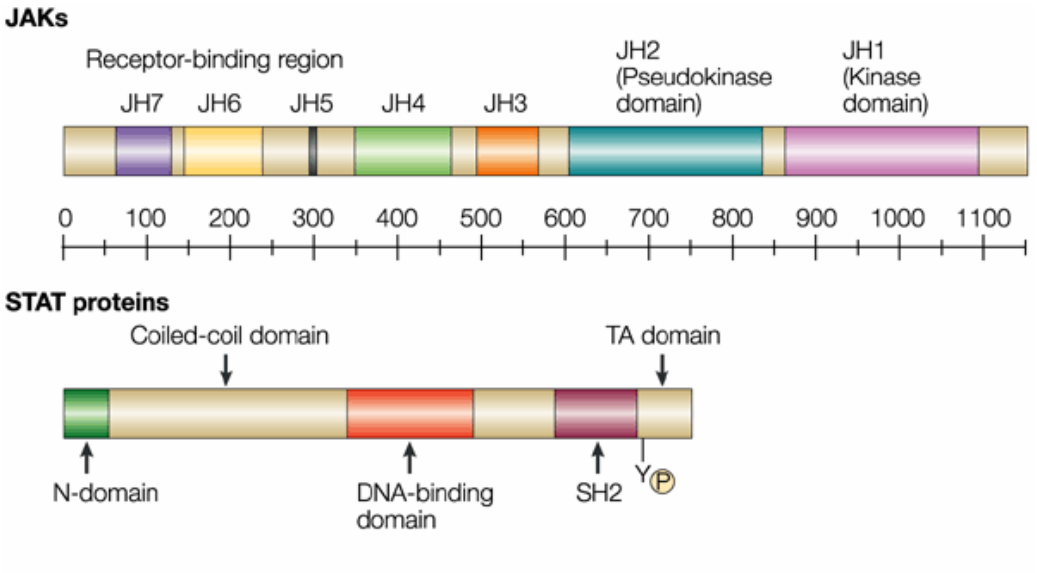
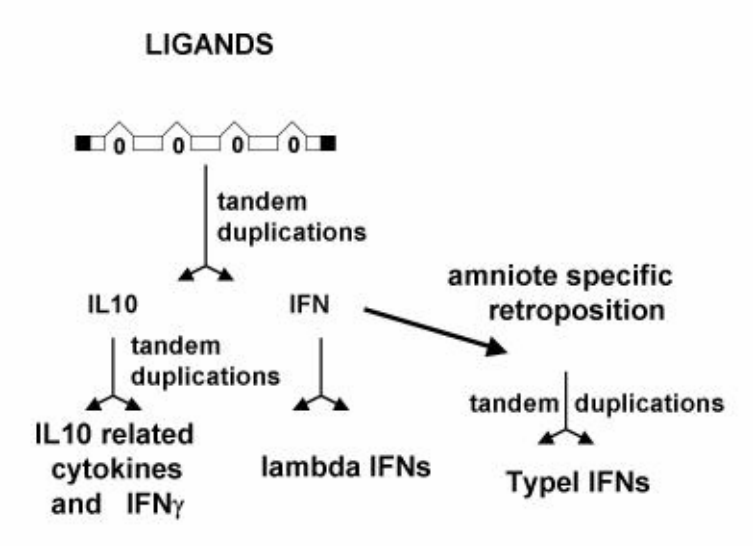


Figure 3. *Structure of Jak and STAT*. Jak binds to the receptor subunit through the N-terminal FERM domain. A catalytic kinase and regulatory pseudokinase are on the carboxy terminus of the protein. STATs interact with receptor subunits through their SH2 domain. (Adapted from (Leonard, 2001)

A



B

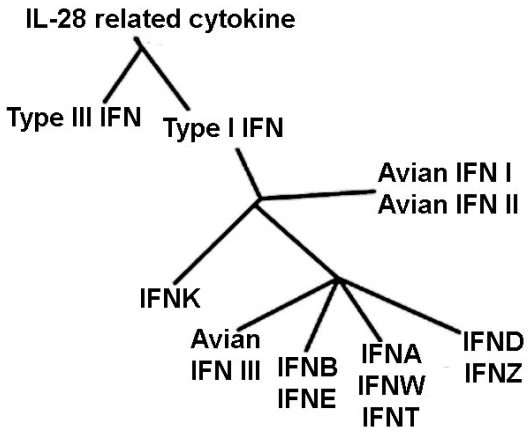


Figure 4. *Independent origin of Type I andType II IFN*. (A) The class II cytokine family all emerged from a single gene that appeared in early chordates. This ancient interleukin duplicated to create an IL-10 and Type III (IFNL/IL-28-29) predecessors. They Type III IFN gave rise to modern Type I IFN after retroposition. IL-10 duplicated into the IL-10 related cytokines subfamily and one of the IL-10 related cytokines evolved into the modern Type II IFN (IFNG) (Adapted from Lutfalla et al. 2003) (B) The evolutionary relationships of avian and mammalian Type I I IFN are illustrated in the schematic. (Adapted from Krauses and Pestka, 2005)

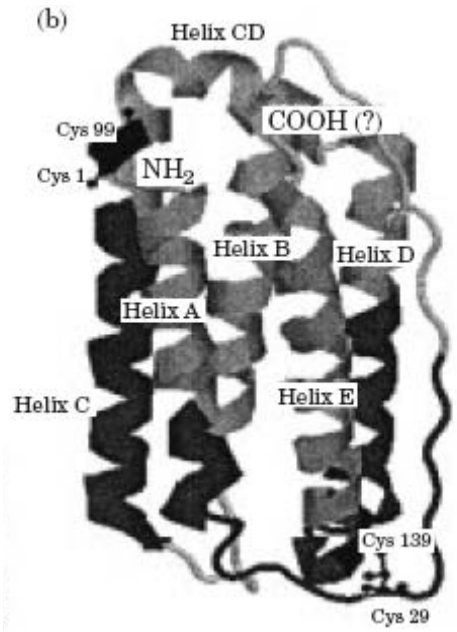
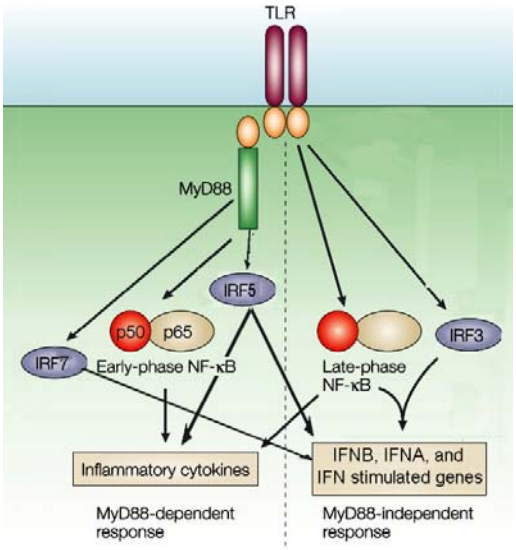


Figure 5. *Structure of Type I IFN based on the molecular model of ovine IFNT.* Type I IFN is contains five alpha-helices (A-E) joined by three short loops and one long loop. (Adapted (Roberts et al., 1999)

A



B

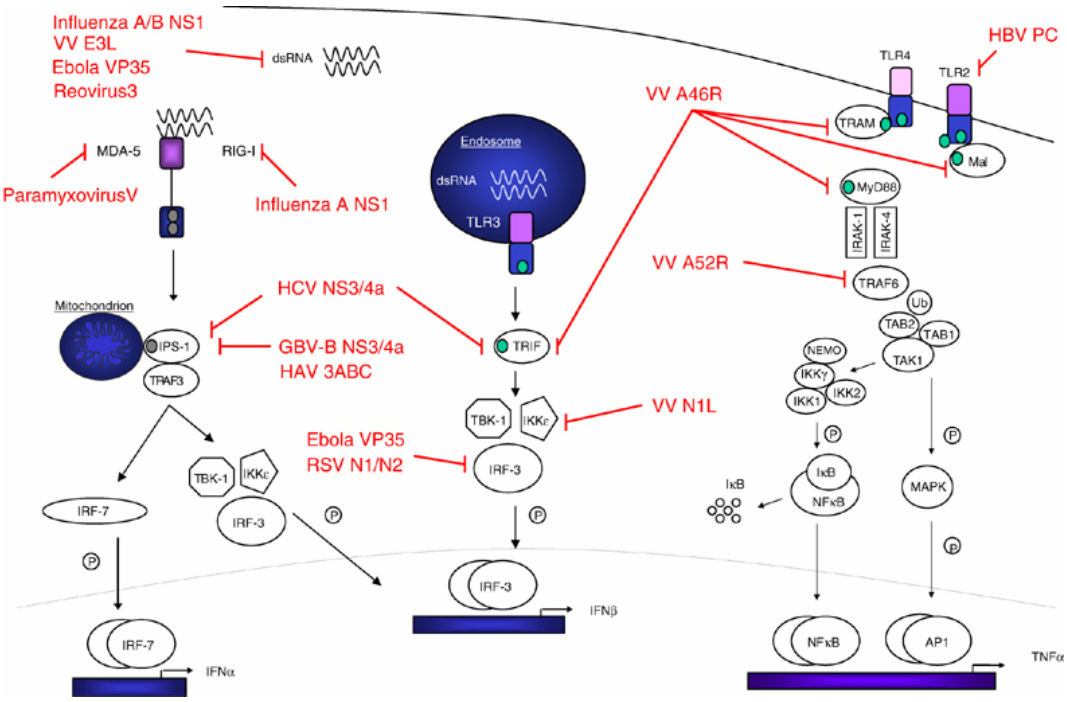


Figure 6. *PRR signaling pathways.*MyD88-dependent and independent signaling (A) (Adapted from Akira and Takeda, 2004) (B) Viruses repress PRR detection at numerous points to prevent IFN induction. (Adapted from Thompson, A., 2007)

Chapter II

Expression of Bovine Interferon-tau Variants According to Sex and Age of Conceptuses

Abstract

Interferon-tau (IFNT) plays a major role in maternal recognition of pregnancy in cattle. Its genes are transcribed in trophectoderm from the time the blastocyst forms until trophoblast attachment to the uterus. It remains unclear how many IFNT genes (*IFNT*s) are transcriptionally active, although at least 12 distinct cDNA have been characterized. Curiously, although the *IFNT* locus is autosomal, female blastocysts produce about twice as much antiviral activity as males. This sex difference is not evident among elongating day14 conceptuses, however. The questions we have addressed here are whether male and female blastocysts differ in the kind and number of *IFNT* they express and whether this pattern changes over development. Blastocysts were produced by standard *in vitro* procedures. Elongating conceptuses were recovered from cattle after slaughter. The blastocysts were bisected at day 8, and one half of each sexed by PCR. Demi-embryos (n=64) were grouped according to whether they were male or female. Each group was divided into two separate pools for RNA extraction and amplification to cDNA by RT-PCR. Individual cDNA were sequenced following shot-gun cloning to provide comparisons between male (M) and female (F) blastocysts, day 14-19 conceptuses, and a cell line (CT-1) derived from an outgrowth of a female blastocyst. Picked clones were sequenced (n=66 F, n=80 M, n=76 CT-1, and n=154 d14-19), and the frequency of expression of different *IFNT* variants determined. No differences were noted between

either the male and female blastocysts or between CT-1 cells and day 8 IVM/IVF blastocysts. The data indicate that female blastocysts do not transcribe a different set of *IFNT*s than males, since the same transcripts are present in similar proportions in both sexes (p=0.54). Moreover, the CT-1 line faithfully reflects the embryo stage at which it was derived (p = 0.24). However, significant differences ($p < 0.001$) were evident among conceptuses of different age, indicating that additional genes may be transcribed as IFNT production increases during development. It remains unclear whether the cDNAs found most abundantly represent IFNT proteins with most potent effects on the endometrium.

Introduction

Interferon-tau (IFNT) is a Type I IFN with a unique transcriptional control that limits its expression to ruminant trophoblasts prior to implantation (Ezashi et al., 2001; Ezashi and Roberts, 2004). The cytokine signals the presence of the conceptus to the dam by abrogating the pulsatile release of prostaglandin-$F_{2\alpha}$ ($PFG_{2\alpha}$) and, thereby, blocking luteolysis (Hansen et al., 1999). Interestingly, sequencing of IFNT cDNA has identified a minimum of 12 *IFNT* variants are expressed by bovine conceptuses despite the apparent singular role of the cytokine in pregnancy maintenance (Ealy et al., 2001). A popular theory is that gene duplication has created numerous *IFNT* variants and evolutionary selection among these variants is on-going to create the most potent signal for maternal recognition of pregnancy. However, as recent microarray data has revealed that IFNT regulates a large number of genes in the endometrium that are not related to prostaglandin synthesis, including ones for such vital functions as angiogenesis and matrix deposition, (Chen et al., 2006), it is possible that different variants are specialized

for distinctive roles in early pregnancy, although there is no solid evidence for such a hypothesis. A third potential reason for why there are so many variants is that they reflect the presence of many genes, which are required for the large scale production of IFNT at a critical time during early pregnancy. Finally, the presence of many cDNA may reflect considerable allelic variance of substantially fewer genes.

One potential role for *IFNT* variants is regulation of progeny sex ratio. Trivers and Willard first suggested altering the predominant sex of the offspring based on the maternal body condition would provide a selective advantage in many species (Trivers and Willard, 1973). This theory has been controversial largely because studies in wild populations attempting to link body condition and sex ratios of offspring do not consistently find a correlation between the two factors (Leimar, 1996; Hewison and Gaillard, 1999; Kruuk et al., 1999; West and Sheldon, 2002). Difficulty controlling experimental variables in wild populations may explain many of the inconsistencies (Clutton-Brock, 2007), although several studies support Trivers and Willard's theory (Burke and Birch, 1995; Landete-Castillejos et al., 2001; Garroway and Broders, 2007; Hellgren and Ruthven, 2007). For example, female red deer (*Cervus elaphus*) in good body condition give birth to a predominance of male offspring, but hinds in poor body condition tend to provide a lower sex ratio, often with a bias towards daughters (Flint et al., 1997). Even more convincingly, a comprehensive review of all relevant studies published prior to 2004 found a significant positive correlation between maternal condition and the predominate sex of the offspring in ungulates ($r = +0.09$) through meta-analysis (Sheldon, 2004). Therefore, some means of sexual selection by the dam in ungulates must be present, but the mechanism for this selection has not been identified.

Two observations on sexual dimorphism in bovine embryos suggested IFNT could play a role in sexual selection of progeny in ruminants. The first concerned glucose concentration in culture medium and its relationship to successful early embryo development. *In vitro* matured/*in vitro* fertilized (IVM/IVF)-derived male bovine conceptuses were found to have a significant advantage over females in making the transition to expanded blastocysts in medium enriched with glucose. However, males had no significant advantage in glucose-free medium (Larson et al., 2001). This result suggests males could have a developmental advantage over females *in vivo*, if the dams consume an energy rich diet and present higher circulating blood glucose levels than less well fed dams. The second observation related to sexual dimorphism in IFNT production. Cultured IVM/IVF bovine female blastocysts release and presumably produce almost twice as much antiviral activity as males in both a glucose-enriched and a glucose-free medium (Larson et al., 2001), although these differences narrow later in development (Kimura et al., 2004a). Analogous observations have been made for red deer conceptuses (Flint et al., 1997). These results indicate that females produce either more IFNT or more potent forms of IFNT, since the sole source of antiviral activity from the ruminant conceptus is IFNT (Hernandez-Ledezma et al., 1992). An earlier observation indicated that IVM/IVF blastocysts predominately express two *IFNT* gene products, bovine *IFNT-3a* and bovine *IFNT-1c* (Ealy et al., 2001). However, the embryo culture medium used at the time of that study contained high glucose and, therefore, the observation was probably, unintentionally, based on a predominately male population. On the other hand, at least twelve different *IFNT* were actively expressed from *in vivo* derived blastocysts (Ealy et al., 2001). Since *in vivo* derived blastocysts likely had a

balanced male to female distribution, there was speculation that females could be producing additional *IFNT* variants compared to males. Together, these studies suggested the intriguing possibility that female conceptuses signal their presence more powerfully than males by releasing either more IFNT or more potent variants of the cytokine than the male. On the other hand, this female advantage might be counteracted by the increased viability of male conceptuses as glucose concentrations rise, a hypothesis strongly advocated by Cameron as a general method of sex selection (Cameron et al., 2008). In summary, the interplay between male advantages in development and female's advantage in signaling through IFNT could potentially allow the dam some capacity for sexual selection of her offspring.

Another possible role for *IFNT* variants is preparation of the endometrium for placentation. Both *IGF-II* and *HIF-1α* mRNA, which have been implicated in stromal epithelial proliferation and angiogenesis, are down-regulated after IFNT administration (Chen et al., 2006). Regulation of genes such as these indicates IFNT has a critical role in the formation of the noninvasive synepitheliochorial placenta of ruminants. Differential temporal expression of *IFNT* variants would be expected if individual variants have specific roles in pregnancy. To assess the relative concentrations of *IFNT* transcripts in conceptuses of different developmental age, Ealy et al. (Ealy et al., 2001) utilized ribonuclease protection assays with ^{32}P- labeled riboprobes for bovine IFNT-1a, 2b, and 3d . Unfortunately, the relative changes in expression of several *IFNT* variants would have been missed by using these probes.

This chapter directly addresses whether or not male and female bovine conceptuses differ in the number and kinds of IFNT transcripts they express and whether

transcript profile changes during development. I have also examined the range of *IFNT* transcript variants found in CT-1 cells, the only accessible, continuously proliferating bovine cell line that has been derived from primary bovine trophectoderm (Talbot et al., 2000) as a comparison with whole conceptuses.

Materials and Methods

Experimental Design

Three experimental analyses were performed: 1. Relative variant expression between sexes: *IFNT* transcript variation was compared among female day 8 IVM/IVF blastocysts (n = 32), male day 8 IVM/IVF blastocysts (n = 32), and CT-1 cells. 2. Relative variant expression over time: *IFNT* transcript variation was compared among *in vivo* derived conceptuses on day 14, day 16, day 18, and day 19 of development. 3. Relative variant expression in CT-1 cells over prolonged passage: *IFNT* transcript variation was compared in two sets of CT-1 cells, one frozen in 2001 was compared to variant expression frequency of CT-1 cells cultured in 2005. The cells were continuously cultured for the majority of the four year period, and they were passaged more than 80 times between 2001 and 2005.

RT-PCR amplification of IFNT transcripts

As PCR with even the best proof-reading polymerase is known to incorporate sequence errors into IFNT amplicons approximately once every 1091 bases for a 30 cycle reaction (Ealy et al., 2001), it was necessary to perform multiple RT reactions and multiple PCR reactions for each RT product to avoid classifying PCR errors as *IFNT*

variants. While the reported error rate for the RT with Superscript III ($3.5 X10^{-5}$) (Potter, 2003) is lower than the PCR error rate reported by Ealy et al., sequencing errors could still be incorporated from the RT itself. An overview of the cDNA amplification protocol used for each analysis is described below:

Relative variant expression between sexes: Blastocysts were derived from slaughterhouse derived ovaries as described below. They were cultured in SOF medium until the morning of day 8 following their removal from insemination medium (Hernandez-Ledezma et al., 1992; Kubisch et al., 1998). Blastocysts were selected and split into halves by my colleague Koji Kimura. One half was sexed by PCR as described below. The other halves were stored individually at -80 C until RNA isolation. After sexing had been completed, the frozen demi-blastocysts were combined into two male and two female groups (n = 16/group). Two reverse transcription (RT) reactions were performed from the pooled RNA from each group, and two PCR reactions were performed for each RT reaction, providing four sets of amplicons for each group.

Relative variant expression over time: Two conceptuses were analyzed for each selected day of development. RNA was isolated from each blastocyst separately. Two RT reactions were performed per RNA sample and two PCRs were performed per RT reaction to provide four sets of amplicons per conceptus.

Relative variant expression from CT-1 cells after passaging: RNA was isolated separately from CT-1 cells I had cultured in 2001 and CT-1 cells I cultured in 2005 (> 80 passages from 2001 to 2005). Four RT reactions were performed for each CT-1 RNA sample and one PCR reaction was performed per RT reaction.

In vitro maturation and fertilization of oocytes and embryo culture

Oocyte collection, maturation, and fertilization were performed as previously described (Hernandez-Ledezma et al., 1992; Kubisch et al., 1998). Cumulus cells were removed from presumptive zygotes 18 h after fertilization by vortexing in HEPES-TL with 0.2% hyaluronidase (w/v) and pipetting vigorously. Zygotes were then cultured in 25 μl drops of D-glucose free SOF medium supplemented with amino acids and 0.5% (w/v) bovine serum albumin (Sigma, St. Louis, MO) overlaid with mineral oil (Sigma, St. Louis, MO) at 39 C in 5% CO_2, 5% O_2, and 90% N_2. On day 8, zygotes that had matured into expanded blastocysts were bisected. Expanded blastocysts were defined as conceptuses in which the blastocoelic cavity filled the zona pellucida and the zona pellucid had apparently thinned and increased in diameter. One half of the blastocyst was stored in RNA STAT-60 reagent (Sigma, St. Louis, MO) at -80 C for RNA isolation and the other half of the blastocyst underwent PCR sexing.

CT-1 Tissue Culture

Continuously cultured bovine trophectoderm cells (CT-1) were provided by Neil Talbot (USDA-ARS, Beltville, MD) (Talbot et al., 2000). STO cells (CRL-1503; American Type Tissue Culture, Rockville, MD) were used to pre-condition the medium in which the CT-1 cells were cultured. The CT-1 culture medium consisted of a 50:50 mixture of DMEM and Ham's F-12 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Harlan, Indianapolis, IN) (Shimada et al., 2001). All CT-1 cells were cultured on flasks pre-coated with Matrigel (BD Biosciences, San Jose, CA) and split at a 1:4 ratio

every two to three weeks. Immediately prior to RNA isolation or PCR sexing, CT-1 cells were dislodged by repeat pipetting and pelleted by centrifugation at 500 g.

Sexing of blastocysts and CT-1 cells by PCR

The sex of the CT-1 cells and blastocysts was determined through PCR amplification of a bovine Y-specific sequence (Peura et al., 1991). A bovine satellite present in both sexes was also amplified to demonstrate the presence of bovine genomic DNA in each sample. The demi-blastocysts and CT-1 cells first were placed in 20 mM Tris-HCl, 0.9% Nonidet P40, 0.9% Tween-20, and 0.4 mg/mL proteinase K for 30 min at 55 C for cell lysis (Canseco et al., 1994). Proteinase K was denatured after lysis by heating the samples to 98 C for 10 min. The reaction was then cooled to the annealing temperature of 62 C and the PCR reaction mix , which consisted of KlenTaq with PFU DNA polymerase (AB Peptides, Inc., St. Louis, MO; Stratagene, La Jolla, CA), Y-specific forward primer (5′-CTCAGCAAAGCACACCAGAC-3′), Y-specific reverse primer (5′-GAACTTTCAAGCTG AGGC-3′), bovine satellite forward primer (5′-TGGAAGCAAA GAACCCCGCT-3′), bovine satellite reverse primer (5′-TCGTAGAAACCGCACACTG-3′), and 100 μM dNTPs, were added to the samples. The 301 nucleotide bovine Y-specific sequence and 216 nucleotide bovine satellite were PCR amplified for 30 cycles (96 C 1 min, 62 C 1 min, 72 C 1 min). Bovine male genomic DNA (isolated from a liver biopsy), bovine female genomic DNA (isolated from an ovary), CT-1 medium, STO cells (that originated from murine spleen cells), and water were included as controls and treated in the same manner as blastocyst and CT-1 cell samples.

Amplified product was visualized by gel electrophoresis at 7 V cm^{-1} in 1.25% agarose gel containing 1 µg/ml ethidium bromide in 1 X TAE (Tris-Acetate-EDTA) electrophoresis buffer under ultraviolet light. Two individuals independently examined all gels and each sample was labeled as "male", "female", or "unclear banding pattern." The "unclear banding pattern" indicated a faint satellite band presumably resulting from insufficient genomic amplification for appropriate sex identification. Blastocysts that were identified as male or female by both examiners were used in the study. Blastocysts identified as opposite sexes by the two examiners or identified as having an "unclear banding pattern" by at least one examiner were removed from the study.

RNA isolation, RT-PCR for IFNT variants, and cloning cDNA

In vivo conceptuses from specific day points were provided by the MU Bovine Genome Project (http://genome.rnet.missouri.edu/Bovine) (McHughes, 2008). Total RNA from CT-1 cells, IVM/IVF blastocysts, and *in vivo* conceptuses was isolated with RNA STAT-60 reagent (Sigma, St. Louis, MO) according to the manufacturer's directions. RNase-free Turbo DNase (Ambion, Foster City, CA) was added to all RNA preparations for 30 min at 37 C to remove genomic DNA. The DNase was then inactivated by incubation with TURBO DNase inactivation reagent for three min at room temperature according to the manufacturer's instructions.

RNA from each group described in the experimental design section was reverse transcribed for 60 min at 50 C by using SuperScript III (Invitrogen, Carlsbad, CA), 250 µM of each dNTP, and an *IFNT* specific primer (5′-CTGAAATGAACACAGGTGAG-3′). The *IFNT* specific RT primer is complementary to a 19 nucleotide region within the

3′ UTR that is 41 base pairs (bp) downstream of the translational stop codon. *IFNT* variants were then PCR amplified with primers specific for a 5′ untranslated region and the transcriptional start site (5′-GGATCCCATCTTC CCCATGGCCTTC-3′) and a 3′ untranslated region (5′-GATTCCATCTTAGTCAGCGAGAGTC-3′), which is 4 bp downstream of the translational stop codon. The regions corresponding to the RT and PCR primers are conserved among all known *IFNT*. Thirty-two cycles of PCR (94 C for 45 sec, 62 C for 30 sec, 68 C for 2 min) were completed by using 250 μM of each dNTP, the PCR primers described above, and KOD HotStart polymerase (Novagen, Gibbstown, NJ). KOD HotStart (Novagen, Gibbstown, NJ) is a high fidelity polymerase and was used to reduce incorporation of nucleotide errors. Reactions identical to the above minus the addition of Superscript III during reverse transcription were performed for each sample to verify no amplification of genomic *IFNT* was occurring.

The amplified PCR products were purified with the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI). A single dATP overhang was incorporated into the PCR products through incubation with 5 U Taq (AB Peptides, St. Louis, MO) and 200 μM dATP for 30 min at 70 C. After a second PCR clean-up, the PCR products were ligated into pcrII TOPO vector (Invitrogen, Carlsbad, CA) and the resultant plasmids transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Cells were plated on Luria broth plates containing ampicillin (50 μg/ml), X-gal (40 μg/ml) and IPTG (1 mM) and incubated at 37 C overnight. Colonies were picked through blue/white selection and grown overnight in Luria broth containing ampicillin (50 μg/ml). Plasmid from the selected colonies was isolated by QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA)

and presence of insert verified by restriction digestion with XhoI and BamHI (Promega, Madison, WI). Insert was sequenced at the University of Missouri-Columbia DNA Core Facility (http://biotech.rnet.missouri.edu/dnacore/) with an M13 forward primer.

Sequence Analysis

All *IFNT* variant sequences were imported into BioEdit version 7.09 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned through ClustalW (Thompson et al., 1994) with previously identified *IFNT* recorded in Genbank (Table 1). All sequences were translated, and *IFNT* variant identity was determined through visual comparison of each translated variant sequenced in the study to the previously identified *IFNT* shown in Table 1. To account for PCR induced sequence errors, sequences were only included in the analyses if they filled one or more of the following requirements: 1, the sequence had previously been identified and entered into Genbank; 2, the sequence had been identified in the annotated bovine genome database; 3, the sequence was found in two separate RT-PCR reactions. It was assumed that the incorporation of errors would be a relatively rare, e.g. one base in ~1000 and incorporated randomly. It would be highly unlikely, therefore, that the same error would re-occur at the same base position.

Statistical Analysis

Sex ratios of IVM/IVF blastocysts were compared to an expected 50:50 ratio by a CHI^2 procedure in GraphPad Prism 4 (Larson, 2000). Blastocysts with an "unclear banding pattern" were removed from the statistical analysis of the sex ratio.

Expression frequency of the variants was calculated by counting the number of times each variant appeared within an RT-PCR reaction. The variant frequencies were small and distributed in a Poisson fashion, meaning the variance was proportional to the mean counts. In order to stabilize the variance, the expression frequency was normalized through the following equation: normalized expression (NE) $= \sqrt{(\text{Count} + 1)}$ (Snedecor, 1989). Data are represented as NE ± Standard Error Measurement (SEM) where each observational group (n) represents one RT reaction. Comparisons of expression frequency for each analysis were performed in Statistical Analysis System (SAS) version 9.1 through ANOVA and $p < 0.05$ was considered significant.

Phylogenetic Reconstruction

The coding sequences for all bovine *IFNT* variants in Genbank and novel IFNT identified in this study were aligned through ClustalW in BioEdit version 7.09. A consensus tree was constructed in MEGA version 4 (http://www.megasoftware.net/) through the Maximum Parsimony method (Erck, 1966) based on the 99 most parsimonious trees. Branches corresponding to partitions reproduced in less than 50% of the trees were collapsed. The percentage of parsimonious trees in which the associated taxa clustered together is shown next to the branches. The branch lengths were calculated by using the average pathway method and based on the number of amino acid changes over the whole sequence. A total of 172 amino acid positions were analyzed, out of which nine were parsimony informative. A phylogenetic reconstruction using the same method was also created based on nucleotide changes among *IFNT* variants. The phylogenetic tree based on the nucleotide changes and amino acid changes were

identical; therefore, only the phylogenetic tree based on amino acid changes has been reported here.

Results

Sexing of blastocysts and CT-1 cells

Of 90 IVM/IVF day 8 blastocysts sexed, 32 were female, 44 male, and 14 indeterminate, i.e. had an "unclear banding pattern." A representative ethidium bromide gel for the PCR sexing is shown in Figure 1A. The sex ratio between males and female blastocysts (n=76) was not significantly different when compared to a 50:50 ratio by a CHI^2 procedure ($p = 0.329$), thereby supporting the previous data illustrating males and females are able to transition to the expanded blastocyst stage in glucose free medium at about the same rate (Larson et al., 2001). The male and female demi-blastocysts that had been stored frozen were sorted into equal groups of 16 to determine *IFNT* transcript expression frequencies.

PCR sexing of CT-1 cells clearly proved that the cells are female in origin (Figure 1B). The authors had some concern that contamination from the STO cells could confound PCR sexing results from the CT-1 cells because the STO cells, which are murine spleen fibroblasts, were used to condition the CT-1 medium. Another rodent cell line, Buffalo rat liver cells, was previously shown to exhibit a faint, non-specific band between the Y and satellite bands (Larson, 2000). Therefore, STO cells were also included among the standard controls for the PCR sexing, which included bovine male genomic DNA, bovine female genomic DNA, culture medium, and water (Larson, 2000).

No bands were present from STO samples; consequently, STO cells could not be a potential error source.

Relative variant expression between sexes

Four replicate groups were used to calculate the relative variant expression frequency of *IFNT* between sexes. A total of 176 full length sequences were analyzed from males and females, but 30 of the176 were discarded because they did not meet our selection criteria to account for PCR induced sequencing error. For the statistical analysis, 80 full length sequences from males and 66 full length sequences from females were compared.

Four variants (*IFNT-1c*, *1d*, *2b*, and *3a*) were identified in day 8 blastocysts. The majority of the transcripts, whether male or female, were *IFNT-1c* and *IFNT-3a* (Figure 2). The normalized expression for these two variants on a scale in which "1" equals no expression, was 4.80 and 4.75 for *IFNT-1c*, and 4.06 and 3.54 for *IFNT-3a* for females and males respectively. In contrast, the mean expression of the next most highly expressed variant (*IFNT-2b*) was 1.41 in both sexes. The predominance of *IFNT-1c* and *IFNT-3a* in this analysis is consistent with previous studies on polymorphic bovine *IFNT* expression (Ealy et al., 2001). The relative degree of variant expression between day 8 IVM/IVF male and female blastocysts was not significantly different ($p = 0.543$). In other words the same *IFNT* variants were expressed in males as in females, thereby ruling out the possibility that females were selectively expressing forms with higher potential anti-viral activity.

The overall expression frequency of *IFNT* variants by CT-1 cells, which were derived from a day 11 conceptus, was not significantly different than day 8 IVF/IVM blastocysts ($p = 0.240$). The CT-1 cells appear to be expressing the same molecular markers as the early stage blastocyst from which they were derived. Two previously unidentified variants were also expressed by CT-1 cells. These two variants, termed *IFNT-3h* and *IFNT-3f*, only differ by one non-synonymous nucleotide change from previously identified *IFNT* variants. *IFNT-3h* is identical to *IFNT-1b* except for a single adenine (A) to guanine (G) change resulting in a glycine to aspartic acid conversion at codon 126. *IFNT-3f* has one nucleotide change (A to G) from *IFNT-3e*, converting a methionine to a valine (codon 146) at the protein level. *IFNT-3h* was also expressed by day 14 and day 16 *in vivo* derived conceptuses in the temporal expression analysis described below. Interestingly, *IFNT-3f* was the only variant identified that matched an *IFNT* from the bovine genome database assembly 2.1, which was based on a single Hereford bull. Surprisingly, *IFNT-3f* never re-occurred among the 453 sequences analyzed in these experiments.

Temporal expression of IFNT variants from in vivo derived conceptuses

A total of 185 sequences from four day points were compared in the analysis of the temporal expression pattern of *IFNT* variants from *in vivo* derived conceptuses. A total of 154 different variants were considered real, and a total of 31 discarded based on the fact that they failed to meet the selection criteria. A significant difference in overall relative *IFNT* variant expression across day points was found ($p < 0.001$) (Figure 3). *IFNT-1c* and *IFNT-3a* were the most frequently expressed variants overall, but their

relative expression decreased over time. Specifically, the normalized expression decreased from 5 to 2.34 for *IFNT-c* and from 4.14 to 2.24 for *IFNT-3a* from day 14 to day 19. The drop in relative expression of *IFNT-1c* and *3a* was compensated for by increases in the expression frequencies of *IFNT-1a*, members of the class 2 family, and five previously unidentified *IFNT* variants. Interestingly, *IFNT-1a* (normalized relative expression 3.0) and *2b* (normalized relative expression 3.6) became the commonest transcripts, surpassing *IFNT-1c* and *3a* by day 19.

As briefly alluded to earlier, five previously unidentified sequence variants were identified from the *in vivo* derived blastocysts. These variants each contained a single non-synonymous nucleotide change that differentiated them from previously known variants deposited in Genbank. A more in depth analysis and classification of these variants is presented below.

Expression of IFNT variants from CT-1 cells analyzed four years apart

The original CT-1 cell isolate of Talbot was monitored for expression of *IFNT* by RT-PCR and Northern blotting over two years of culture and 76 passages in order to verify that the trophoblast phenotype was not being lost over time (Talbot et al., 2000). However, the change in temporal expression pattern of *IFNT* variants noted in *in vivo* derived blastocysts raised new concerns that relative *IFNT* variant expression might be subtly changing as CT-1 cells were passaged. Fortunately, CT-1 RNA had been isolated from CT-1 cells cultured in 2001 and could be compared with contemporary material from 2005 cells which had been in nearly continuous culture for four years and over 80 passages. For this analysis, we compared a total of 41 sequences from 2001 cells and 35

from 2005 cells (after discarding 16 sequences that did not meet our criteria). A total of six transcripts (*IFNT-1a*, *1c*, *3a*, *3e*, *3f*, and *3h*) were identified from CT-1 cells. The major transcripts were *IFNT-1c* and 3a with a normalized expression of 4.49 (*1c*) and 4.69 (*3a*) for 2001 and 4.0 of both for 2005. The next most frequently identified variant is *IFNT-3e* was only identified in CT-1 from 2005 with a normalized expression of 2.24 but was not identified from CT-1 cultured in 2001. No significant difference in overall *IFNT* variant diversity was noted (p=0.124) (Figure 4). Thus the CT cells are maintaining molecular markers of early trophectoderm despite four years of passage.

Novel IFNT variants

Several novel *IFNT* variants were identified during the course of this study. All of the variants, except *IFNT-3f*, were sequenced from separate conceptus groups. *IFNT-3f*, as previously discussed, was previously identified in the bovine genome assembly 2.1. Interestingly, the same variant did not appear in the most current bovine genome assembly (3.1).

All the novel *IFNT* transcripts identified during this study bear strikingly close nucleotide sequence identity to previously identified *IFNT* (Figure 5). Single non-synonymous nucleotide changes are responsible for each of the new "variants" identified. Only nine codons are not conserved in all variants that were sequenced in this study. These nine codons are denoted with a blue box in Figure 5 and are termed variable sites. At each variable site, only two different amino acids were identified. The codon positions of the variable sites with their respective amino acids are as follows: 5 (D/N), 46 (N/S), 65 (L/F), 69 (Y/H), 70 (T/I), 102 (P/Q), 105 (G/E), 126 (G/D), and 146 (V/M).

Bovine *IFNT* variants were previously separated into three classes based on their inferred evolutionary history through a Maximum Parsimony phylogenetic reconstruction (Figure 6) (Ealy et al., 2001). A new Maximum Parsimony phylogenetic tree was constructed here based on the amino acid sequence of the previously identified IFNT and the new IFNT found in this study in order to improve our understanding of this subfamily. Unfortunately, the bootstrapping for most branches scored below 10 because the novel variants have a very high amino acid identity with previously defined variants . Bootstrapping scores below 20 have virtually no statistical significance in understanding the phylogenetic relationship of proteins (Sanderson and Wojciechowski, 2000). Consequently, we chose to use a slightly different procedure for phylogenetic reconstruction than in the 2001 paper. The Maximum Parsimony method was still utilized to create initial trees, but a consensus tree was then created from the 99 most parsimonious trees. This approach collapsed some branches that are present in the 2001 tree when they appeared in less than 50 % of the 99 phylogenetic reconstructions (Figure 7). Based on this consensus tree, sub-group 1 can be split into two groups, one containing *IFNT-1a* and two of the novel variants, *1f* and *1g*, and the second comprised of *IFNT-1c* and *1d*. *IFNT-1b* is an intermediate protein and could not be placed with confidence in any grouping. Class 2 branched at the same place in all 99 of the parsimonious trees analyzed, supporting the continued placement of the three known *IFNT2* variant*s* into their own class. Class 3 has several new members, *IFNT-3f*, *3g*, *3h*, and *3i*, based on the new phylogenetic tree.

Discussion

The main goals of this study were first to conduct an extensive sequence coverage of *IFNT* transcripts from bovine embryos of different developmental age to define as completely as possible the full range of sequence variation of *IFNT* in the *Bos taurus* genome. It was also hoped that this exercise would provide insight into how many transcriptionally active *IFNT* are present in the genome, as this issue remains controversial, with the most recent assembly suggesting that there are only three. A second goal was to determine whether male and female blastocysts could be distinguished according to the complement of transcripts they expressed. In particular, we sought an explanation for why female blastocysts produce almost twice as much IFNT as their male counterparts at the blastocyst stage of development when sexual dimorphism in IFN production has been confirmed to exist (Kimura et al., 2004a). A third goal was to determine whether the pattern of *IFNT* expression changes over development age. For example, are their "early" and "late" genes that might reflect the need for certain variants at particular stages of trophoblast development?

Previous cDNA sequencing efforts identified at least twelve *IFNT* variants expressed in bovine conceptuses over a range of developmental ages, raising the possibility that there were either multiple genes or considerable allelic variation. Of these variants, *IFNT-1c* and *3a* predominated (Ealy et al., 2001), an outcome that suggested that all genes and their allelic variants were not transcribed at similar rates. A similar heterogeneous pattern of *IFNT* variant expression was observed in the current study, in which fourteen *IFNT* variants were confirmed, six of them new. Again the predominately expressed variants were *IFNT-1c* and *3a*. Four variants [*IFNT-2c*

(AF196323), *3b* (AF196325), *3c* (AF196326), and *3d* (AF196327)] previously reported by Ealy et al. (Ealy et al., 2001) in a study that also controlled for the possibility of PCR error were not found in the present study, suggesting 18 protein variants actually exist in cattle. As pointed out above, it seemed likely that some, possibly the majority of these variants are allelic and possibly breed specific. Based on genomic analysis obtained through *in situ* hybridization and restriction fragment length hybridization others have inferred six *IFNT* to be present in a mixed breed cow (Iannuzzi et al., 1993; Ryan et al., 1993). However, the recent annotation of Type I IFN locus in the bovine genome suggested that only three genes are present in the genome of the Herford bull analyzed. One possibility is that the genes are so similar in sequence that some have been "lost" during the assembly process (particularly as genes have come and gone between assemblies). A careful examination of the original traces obtained in the 6-fold coverage of the bovine genome, revealed that 45 *IFNT* sequences were read, raising the possibility that the true number of IFNT is higher than three. Regardless, at least six different *IFNT* are expressed by a single Angus conceptus and CT-1 cells, a cell line derived from a single female blastocyst of unknown breed (Figure 3). Unfortunately, the phylogenetic analysis (Figures 5 and 6) provided little further insight into the number of genes present. We had hoped, despite the close similarity of the variant forms, that the main branches of the tree might represent genes and that the variants within each branch might represent alleles. On such a basis, up to six genes might be present, although only three groups (1, 2, and 3) can be defined with confidence. Oddly, one of them, group 2, is not represented at all in the current genome assembly and was not among the sequences represented in the 45 original traces. Indeed group 2 variants, although a well defined group based on

their sequences, are expressed by some conceptuses and not by others. Although it is possible that the group 2 gene(s) remain silent in some conceptuses, this seems unlikely in view of the absence of these genes in the genome assembly. An alternative hypothesis is that group 2 is actually absent in some animals but present in others. Such variability in gene complement has been reported for other large gene families, including the odorant genes (Nozawa et al., 2007).

The distribution of *IFNT* variant transcripts among male and female blastocysts did not explain the sexual dimorphism in IFNT production. Females neither expressed more kinds of transcripts than males nor a different group of transcripts. Males and females predominantly expressed *IFNT-1c* and *3a*, while the other variants, although not ubiquitous, showed no bias towards one sex or the other. Certainly the results do not explain why female blastocysts produce almost twice as much antiviral activity as males. Increased antiviral activity in the female culture medium must result from increased overall *IFNT* expression and not from the presence of more potent *IFNT* variants being expressed by the female. Kimura et al. (Kimura et al., 2004b) proposed that the sexually dimorphic difference in IFNT production was likely due to the higher redox state of the female trophoblasts resulting from the presence of two transcriptionally active X-chromosomes and, specifically, two active copies of the gene encoding glucose 6-phosphate dehydrogenase (G6PDH). The resulting accelerated metabolism of carbohydrate through the oxidative arm of the pentose phosphate pathway would be expected to raise NADPH concentrations and the redox state of the cells, possibly favoring the activities of crucial transcription factors. On the other hand, unusually high glucose concentrations in the medium might be expected to exacerbate this metabolic

state and lead to increased embryonic death of females through generation of free radicals and specifically reactive oxygen species.

The most notable feature of *IFNT* variant expression in relation to conceptus age was that the relative quantities of *IFNT-1c* and *3a* transcripts declined over time, while the total number of less common variants increased their representation. It is unclear how to interpret these observations; although it is conceivable that the different isoforms possess somewhat different specificities and that the more unusual variants have been selected for specialized roles in modulating endometrial gene expression that cannot be matched by the two commoner isoforms. Among human IFNA, it is well established that the various variants can display different activities, e.g. as antiviral and anti-proliferative agents, despite operating through the same Type I IFN receptor (Yamaoka et al., 1999). Similarly ovine *IFNT* variants are known to vary in antiviral activities (Winkelman et al., 1999), although it is difficult to rule out the possibility that some of the disparity is due to refolding efficiency of the bacterial recombinant products. More relevant to this work is the observation that individual bovine IFNT variants differentially regulate prostaglandin synthesis in endometrial cells (Parent et al., 2003). While bovine IFN-2b inhibited both prostaglandin (PG) $F_{2\alpha}$ and PGE_2 production in primary uterine epithelial cells, bovine IFNT-3b had an opposite effect; it induced more prostaglandin formation. Interestingly, bovine IFNT-1a inhibited prostaglandin synthesis at low concentrations (< 1 μg/ml), but promoted synthesis at high concentrations (> 1 μg/ml). The downstream actions of IFNT can clearly be complex, possibly through the distinctive abilities of variant forms to trigger the assorted signaling pathways that ultimately guide endometrial responses.

Changes in relative *IFNT* variant expression based on the age of the conceptus has significant implications for any model system used in the study of IFNT. The most commonly employed bovine trophectoderm model system for our and other laboratories is a bovine line of continuously cultured trophectoderm (CT-1) cells originally derived by Neil Talbot (Talbot et al., 2000; Michael et al., 2006; Ezashi et al., 2008). It is conceivable that CT-1 cells could slowly been changing their relative expression of specific *IFNT* variants as they are passaged since temporal variant expression changes are occurring as trophectoderm cells mature. This subtle change was not observed in our work though. In fact, the variant profile most closely resembles the early stage blastocyst (day 11) from which they were derived.

The mechanism that leads to differential expression of individual *IFNT* variants is difficult to explain. As far as can be discerned, the main control elements for the IFNT lie in the proximal 5/ UTR, and are concentrated within a complex enhancer element centered around the binding sites for three transcription factors (DLX3, ETS2, and AP1) (Das et al., 2008). The promoters of four bovine *IFNT* variants, *IFNT-1a* (M60903), *1c* (AF339094), *2b* (AF339095), and *3b* (AF339096), have been fully sequenced for approximately 430 bp upstream of their transcriptional start site. All four retain the complete DLX3/ETS2/AP1 enhancer and are > 99% conserved over the entire 430 bp sequence (Ealy et al., 2001). *IFNT* promoters annotated from the bovine genome database assembly 3.1 have also been compared (and discussed in detail in the following chapter) and are 98.3% to 99.9% identical to one another for at least 900 bp upstream of the transcriptional start-site. Such conservation is in itself remarkable and attests to the recent origins of the duplications that produced the variant genes, but provides little

insight into how differential expression could be achieved. One possible explanation is that an additional level of transcriptional control, particularly temporal control, operates from beyond the 900 bp cut-off, much as it does, for the β-globin genes during human and mouse embryonic development (Li et al., 2002). In this and other examples, expression from groups of linked genes is controlled, not just by elements proximal to the genes, but by *cis*-acting sequences usually named locus control regions (LCR) far upstream, that provide longer range interactions, In the case of the *IFNT,* a *cis*-oriented LINE tandem with a SINE is invariably present 660 bp upstream of the transcriptional start site, and could conceivably provide some measure of additional control, as other genes are known to be regulated by *cis*-oriented LINEs (Britten, 1996; Shapiro, 2005). As most LCRs have been discovered through experiments with transgenic mice, when normal, "physiological" expression was only achieved by including far-upstream elements in the transgene construct, it may be extremely difficult to conduct a similar analysis in cattle. Consequently, the controls operating on IFNT are likely to remain mysterious for some time to come.

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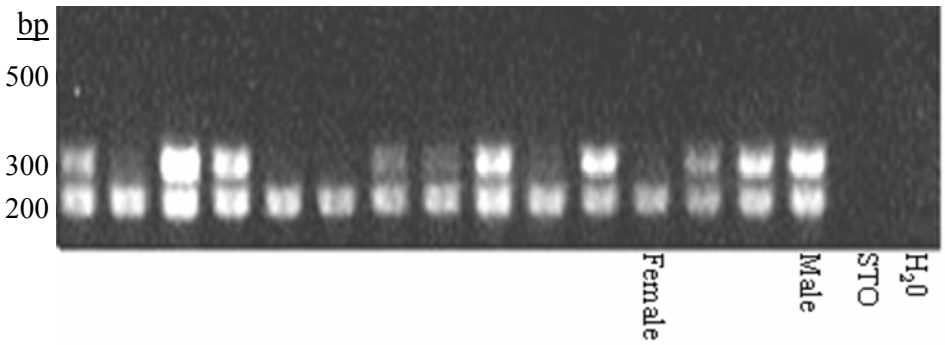
Gene	Accession No.	Gene	Accession No.
IFNT-1a	M31557	IFNT-3a	AF196324
IFNT-1b	M60913	IFNT-3b	AF196325
IFNT-1c	AF196320	IFNT-3c	AF196326
IFNT-1d	M60908	IFNT-3d	AF196327
IFNT-2a	AF196321	IFNT-3e	AF270471
IFNT-2b	AF196322		
IFNT-2c	AF196323		

Table 1. *IFNT variants previously annotated in Genbank.* Transcripts that matched the previously identified IFNT, shown in the above table with their Genbank numbers, were included in this study. Transcripts that matched IFNT identified in the bovine genome database or that were identified in two separate RT-PCR reactions were also included.

A.



B.

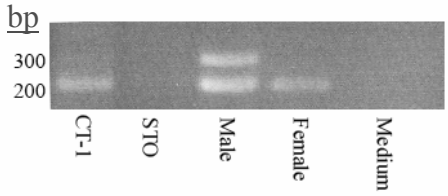


Figure 1. *PCR sexing of blastocysts and CT-1 cells.* These are representative ethidium bromide stained agarose gels for PCR sexing of the IVM/IVF day 8 blastocysts (A) and CT-1 cells (B). A 301 base pair bovine Y-specific sequence and a 216 base pair bovine satellite were PCR amplified after sample lysis. The latter served as an internal control for the presence of bovine genomic DNA.

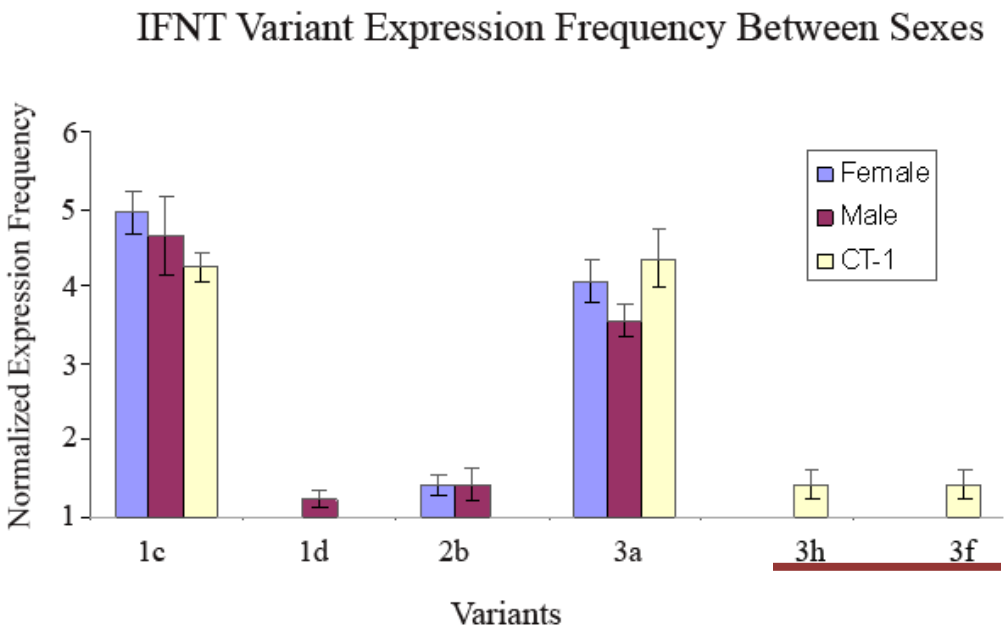


Figure 2. The normalized expression frequency was plotted for each *IFNT* variant sequenced. No significant difference in *IFNT* variant expression frequency was present between male and females blastocysts ($p = 0.543$). CT-1 cells, which were derived from a day 11 female blastocyst, did not have a significantly different *IFNT* expression profile than IVM/IVF day 8 blastocysts ($p = 0.240$). Two novel variants, *3f* and *3h*, were sequenced from CT-1 cells and are indicated by a red line below the X-axis on the right side of the graph.

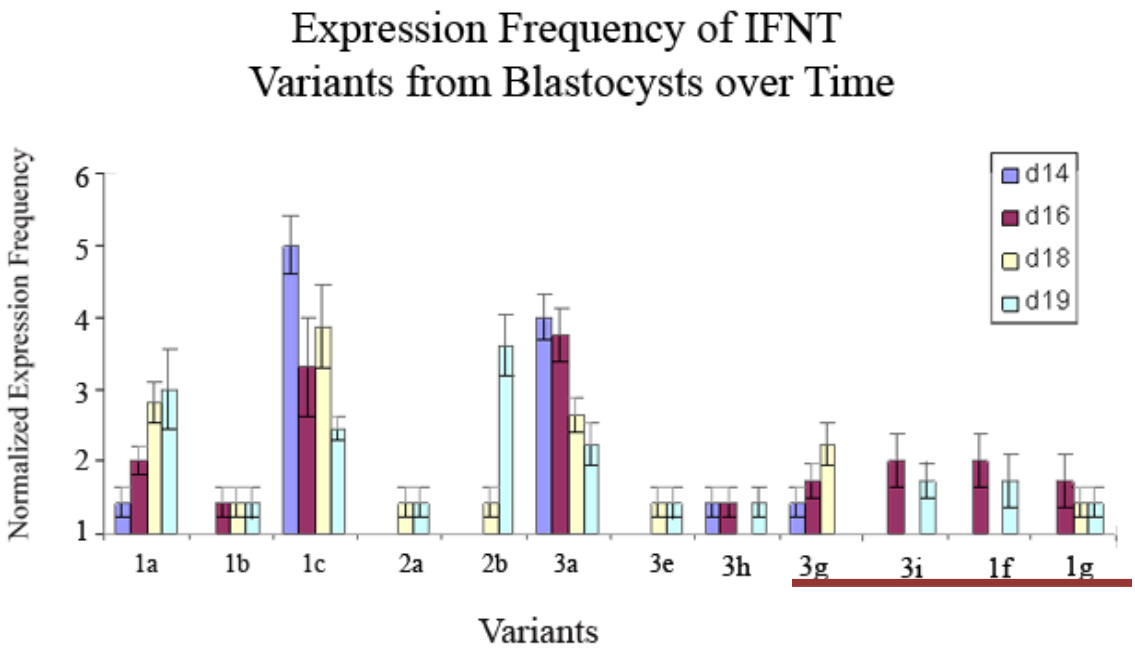


Figure 3. Normalized expression frequency of *IFNT* variants from *in vivo* derived day 14, 16, 18, and 19 blastocysts was calculated. The overall *IFNT* variant expression frequency profile was significantly different ($p < 0.001$) among day points. *IFNT-1c* and *IFNT-3a* decreased in expression frequency from day 14 to 19. On the other hand, *IFNT-1a*, members of the class 2 family, and several novel variants increased in expression frequency from day 14 to day 19. The novel variants are indicated by a red line below the X-axis.

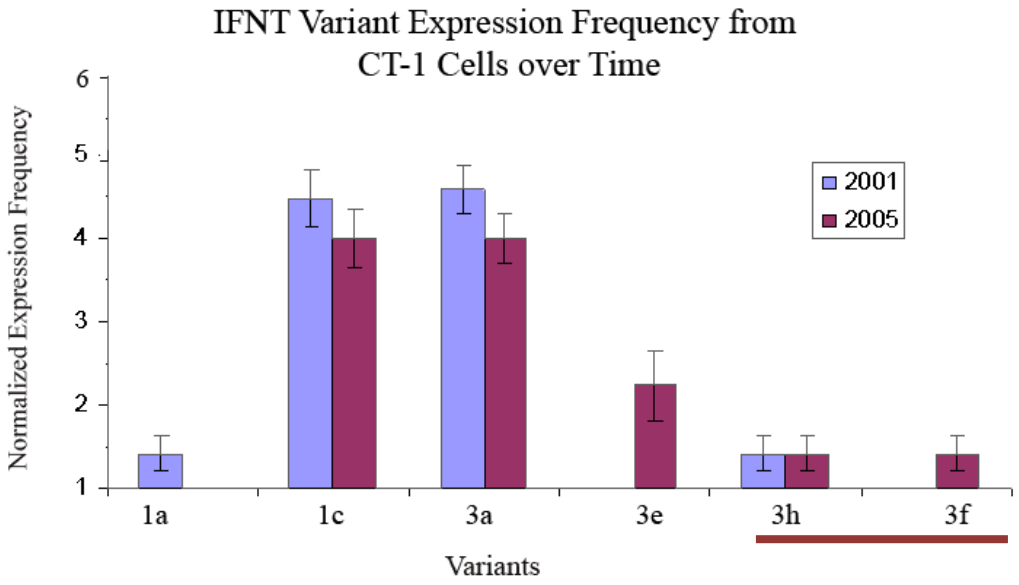
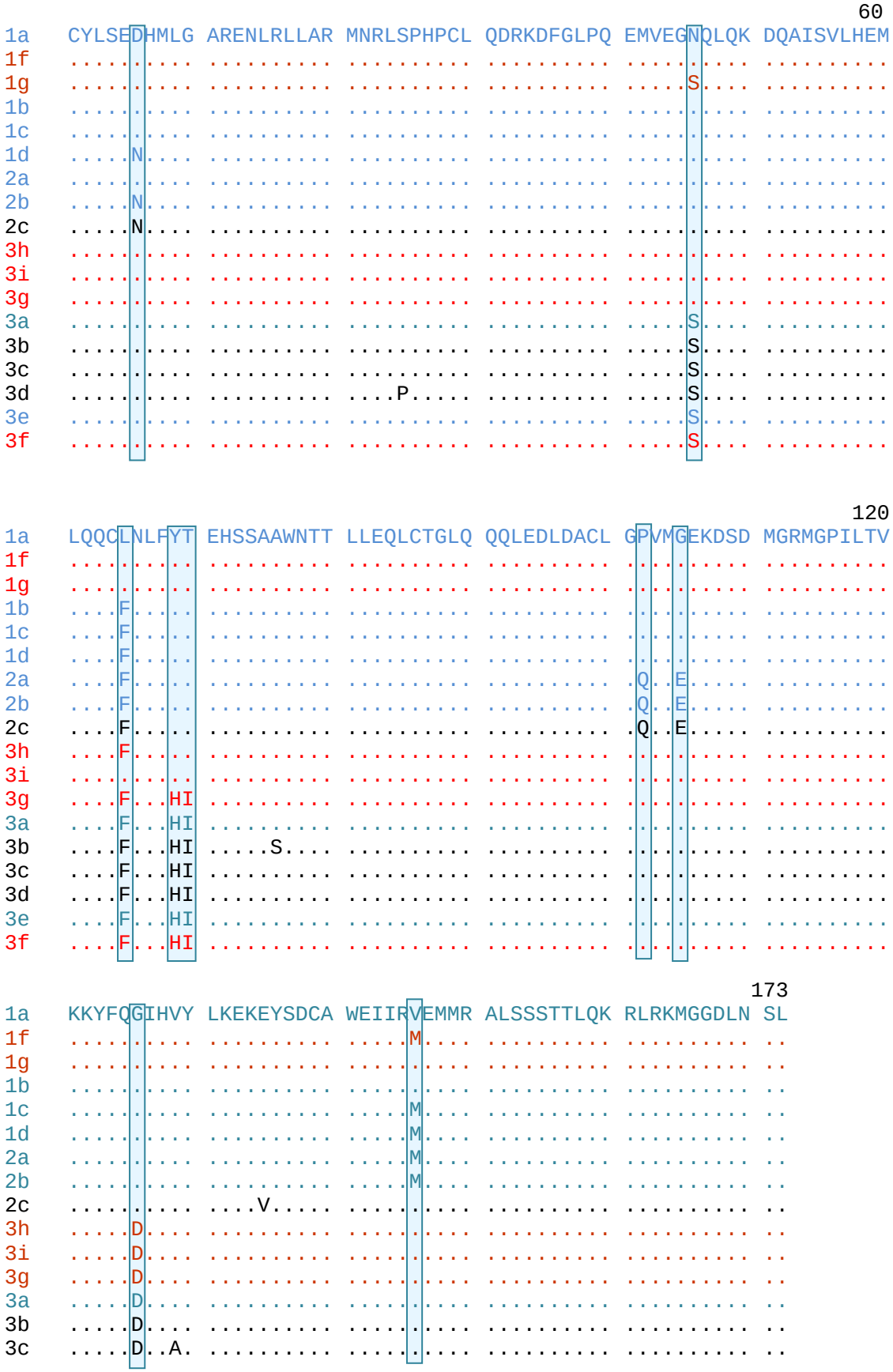


Figure 4. Normalized expression frequency of *IFNT* variant from cells cultured in 2001 was compared to cells cultured in 2005. No significant difference was found in *IFNT* variant expression profile by CT-1 cells after four years of culturing(> 80 passages) ($p = 0.124$).


60
1a CYLSEDHMLG ARENLRLLAR MNRLSPHPCL QDRKDFGLPQ EMVEGNQLQK DQAISVLHEM
1f
1g S
1b
1c
1d N
2a
2b N
2c N
3h
3i
3g
3a S
3b S
3c S
3d P S
3e S
3f S
120
1a LQQCLNLFYT EHSSAAWNTT LLEQLCTGLQ QQLEDLDACL GPVMGEKDSD MGRMGPILTV
1f
1g
1b F
1c F
1d F
2a F Q E
2b F Q E
2c F Q E
3h F
3i
3g F HI
3a F HI
3b F HI S
3c F HI
3d F HI
3e F HI
3f F HI
173
1a KKYFQGIHVY LKEKEYSDCA WEIIRVEMMR ALSSSTTLQK RLRKMGGDLN SL
1f M ..
1g ..
1b ..
1c M ..
1d M ..
2a M ..
2b M ..
2c V ..
3h D ..
3i D ..
3g D ..
3a D ..
3b D ..
3c D A ..

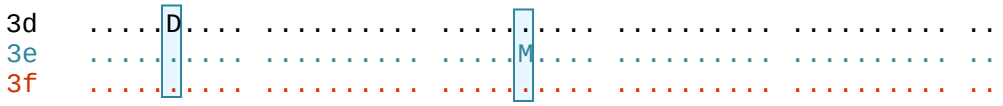


Figure 5. *Amino acid alignments of all known IFNT variants*. Amino acid sequence minus the signal peptide for all translated *IFNT* previously recorded in Genbank and all novel *IFNT* identified in this study were aligned through ClustalW. IFNT-1a is the reference sequence in this alignment and dots represent amino acids conserved with IFNT-1a. The sequences are listed in agreement with their class organization based on Figure 7. Variants identified for the first time in this study are shown in red. Variants previously submitted to Genbank and also observed in this study are shown in blue. Variants previously submitted to Genbank, but not observed in this study are shown in black. Blue boxes are placed around sites where amino acid substitutions have occurred. These sites are also the parsimony informative sites that form the basis of the phylogenetic tree in Figure 7.

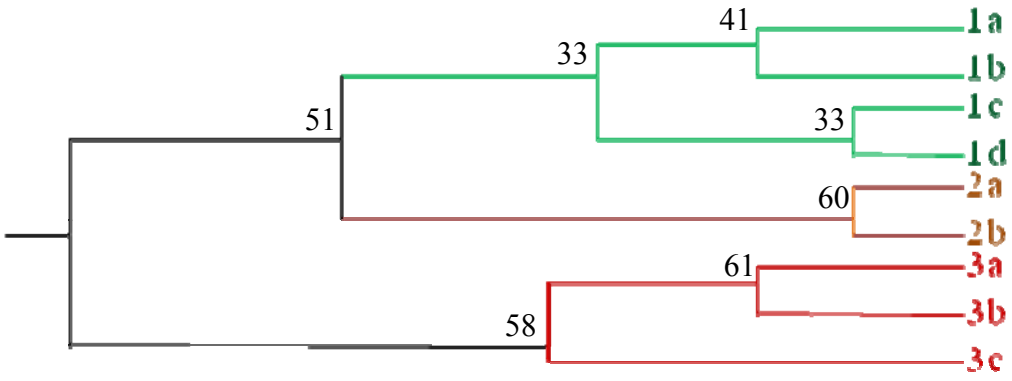


Figure 6. *Phylogenetic reconstruction of IFNT variants based on Ealy et al. (2001).* IFNT variants were previously named based on their evolutionary relationship inferred through a Maximum Parsimony tree reconstructed here. Three classes, class 1 illustrated in green, class 2 illustrated in brown, and class 3 illustrated in red, were evident through this evolutionary analysis. Each class separated into a unique clade when analyzed at the amino acid or nucleotide level despite the bootstrapping scores consistently being below 80.

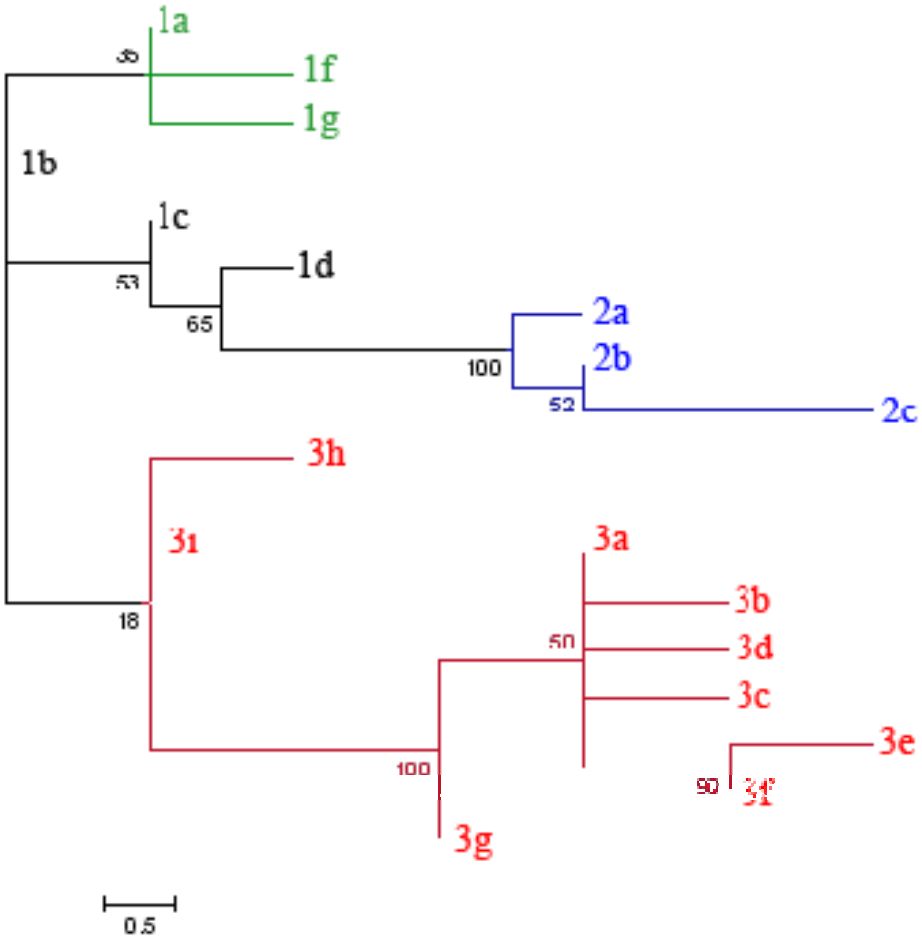


Figure 7. *Phylogenetic reconstruction of all IFNT variants.* The phylogenetic tree of *IFNT* was altered by inclusion of the additional variants. The high identity between the variants resulted in bootstrapping scores that were consistently below 10 when analyzed through the same Maximum Parsimony method utilized in *Ealy et al 2001*. Therefore, a more statistically robust method was employed in this study. A consensus tree based on the 99 most parsimonious trees was created and branches corresponding to partitions reproduced in less than 50% of the trees were collapsed. The percentage of parsimonious trees in which the associated taxa clustered together is shown next to the branches. Class 3, shown in red, has expanded and class 1 has become broken into two subclasses, illustrated in black and green, based on this phylogenetic reconstruction.

Chapter III

Characterization of the Bovine Type I IFN Locus: Rearrangements, Expansions, and Novel Subfamilies

Abstract

The Type I interferons (IFN) have major roles in the innate immune response to viruses, a function that is believed to have led to rapid expansion in the number and complexity of their genes. The locus encompassing these genes has remained intact during its evolutionary history. A highly conserved synteny is observed in mouse and human in which it has so far been examined. Specifically, *IFNB* and *IFNE* define the limits of the locus, with all other Type I IFN genes except *IFNK* distributed between the boundaries imposed by these two most ancient genes, strongly suggesting that the locus has broadened as IFN genes duplicated and then evolved into a series of distinct families. Here we provide the first comprehensive annotation of the Type I IFN locus in Bos taurus, thereby providing an insight into the functional evolution of the type IFN in ruminants. The locus has undergone significant rearrangement and expansion in bovine compared to mouse and human, with the constituent genes separated into two sub-loci separated by a > 700 kb region. The *IFNW* subfamily is greatly expanded compared to other species, comprising 24 potentially functional genes and at least 8 pseudogenes. Selective pressure analysis reveals the regulatory regions of the *IFNW* are diverging faster than the coding regions. *IFNA* and *IFNB* are also present in multiple copies with 13 *IFNA* and 6 *IFNB* in the current genome annotation. Interestingly, only three *IFNT*,

which encode a ruminant-specific IFN secreted by the pre-implantation conceptus, are present in the current assembly despite previous work predicting that a greater number of copies is likely to be found. The differentiation of *IFNT* from *IFNW* can be traced in the regulatory regions of an *IFNW/T* gene and two pseudogenes with properties intermediate between *IFNT* and *IFNW*. The identification of a new Type I IFN subfamily of four members, one of which is a pseudogene, apparently derived from the *IFNA* lineage at least 83 million years ago is the most striking finding of this study. This subfamily appears to exist in the horse but not in the other species whose genomes have been so far examined. At least one member of this previously unidentified gene subfamily is expressed in virally challenged bovine kidney cells, suggesting that the genes are virally inducible.

Introduction

Viruses are constantly evolving to find more effective means to survive and multiply in their host species (Goodbourn et al., 2000; Iannello et al., 2006; Randall and Goodbourn, 2008). The immune defense system, in turn, exists in a perpetual state of co-evolution with the pathogens to limit infectious disease, a circumstance often likened to an "arms race." The primary defense mechanism against viruses in vertebrates is Type I IFN (interferon) of the innate immune system (Stark et al., 1998). It can reasonably be argued that complex organisms like mammals can only survive as long as immune defenses can adjust to the strategies of invading pathogens. Accordingly, a rapidly evolving, adaptable IFN system is essential to mammals if they are to endure viral infections. Type I IFN are also pleiotropic cytokines, with significant roles in modulating

adaptive immunity, cell proliferation and cell death, and numerous other processes vital to mammalian health and survival (Goodbourn et al., 2000). Most likely as a response to these challenges Type I IFN demonstrate a complex evolutionary history that has resulted in the divergence of eight distinct subfamilies: IFN-kappa (IFNK), IFN-beta (IFNB), IFN-epsilon (IFNE), IFN-delta (IFND), IFN-zeta (IFNZ), IFN-alpha (IFNA), IFN-omega (IFNW), and IFN-tau (IFNT) (Krause and Pestka, 2005).

Mammalian Type I IFN probably emerged during tetrapod evolution from an older cytokine family, Type III IFN, which provides the primary viral defense mechanism in fish (Lutfalla et al., 2003; Levraud et al., 2007). It is difficult to determine exactly when Type I and Type III IFN diverged because no Type I IFN has been identified in amphibians, but the split definitely occurred prior to the divergence of birds and mammals approximately 310 million years ago (MYA) (Hedges, 2002; Krause and Pestka, 2005). Type III IFN, known more commonly in mammals as either IFN-lambda (IFNL)1-3 or interleukin (IL)28 and IL29, is encoded by a five exon gene, opposed to the single exon Type I IFN, and acts through a different receptor complex than Type I IFN. Despite these differences, both Type I and Type III IFN have similar mechanisms of induction, activate the same signaling pathways, and trigger the same biological actions in the target cell. Type III IFN has been retained in some mammalian species including humans and mice but has been lost in others (Kotenko et al., 2003). Even when present, it appears to have assumed a less dominant role as an anti-viral agent and even been supplanted with the emergence of the many contemporary Type I IFN.

All Type I IFN have antiviral properties, but some may play a more dominant role as first responders than others. IFNA and IFNB were the first Type I IFN to be

characterized in human and have been assumed to constitute and the primary viral defense mechanism (Rubinstein et al., 1979; Stein et al., 1980). IFNA is released by almost all cell types and a few of its family members, specifically human IFNA2a and IFN2b, are currently approved for treatment of a range of viral diseases including hepatitis B and C, condylomata acuminate (genital warts), and AIDS-related Kaposi sarcoma (Pestka, 2007). IFNB is the main IFN secreted by fibroblasts in response to a viral challenge, but is clearly produced by multiple cell types (Pestka et al., 2004). It acts in the immediate antiviral response and helps regulate the later expression of several *IFNA* (Barnes et al., 2002). IFNW and IFNZ both appear to have developed specific niches in antiviral protection for certain species. IFNW has been implicated in protection against specific viruses, such as parvovirus, particularly in cats (de Mari et al., 2003; Paltrinieri et al., 2007), while murine IFNZ provides a unique combination of high antiviral activity with relatively low lymphomyeolosuppresive activity (Oritani et al., 2000) and suggesting it may act to suppress viruses targeting the bone marrow and spleen like mouse leukemia virus. IFNK is predominately expressed in keratinocytes where it is acts through a unique cell-associated viral protection mechanism (LaFleur et al., 2001; Buontempo et al., 2006). Antiviral activity cannot be detected in conditioned medium from keratinocytes or IFNK-transfected cells, but groups of virally protected cells form clusters around IFNK expressing cells suggesting that IFNK may function in a juxtacrine rather than a paracrine manner. IFNE is expressed in a variety of cell types, but has been suggested on the basis of rather meager evidence to serve a specific role in reproductive tissues either in viral protection or early placental development (Krause and Pestka, 2005; Matsumiya et al., 2007). IFND and IFNT, on the other hand, are not induced by viruses

but instead are released by the early pre-implantation embryos of swine and ruminant species, respectively, where they appear to trigger responses in maternal uterine endometrium that allow the pregnancy to become established (Lefevre et al., 1998; Roberts et al., 2008).

The arrangement of Type I IFN genes within the locus likely reflects the origins and subsequent evolution of individual family members. All Type I *IFN* in human and mouse are clustered in an approximately 400 kb length of DNA, located on the short arm of chromosome 9 (9p21) in human and on the centromere-proximal region of chromosome 4 (4C4) in mouse (Trent et al., 1982; Diaz et al., 1994; Hardy et al., 2004). Two genes of ancient origin, *IFNB* and *IFNE,* define the outer limits of the locus. All the other Type I IFN genes, except *IFNK*, are distributed between these two ancient genes, indicating the locus has expanded internally as IFN genes duplicated and then evolved into their respective families (Hardy et al., 2004). However, species-specific expansion and contraction of families has occurred, with some IFN families only existing in certain taxonomic groups. For example, *IFND* has only been identified in the pig and is absent in the mouse and human, while *IFNZ* is represented in the mouse, but only remnants of the gene has been found in rats, while it is completely absent in humans (Lefevre et al., 1998; Oritani et al., 2000; Takahashi et al., 2001). The IFNW, which are considered to have arisen from the IFNA at least 129 MYA (Hughes, 1995; Pestka et al., 2004), constitute a particularly variable grouping. While the mouse and dog lack *IFNW*, there are seven *IFNW*, six of which are pseudogenes, in the human (Diaz et al., 1994). On the other hand, ruminant species, such as cattle, are known to possess several, apparently functional, *IFNW* (Capon et al., 1985; Leaman and Roberts, 1992). There is also one

example of a Type I family, the *IFNT*, that arose relatively recently (36 MYA) in the lineage to the ruminant artiodactyls. As a consequence, the *IFNT* are absent from all species except those in the sub-order Ruminantia (Leaman and Roberts, 1992; Roberts et al., 1998). Together, these data suggest that novel IFN genes can be gained and existing genes discarded in response to specific environmental challenges, which most likely include threats from emerging new pathogens. In addition, existing IFN may become co-opted into new roles unrelated to viral pathogenesis, as has occurred in the case of the IFND (Lefevre et al., 1998; Oritani et al., 2000; Takahashi et al., 2001).

Although it has been clear for some time that there are similarities in the organization of the Type I IFN locus of cattle and that of other species (Iannuzzi et al., 1993; Ryan et al., 1993), it was equally evident that the bovine locus must have some unique features, most notably because of the existence of the *IFNT*, genes unique to ruminant species whose protein products, although active in antiviral assays, have a primary role as hormones of pregnancy (Roberts et al., 1992). Cattle also have multiple *IFNB* while all non-ruminant species so far examined possess only a single copy *IFNB* (Krause and Pestka, 2005). Together these findings suggest either a decreased restriction on duplication of Type I IFN genes in cattle or evolutionary pressure to acquire additional genes. The recent sequencing of the bovine genome has provided the first opportunity for a detailed study of the Type I *IFN* locus in a ruminant species. Here we provide a detailed description and full annotation of the locus and some inferences about its evolutionary history.

Methods

Annotation

Most of the *IFN* gene candidates were identified through the National Center for Biotechnology and Information (NCBI)'s bovine genome resource by using the basic local alignment search tools (BLAST) (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9913). Additional searches were performed through NCBI by using the appropriate genome resource (http://www.ncbi.nlm.nih.gov/mapview/) for other species, which are discussed later in this section, and by using the basic nucleotide BLAST suite (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) (Ye et al., 2006). Several combinations of BLAST algorithms and databases within NCBI were utilized for this work and are described below (McEntyre, 2002).

BLAST algorithms

1. MegaBLAST was designed to compare highly related nucleotide sequences and works best when the target sequence has a 95% identity or higher to the query sequence.
2. Cross-species megaBLAST, also referred to as discontiguous BLAST, is a derivative of megBLAST that ignores certain bases, thereby allowing mismatches. It was designed to compare nucleotide sequences from one species to nucleotide sequences in another species.
3. BLASTN also compares nucleotide query sequences to a nucleotide database. This algorithm is slower than megaBLAST, but it can identify shorter

sequence matches than megaBLAST. It was not specifically designed for cross-species comparisons.

4. TBLASTN was designed to compare a protein sequence with a nucleotide database dynamically translated in all reading frames.

NCBI databases

1. The "genome (reference)" database represents the most current publicly available assembly of a genome. The most current assembly of the bovine genome at the time this work was completed was assembly 3.1. The most current assembly for other species examined in this work are placed in parenthesis here – human (36.2), mouse (37.1), horse (1.1), and dog (2.1).
2. The "WGS contigs" database contains the contigs, or overlapping unassembled sequences, that forms the basis for the assembled genome. Both pig and cat do not have an assembled genome available at this time and only the "WGS contigs" database can be searched for genomic information for these species.
3. The "traces-WGS" database contains the trace data for whole genome shotgun sequence (WGS) bacterial artificial chromosome (BAC) end sequencing. This database contains single pass sequencing reads that are not trimmed based on quality or vector contamination.
4. The "nucleotide collection (nr/nt)" database contains all Genbank, RefSeq, EMBL (Europe's primary nucleotide database), DNA Database of Japan (DDJB), and many Protein Databank (PDB) sequences. The "nucleotide (nr/nt)" database is subdivided into "human nucleotide (nr/nt)," "mouse

nucleotide (nr/nt)," and "others nucleotide (nr/nt)" databases. The "others nucleotide (nr/nt)" database does not contain any mouse or human sequences.

Bovine *IFNB*, *IFNA*, *IFNW*, and *IFNT* cDNA sequences (Table 1) from GenBank were used to perform a megaBLAST search in the bovine "genome (resource)" database. Human *IFNE*, murine *IFNZ*, porcine *IFND*, human *IFNK*, and human *IFNL/IL28-29* sequences (Table 1) were queried with cross-species megaBLAST in the bovine "genome (reference)" database because no bovine homologues for the latter group of genes have been reported. The translated sequence for each of the non-bovine *IFN* cDNA were also queried with TBLASTN in the bovine "genome (reference)" database because the TBLASTN algorithm can often identify homologues that are not detected through other searches. *IFNL/IL28-29* nucleotide and amino acid sequence were also queried in the bovine "WGS contig" database by using a cross-species megaBLAST and TBLASTN search, respectively, to verify no sequence with high identity to *IFNL/IL28-29* in the bovine genome was missed. Specific genes were analyzed in the bovine "traces-WGS" database to verify frameshift mutations or nucleotide variations from the query sequences.

The bovine genome consortium created a consensus predicted gene set through an algorithm, termed GLEAN, developed during the annotation of the honey bee that used latent class analysis to automatically combine disparate gene prediction evidence (Elsik et al., 2007). Since the majority of positive megaBLAST, cross-species megaBLAST, and TBLASTN matches were clustered on two scaffolds, Chr8.25 and Chr8.34, all GLEAN models on those two scaffolds were also annotated through Apollo

(http://apollo.berkeleybop.org/current/index.html) (Lewis et al., 2002). GLEAN models present on scaffolds Chr8.25 and Chr8.34 that had not been identified in the aforementioned searches were queried through BLASTN and discontiguous megaBLAST in the "others nucleotide collection (nr/nt)" and "human nucleotide collection (nr/nt)" databases to verify their status as IFN genes or another gene family. Discontiguous megaBLAST and TBLASTN searches in human, mouse, equine, porcine, feline, and canine "genomic (reference)" and "WGS contigs" databases were performed for the unique IFN family discovered during the annotation of Chr8.34.

The 64 identified IFN genes and pseudogenes and the original query cDNA from Genbank (Table 1) were aligned through CLUSTALW in BioEdit version 7.09 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) (Thompson et al., 1994). A pairwise comparison to known *IFN* nucleotide sequences was performed through the Maximum Composite Likelihood method in MEGA version 4 (MEGA4) (http://www.megasoftware.net/) to determine the IFN family for each gene (Tamura et al., 2004; Tamura et al., 2007).

IFNT was queried with megaBLAST in the bovine "traces-WGS" database to validate the number of IFN genes present in the genome. Bovine sequence matches that had greater than 94% sequence identities to the query *IFNT* for more than 400 bp were visually inspected. An *IFNT* match was counted as a positive if the sequence had greater than 98% identity to an *IFNT* cDNA in the portion of the trace with a quality score, available through NCBI, higher than 40 on a scale between 0 and 100. The total number of *IFNT* matches in the WGS contig database was divided by the bovine genome coverage to approximate the total IFNT gene number.

Selective Pressure Comparisons and Evolutionary analysis of IFNW and IFNT

When the annotated bovine IFN were mapped, all *IFNW* and *IFNA* grouped into three clusters (Figure 2). In order to determine if nucleotide divergence rate differed between clusters, *IFNW* and *IFNA* nucleotide sequences were divided into three groups, corresponding to clusters 1-3, per subfamily through MEGA4. The nucleotide divergence within each cluster then was calculated in pairwise comparisons by using the Maximum Composite Likelihood method with 1000 bootstrap replicates in MEGA4. All codon positions were included in the analysis, and all positions containing gaps were eliminated in pairwise comparisons (Pairwise Deletion Option).

The selective pressure on the *IFN* coding regions within the locus was determined by comparing the rate of nonsynonymous changes per nonsynonymous site (dN) to the rate of synonynomous changes per synonymous site (dS) where dN:dS > 1 is positive selection, dN:dS = 1 is neutral selection, and dN:dS < 1 is purifying selection (Hughes, 1994). Pairwise analysis of the coding region for each Type I IFN family was conducted by using the Nei-Gojobori (p-distance) method in MEGA4 to calculate dS and dN. The significance of the selective pressure was additionally verified through a codon based Z-test (large sample) for the individual subfamilies (Overall Average Option) and a codon basd Z-test (large sample) for gene pairs (In Sequence Pairs Option) conducted in MEGA4 (Nei and Gojobori, 1986). The variance of the difference was computed by using the bootstrap method (1000 replicates). All positions containing alignment gaps were again eliminated in pairwise sequence comparisons (Pairwise Deletion Option).

The selective pressure on the *IFNW* promoters was evaluated through three separate tests: 1. The number of base differences per site (p-distance) of the *IFNW*

promoter was compared to the dS of the coding sequence, 2. *IFNW* promoter p-distance was compared to open reading frame (ORF) p-distance, and 3. *IFNW* promoter p-distance was compared to the 3′ untranslated region (UTR) p-distance. The p-distances for the promoter, ORF, and 3′ UTR were calculated through pairwise comparisons in MEGA4 by means of the pairwise deletion option. For the first test, the assumption was made that the dS for each gene pair was the neutral mutation rate. Therefore, promoter p-distance:ORF dS < 1 is purifying selection and promoter p-distance:ORF dS > 1 is positive selection. *IFNW* promoter p-distance:ORF dS was calculated for two promoter lengths, one extending 900 and the other 570 nucleotides upstream of the transcriptional start site. The second test, ORF p-distance compared to promoter p-distance, provided additional support for the findings in test 1. The third test assumed the 3′ UTR p-distance for each gene pair approximated the neutral mutation rate. Consequently, similar to the previous tests, the promoter p-distance:3′ UTR p-distance = 1 is neutral selection, promoter p-distance:3′ UTR p-distance < 1 is purifying selection, and promoter p-distance:3/UTR p-distance > 1 is positive selection. Two genes (GLEAN 09996 and GLEAN 09997) were removed from the analysis of the 3′ UTR because both exhibited sequencing gaps within the region under analysis.

The data from the selective pressure tests for the *IFNW* promoters discussed in the previous paragraph fell into three groups when graphed (Figure 8-10). Statistical analysis of the groups was conducted through ANOVA. The intra-group y:x was first calculated where y equaled promoter p-distance and x equaled ORF dS, ORF p-distance, or 3' UTR p-distance. Inter-group ANOVA was performed on groups where the intra-group x:y did not deviate from 1 at $p < 0.01$.

Phylogenetic Reconstruction

Alignments for the genomic *IFNW* ORFs, 570 bp promoters, and 3/ UTRs were created through ClustalW in BioEdit version 7.09. Individual genes were denoted by their GLEAN numbers. Phylogenetic trees were constructed in MEGA4 through the Neighbor-joining (NJ) method with bootstrapping test (1000 replicates) and *IFNA* was included as an outgroup for all trees (Krause and Pestka, 2005). A uniform rate of change was used to calculate all *IFNW* trees.

The coding sequences for all bovine *IFN* identified in the genome database were treated the same as above except the phylogenetic tree was rooted to *IFNK* (Krause and Pestka, 2005) and a second tree was created with the assumption of a non-uniform rate of change between sites (gamma = 1).

Identification of repetitive elements

The localization and identity of all repetitive elements were determined using the RepeatMasker program (http://www.repeatmasker.org/), which uses the RepBase library of repeat elements (Jurka et al., 2005). Sub-locus 1, corresponding to 20000-711500 bp in scaffold Chr8.25, and sub-locus 2, corresponding to 2000-446000 basepairs (bp) in scaffold Chr8.34, sequences were first selected through Apollo and imported into an editable word program. All gaps within the scaffolds, which are represented by an "N" in the bovine assembly, were removed manually. IFN sub-loci sequences were then analyzed in RepeatMasker version 3.1.9 run in default mode with blastp version 2.0MP-WashU (http://blast.wustl.edu/) to determine the percentage of repetitive elements. *Bos taurus* was set as the assumed species within the program parameters. Simple repeats

and low complexity regions were not masked (Morgulis et al., 2006), which means they were not excluded as start sites for a BLAST match, and the matrix was optimized for 42% GC content based on sub-loci optimization pre-runs. The same protocol was used to identify repetitive elements within individual *IFNW* and *IFNT* genes by utilizing the appropriate sequence fragments.

Cell culture and RNA isolation

Mardin Darby bovine kidney (MDBK) cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin. MDBK cells at 75% confluency were incubated with vesicular stomatitis virus (VSV) (Alexenko et al., 1997) overnight. After virus exposure, medium was removed and RNA extracted from the cells with TRI Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's directions. RNA was then ethanol precipitated and the pellet was dissolved in RNase-free water with 0.01% (v:v) RNaseOUT (Invitrogen, Carlsbad, CA). RNA concentration was assessed on a Nanodrop N-1000 (Wilmington, DE) at 260 nm and RNA was stored at -80 C until use.

RT-PCR, partial cDNA cloning, and sequence analysis of IFNX

700 ng total RNA was first heated to 70 C for three minutes (min) and then cold-shocked on ice for three min to enhance DNA accessibility. Genomic DNA in the total RNA was digested with TURBO DNase (Ambion, Foster City, CA) for 25 min at 37 C. Turbo DNase was then inactivated with TURBO DNase inactivation reagent for three min at room temperature. Reverse transcription (RT) was performed at 55 C for 60 min using Supscript III (Invitrogen, Carlsbad, CA), a gene specific primer (5'-TGCTGCTGGCCCTGGTGATGCTC-3'), and 250 µM each of dATP, dCTP, dTTP, and

dGTP. Thirty-six cycles of PCR (95 C for 45 sec, 59 C for 30 sec, 72 C for 1 min) were completed by using PCR MasterMix (Promega, Madison, WI) and bovine *IFNX* specific primers. The forward (5'-CACACGTTGGAGTCAGATGGAG) and reverse (5'-AGGAATCTGTCCAGGAGGCTCT-3') *IFNX* PCR primers corresponded to regions conserved for all three potential IFNX genes but not to any other IFN genes. They were designed to amplify a 210 (bp) fragment that could differentiate the three potential IFNX genes. Non-reversed transcribed Turbo DNase treated RNA was used as a control for genomic DNA contamination for the RNA preparation. Amplified product was analyzed by gel electrophoresis at 7 V cm^{-1} in a 1.5% agarose gel containing 1 µg/ml ethidium bromide in 1 X TAE (Tris-acetate-EDTA, pH 7.5) electrophoresis buffer and visualized under ultraviolet light.

The amplified PCR product was purified by using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) and was A-tailed by incubation with 5 U Taq (AB Peptides, St. Louis, MO) and 0.2 mM dATP for 30 min at 70 C. The Wizard SV Gel and PCR Clean-up System was employed again prior to subcloning the IFNX PCR product. A-tailed PCR product was ligated into pcrII TOPO (Invitrogen, Carlsbad, CA) and the resultant plasmid was transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Cells were plated on Luria Broth plates containing ampicillin (50 µg/ml), X-gal (40 µg/ml), and IPTG (1mM). Colonies were picked through blue/white selection and were grown overnight in Luria Broth containing ampicillin (50 µg/ml). Plasmid was isolated by QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Presence of insert was verified by

restriction digestion with XhoI and BamHI, and the inserted sequenced at the University of Missouri-Columbia DNA Core Facility.

Results and Discussion

IFN Gene Families in Bovine

Evidence for the presence of all previously known Type I IFN subfamilies except *IFNZ* was found on chromosome 8 of the bovine genome assembly 3.1 (Table 2). *IFNZ* has only been reported in mouse (Oritani et al., 2001; Hardy et al., 2004), so that its absence in bovine was not unexpected. Both *IFNK* and *IFNE* are present as single genes with intact ORF and are assumed to be functional, providing the first evidence that either of these subfamilies is present in ruminants. Bovine *IFNK* and *IFNE* have 81.2% and 84.7% nucleotide identity respectively when compared to their human orthologues, which is similar to their conservation between human and species such as cat, dog, and pig (Walker, unpublished data). The *IFNW* family is greatly expanded compared to other species that have so far been examined. There are 24 potential *IFNW* and at least 8 pseudogenes. The query *IFNW* (M11002) (Capon et al., 1985) exactly matches two *IFNW* annotated from the genome database (GLEAN 09983 and GLEAN 10004). The remaining *IFNW* range in sequence identity from 86% to 96% relative to the query sequence. The *IFNA* and *IFNB* are also present in multiple copies, with 13 and 6 genes, respectively, although neither family is as large as the *IFNW*. An apparent *IFNB* pseudogene, deemed nonfunctional due to a frameshift deletion, also exists. *IFND* are only represented as three pseudogenes, a not unexpected finding as a functional gene has only been reported previously for the pig (Lefevre and Boulay, 1993). Three apparently

functional *IFNT* are found within the locus. Surprisingly none of these provide an exact match for any of the many cDNA and gene sequences that have previously been reported. One particular sub-family, the *IFNT2* grouping (Ealy et al., 2001), is not present in the assembly. Finally, we detected a novel Type I IFN, which, as we shall discuss later, appears to have diverged prior to the *IFNA/IFNW* split, and consists of three potentially functional genes and one pseudogene, none of which provides a close sequence match with any previously described Type I *IFN*. For convenience, and until an appropriate nomenclature is approved, this new family will be termed *IFNX*.

IFNT, encoding a ruminant specific IFN required for early embryonic survival, is also present in the assembly. Previous mRNA sequencing of the IFNT family had indicated that over 10 bovine *IFNT* might exist (Ealy et al., 2001) and my own work has shown the presence of as many18 cDNA (Walker et al., unpublished data). Only three *IFNT* are present in the bovine genome assembly 3.1, however. Additional analysis revealed 45 acceptable matches to *IFNT* in the WGS contig database. Since the bovine genome at this time has 7.1X coverage, the number of IFNT matches divided by this coverage value suggests the possibility of around six *IFNT* . One explanation is that these "extra" genes have been lost in the assembly process, a value still significantly lower than the 10 to 18 *IFNT* previously believed to exist. Some of the latter are most likely alleles.

A weak sequence identity to IFNL genes was found on chromosome 13, specifically located on scaffold Chr13.80 (NW_001493172) from 635,850 to 636,120 bp. This sequence appears not to encode a functional gene in either the 3.1 assembly or the WGS contig database. These data suggest that the Type III IFN family exists only as a relic and is no longer a functional component in bovine pathogen defenses.

Locus Map

The Type I IFN locus is organized similarly in mouse and human, and possibly also in pig, i.e. there is relatively conserved synteny across rodents and, primates, and swine. Two mammalian IFN genes of ancient origin, *IFNB* and *IFNE,* define the outer limits of the locus, with all the other genes, except *IFNK,* distributed between these two markers. The genes are predominantly (but not exclusively) localized on one strand and transcribed in the same direction as the *IFNB* and *IFNE* (Hardy et al., 2004). The relative arrangements of the murine and human *versus* the bovine *IFN* locus are illustrated in Figure 1. The bovine Type I IFN locus is clearly organized differently than that of the other two species. Instead of a single stretch of DNA defining the locus, cattle have two sub-loci (1 & 2) encompassing 701 kb and 441 kb, respectively, separated by a gap estimated to be approximately 11 megabases (Mb) in assembly 3.1 (but ~750 kb in assembly 4) (Figure 2). The gene density based on the both predicted open reading frames (Gene Sequence map) and bovine EST and mRNA (BT UniG map) alignments with the assembled sequence is lower in the region between the two IFN sub-loci than much of the rest of chromosome 8, but many genes are present and actively transcribed (Figure 2).

The following explanation of the locus organization has been based on the chromosome map assigned during the assembly process and reported by NCBI (Figure 2). An *IFNB* defines the distal end (relative to the start of the chromosome map) of sub-locus 2 (Figure 1 and 3). *IFNE,* while present in the bovine, is located towards the distal end of sub-locus 1. The majority of the genes in both bovine sub-loci are transcribed in the same direction as the distally placed *IFNB,* except one cluster of *IFNW* and *IFNA* and

the solitary *IFNE*, which are transcribed in the opposite direction . *IFNK* is present in a single "copy" nearer to start of the chromosome map and well separated (6.044 Mb) from the closest sub-loci (sub-loci 1) (Figure 1). The bovine *IFNK* location is very similar to human where a single *IFNK* is located 6.5 Mb from the Type I IFN locus (LaFleur et al., 2001) (Figure 1).

There are three clusters of *IFNA/IFNW*. Two of them are on sub-locus 1, one at the proximal end, the second placed about half way along (Figure 3). A gene set in the first *IFNA/IFNW* cluster 1 is a palindrome to one in the second cluster. The corresponding gene pairs have complete nucleotide identity within their coding regions, suggesting that the duplication or gene conversion event that led to their formation occurred quite recently. The third cluster of *IFNA/IFNW* is at the distal end of sub-locus 2, but lacks the duplicated group of four genes in *IFNA/IFNW* clusters 1 and 2.

Only one non-IFN gene is detectable within sub-loci 1 and 2. An intronless kelch-like 9 (*KLHL9*) is located 33.5 kb proximal to *IFNA/IFNW* cluster 2 in sub-locus 1 (Figure 3). The orthologous *KLHL9* gene can be found in the human and mouse Type I IFN locus approximately 25 kb from the nearest functional *IFN* (*IFNA8*) in mouse (Hardy et al., 2004) and 29 kb from the nearest *IFN* (*IFNA6*) in human (Figure 4). The fact KLHL9 has resisted duplication despite residing close to genes undergoing multiple duplications is noteworthy and possibly indicates that multiple copies of this gene are not well tolerated.

The presence of KLHL9, which appears to be under different evolutionary constraints than *IFNW* and *IFNA*, close to *IFNA/IFNW* cluster 2 suggested the cluster as whole might be under different evolutionary control than cluster 1 or 3. Evolutionary

divergence rates do not indicate that this is the case, however (Table 3). While cluster 1 does have slightly higher divergence rate than cluster 2, all three clusters are relatively constant in their rate of change. Inter-cluster divergence is actually very low in all three of the *IFNA/IFNW* clusters.

The *IFNT* and two *IFNW* pseudogenes are clustered at the distal end of sub-locus 1, suggesting that this cluster of genes originated from an *IFNW* that had become isolated from other *IFNW* before the divergence of the *IFNT*. Its unique position outside the *IFNA/IFNW* clusters and close to the edge of the sub-locus may have permitted the rapid expansion and evolution of the *IFNT* family without the restraints placed on the clustered *IFNW*.

All non-ruminant species examined to date, including mouse, human, cats, dogs, rabbits, and pigs, contain only one *IFNB* (Pestka et al., 2004). In cattle, this family has clearly expanded and extends from the distal end of *IFNA/IFNW* cluster 3 to the end of sub-locus 2. Interspersed within these multiple *IFNB* are members of the previously unidentified *IFN* family, *IFNX*. Again, it is tempting to hypothesize that the *IFNX* and expanded *IFNB* family were able to emerge due to their location on the edge of the sub-locus 2, as suggested for the *IFNT* in sub-locus 1.

Repetitive Elements within Sub-loci

Repetitive elements have been implicated in gene duplication by creating regions predisposed to homologous recombination (Fiston-Lavier et al., 2007; Yang et al., 2008). Over one third of the bovine Type I IFN locus consists of interspersed repeats, rather less than the 43% assessed for the murine Type I IFN locus (Hardy et al., 2004). Repeats are

more enriched in sub-locus 1 largely because of the presence of a greater number of long interspersed nucleotide elements (LINE) and long terminal repeats (LTR) (Table 4). LINE2 elements are absent in both sub-loci. Short interspersed nucleotide elements (SINE)s are present in similar proportions, approximately 15%, for both sub-loci. The arrangement of these elements provides no clue as to whether they were involved in gene duplication events.

Palindromic IFN within IFNA/IFNW clusters 1 and 2

As mentioned previously, a gene set located on the distal end of *IFNA/IFNW* cluster 1 is a palindrome to a gene set on the distal end of cluster 2 (Figure 5A). Two *IFNA* and two *IFNW* are present in each gene set and these are designated as *A(1)* and *A(2)* for the two *IFNA* and *W(1)* and *W(2)* for the two *IFNW* in cluster 1. *A(1)* and *A(2)* are located at the proximal and distal ends of the gene set in cluster 1, respectively. *W(1)* and *W(2)* are located from proximal to distal between A(1) and A(2). The genes with identical ORFs in cluster 2 are designated as *A(1)′*, *A(2)′*, *W(1)′*, and *W(2)′* (Figure 5B). The ORF and the first 350 bp of the 3′ UTR for the two *IFNA* and *IFNW* gene pairs are identical, and an approximately 550 bp promoter for both IFNA gene pairs and the *W(2)/W(2)′* pair are also identical. The promoters of the *W(1)/W(1)′* pair, while closely similar (99 %), are not identical, however. All nucleotide differences between the *W(1)/W(1)′* gene pair are within a region between 300 bp and 400 bp upstream of the transcriptional start site.

Two different evolutionary processes, gene duplication or gene conversion, could explain the existence of the palindromic gene sets. Gene duplication involves the

formation of a new gene copy and, regardless of its role in the generation of the palindromic genes, is certainly involved in the expansion of the *IFNW* and *IFNB* families in bovine. Gene conversion, on the other hand, does not generate new gene copies and homogenizes the existing genes within a species. Both gene duplication and gene conversion have been specifically implicated in the evolution of the *IFNA* in human, chimpanzee, dog, rhesus monkey, rat, and mouse (Miyata et al., 1985; Hughes, 1995; Woelk et al., 2007). Gene conversion, specifically, was predicted by two different statistical programs, GARD and GENECONV, in humans, chimpanzee, rhesus monkey, and mice. Furthermore, despite IFNA genes aligning in conserved positions on a locus map for chimpanzees and humans, the subfamily separated into species-specific clades on phylogenetic analysis (Woelk et al., 2007).

Gene duplication cannot be unambiguously distinguished from gene conversion (Chen et al., 2007). However, when the sequence tract involved is "too large" for gene conversion, gene duplication is assumed to be involved (Blanco et al., 2000). Conversion events seldom involves sequence longer than 1 kb in mammals, with 3 kb considered the maximum length (Chen et al., 2007). The palindomic gene set involves at least a 27 kb tract far exceeding this size limit and reducing the likelihood of a conversion event. Therefore, a segmental duplication event, which is a specific type of gene duplication that involves a large segment of a locus, combined with an inversion is the best explanation for the palindrome (Hurles, 2004).

Selective Pressure on the ORF of Type I IFN Subfamilies

Comparison of the rate of non-synonymous nucleotide change relative to the rate of synonymous change can provide information about the type of selection operating on the members of a multigene families (Hughes, 1994). If neutral selection is occurring, then all nucleotides in a sequence are equally likely to change. Consequently the rate of synonymous nucleotide changes (dS) will be equal to the rate of non-synonymous changes (dN) and dS:dN will equal 1. Rapid change in the amino acid sequence is the desired endpoint for positive selective pressure. Hence, in this scenario, dN will exceed dS, and dN:dS will be greater than 1. Conversely, if strong selection against amino acid change is present (purifying selection), dN will be less than dS and dN:dS will be less than 1. Virtually all pairwise comparisons within *IFNA*, whatever the species (Cheng et al., 2006; Woelk et al., 2007), and *IFNT* (based on cDNA sequences) (Ealy et al., 2001) have shown the overall value for dN not to be significantly higher than dS. Indeed, dN values have been generally calculated to be lower than dS, consistent with the conclusion that there has not been strong positive selection for amino acid change within the coding regions of these subfamilies of IFN.

The dN:dS for all multigene bovine *IFN* subfamilies, including only *IFN* annotated during this work, is illustrated in Figure 6. Similar to previous studies, no evidence for positive selection on *IFN* subfamilies can be found. No multigene IFN subfamily (*IFNA*, *IFNB*, *IFNW*, or *IFNT*) in bovine has a dN significantly exceeding dS. In fact, bovine *IFNA* and *IFNW* provide strong evidence for purifying selection (Figure 6). The significance of purifying selection within *IFNW* and *IFNA* subfamilies may also be verified through a codon based Z-test ($p < 0.001$) (Table 5). Four *IFNB* pairs out of

15 pairwise comparisons examined provide some evidence for purifying selection on the basis of the pairwise codon based Z-test (Table 6), but the subfamily as a whole does not appear to be undergoing significant purifying selection ($p = 0.171$). On the other hand, a codon based Z-test for neutral selection could not find significant evidence that no selective pressure is operating on the *IFNB* subfamily either ($p = 0.343$), indicating the six genes comprising the bovine *IFNB* subfamily are too few for a meaningful analysis. Since only three *IFNT* were identified, statistical analysis of this subfamily from the genomic data was not possible.

Phylogenetic reconstruction of the IFNW ORF and Regulatory Regions

When the bovine *IFNW* ORFs were compared through phylogenetic reconstruction, no distinct classes emerged, and the bootstrapping support for most branches was weak, i.e. bootstrapping support was below 60 (Figure 7A). On the other hand, a phylogenetic reconstruction of the *IFNW* promoters, which were defined as sequence extending 570 bp upstream of the transcriptional start site, divided the *IFNW* into three classes, termed class P1, P2, and P3 (Figure 7B). Class P1 was the most evolutionary ancient class and had the fewest members ($n = 4$). Class P2 was large and could be additionally partitioned into two subclasses, termed subclass s1 and s2, containing seven and nine members respectively. Class P3 comprised a single *IFNW-T* intermediate and will be discussed in greater length later in the chapter. Interestingly, none of the classes designated by their promoter characteristics provided an analogous grouping in the tree based on the ORF. A third phylogenetic tree could be constructed based on the 3′ UTR. Interestingly, this reconstruction divided the *IFNW* into well

supported classes based on bootstrapping scores (Figure 7C). However, the classes formed in the analysis of the 3/ UTR did not correlate with the classes formed in either the promoter tree or ORF tree.

Selective Pressure on IFNW Regulatory Regions

The standard model of gene duplication states that after a duplication event one gene continues to perform the ancestral function while the second either rapidly evolves to fill a new niche or becomes inactive (Ohno, 1970; Walsh, 1995; Hurles, 2004). As a consequence gene duplication is usually followed by a period in which there is an acquisition of non-synonymous nucleotide changes in one of the two genes, leading to a divergence in amino acid sequence. This temporary relaxation of purifying selection permits the gene to become fine-tuned to its new role. Such a sequence of events does not appear to have occurred during the large scale expansion of the *IFNW* family where there is strong evidence for purifying selection operating on the coding regions of the genes when they are analyzed in their entirety (Figure 6 and Table 5). One potential explanation is that sub-functionalization is occurring through alterations in the manner in which these genes are subjected to transcriptional control rather than in the structure of the protein itself. Determining the selective pressure operating on non-coding regions is difficult. Despite the likelihood that certain regions of the promoter are more critical for transcriptional control of a gene than others, it is tricky to define truly "neutral" sites where mutations have no effect. Here I have made the assumption that dS within the coding region for each gene pair in a pairwise comparison also equals the neutral mutation rate in the promoter.

The nucleotide divergence in the promoter between individual *IFNW* gene pairs was therefore compared to ORF dS for the same gene pair (Figure 8). The promoter p-distances, which equal the total nucleotide diversity, were calculated with both a 900 bp promoter and a shorter 570 bp promoter that corresponds to the length of the ORF. The results were statistically equivalent for both promoter lengths. The ORF dS was close to 0.133 in all gene pair comparisons (n = 210), but the total nucleotide divergence in the promoter, i.e. promoter p-distance, was quite variable and fell into three main groups on the selective pressure graph. The groups are labeled 1, 2, and 3, reflecting the highest promoter p-distance: ORF dS ratio to the lowest, respectively. The mean promoter p-distances based on the 570 bp promoter for the three groups are 0.411, 0.170, and 0.053, values that are statistically different from one another by ANOVA ($p < 0.0001$).

Group 1 p-distance is approximately three-times dS, indicating that these promoters are diverging much faster than the coding regions and suggesting strong positive selection might be occurring in order to diversify promoter nucleotide sequences. All group 1 pair-wise comparisons (n = 85) represented inter-class relationships (Figure 5B). The average promoter p-distances for the short promoter for the inter-class comparisons were 0.508 for P3 *versus* P1 (n = 4), 0.410 for P3 *versus* P2 (n = 64), and 0.410 for P2 *versus* P1 (n = 17).

Group 2 had a p-distance:dS that was close to 1 (1.27), implying that this relatively large group of pairwise comparisons (n = 99) involves promoters operating under neutral selection. Group 2 included all 63 inter-subclass pairwise comparisons (p-distance = 0.170), 27 intra-subclass P2s2 pairwise comparisons (p-distance = 0.166), 6 intra-subclass P2s1 pairwise comparisons (p-distance = 0.148), and 3 intra-class P1

pairwise comparisons (p-distance = 0.201). The majority of the P2s2 subclass pairwise comparisons (75%) fell into group 2, but, for subclass P2s1, only intra-subclass comparisons involving GLEAN 24308, the most ancient member of the P2s1 subclass, fell into group 2, indicating different selective pressures have been acting on these two subclasses.

Group 3 had promoter p-distances lower than the ORF dS, suggesting a subset of promoters have been undergoing purifying selection. Group 3 contained all intra-subclass P2s1 pairwise comparisons that did not involve *GLEAN 24308* (n = 15), select intra-subclass P2s2 pairwise comparisons (n = 9), and half the intra-class P1 pairwise comparisons (n = 3). The majority of the P2s1 pairwise comparisons (71.4%) fell into group 3 emphasizing this subclass is evolving under different evolutionary constraints than subclass P2s2 and class 1. Comparisons involving *W(1), W(1)′, W(2), and W(2)′* from the recent, presumptive, gene duplication event comprise a large portion of group 3. Specifically, 60% of the subclass P2s2 comparisons, and 55% of the subclass P2s1 pairwise comparisons involved the duplicated *IFNW*, suggesting these genes are a focal point for purifying selection.

A second test was performed in which the p-distance rather than dS of the ORF, i.e. the number of nucleotide changes per nucleotide position, was compared to the p-distance of the promoters (Figure 9). These results largely mirrored the previous analysis, i.e. three groups of values were obtained containing the same pairwise comparsions as in Figure 4, although a more pronounced difference in the divergence patterns between the coding sequence and the promoter regions of these genes can be observed as a result of selection operating against non-synonymous nucleotide changes in

the ORF. The three graphical groups present in this analysis were statistically different through ANOVA ($p < 0.0001$).

In the third test we compared promoter p-distance to the 3′ UTR p-distance. This analysis, whose validity is perhaps more questionable than the other two, was more difficult to interpret. Clearly some 5′ promoter regions are diverging much faster than the 3′ UTR, which, for the sake of this analysis, was assumed to be under no selective pressure (Figure 10). The range of nucleotide diversity in the 3′ UTR, however, is much broader than observed for dS suggesting that the 3′ UTR may not be the best standard for a neutral mutation rate. Despite this concern, all three tests support the hypothesis that divergence between promoter classes is occurring at a faster than assumed neutral mutation rate and much faster than the ORF. It also supports the division of *IFNW* into three classes, with class 2 subdivided, according to the number of nucleotide changes accumulated. Class P1 and subclass P2s2 evolved largely, but not exclusive, under neutral selection, while subclass P2s1 evolved primarily under purifying selection.

The broad range of nucleotide divergence within the 3′ UTR was then analyzed more closely, since this region of the gene can affect mRNA stability and relative rates of translation, and also, like the 5′ UTR, have a regulatory role in gene expression (Brewer et al., 2003; Malquori et al., 2007). A comparison of the 3′ UTR p-distance to the corresponding ORF dS reveals that a portion of the genes were diverging much faster in their 3′ UTR than the assumed neutral mutation rate, dS (Figure 11). While pairwise comparisons of the promoters formed three groups concentrated in certain p-distances, discussed in detail in the above paragraphs, pairwise comparisons of the 3′UTR did not

form distinct groups. The nucleotide diversity ranged from around 0.41 to 0.05 in a continous band.

Graphing the ORF dS, ORF p-distance, promoter p-distance, and 3′ UTR p-distance for each gene pair (where each gene pair represents one point on the horizontal axis) also illustrates the broad range of nucleotide diversity within the 3′ UTR compared to the other regions of the gene (Figure 12). A clear pattern can be observed, with the low ORF p-distance illustrating it is the least divergent portion of the gene while certain promoter pairs are the most divergent. ORF dS, the best estimate for the neutral mutation rate, is only slightly higher than the total nucleotide diversity in the ORF. The 3′ UTR demonstrates heterogeneity in nucleotide diversity ranging from low values roughly equivalent to ORF p-distance to high values approaching the most extreme promoter p-distances. The promoters are clustered either slightly higher than dS, i.e. displaying a neutral mutation rate, or represent the most highly divergent genomic region.

Together, these results suggest rapid divergence in the regulatory regions of the *IFNW* compared to the ORF. Analysis of the promoter suggests that there has been strong selective pressure for change at some nucleotide positions, but that changes at other positions have not been permitted. In other words, positive selective pressure is acting on the IFNW promoter, but deleterious changes are quickly removed from the active gene pool. The 3′ UTR, on the other hand, has experienced strong selective pressure without major restriction on the types of changes allowed. Consequently, the 3′ UTR analysis did not create any clear groupings.

This close examination of divergence patterns within the *IFNW* subfamily alters the current train of thought on evolution of Type I IFN. Previous evolutionary studies

have focused on *IFNA* because it is the only multigene Type I IFN subfamily in most species. These studies on the *IFNA* only examined divergence patterns within the coding sequences where specific amino acid changes were linked with subtle alterations in biological activity (van Pesch et al., 2004; Cheng et al., 2006; Woelk et al., 2007). Our work, however, suggests that coding regions contain the least amount of information about the evolution of IFN subfamilies. Divergence within the regulatory regions, particularly the promoter, is occurring at a much faster rate than within the coding region. Therefore, any attempts to describe the evolutionary history of IFN families (and possibly other large, multigene families) should focus more heavily on promoter analyses rather than on the divergence of the ORF. It is interesting to note that all attempts to link specific changes in the coding sequence of *IFNW* with specific changes in the promoter failed. In other words, divergence of the promoters was not only faster than that of the ORF, but the two processes appeared to proceed independently. Careful consideration of the interaction between pathogens and IFNs logically explain the differences in selective pressure on the regulatory versus coding regions of the gene. Many viruses evade the IFN defense system by preventing IFN induction (Randall and Goodbourn, 2008). A means to change the promoter of these genes quickly is advantageous to counter attempts at viral evasion of IFN induction. On the other hand, all Type I IFN act through the same receptor; consequently, radical changes within the coding sequence would more than likely reduce IFN potency.

Emergence of IFNT

IFNW is a relatively ancient gene family emerging about 129 MYA (Capon et al., 1985; Hughes, 1995). The *IFNW* is also the parent group of another Type I IFN, the *IFNT*, which diverged from the *IFNW* approximately 36.5 MYA in pecoran ruminants where it serves a role in maternal recognition of pregnancy (Roberts et al., 1998). Twelve to eighteen bovine *IFNT* have previously been identified through cDNA library analysis and RT-PCR of transcripts in early trophoblast. In the case of bovine IFNT all variants have over 98% nucleotide identity to one another (Ealy et al., 2001). Surprisingly, the bovine Type I IFN locus contains only three recognizable *IFNT*, plus two pseudogenes, and one potentially functional gene (*GLEAN 09993*) whose ORFs are intermediary in sequence of their ORF between the queried *IFNW* and the queried *IFNT*. The two pseudogenes, termed *W-Tp1* and *W-Tp2*, have somewhat closer identity to *IFNW* (88% and 86% respectively) than to *IFNT* (85% and 83% respectively). *GLEAN 09993* is more similar to *IFNT* (92% identity) than to the pseudogenes, but this value is well below the 98% identity exhibited within the *IFNT* subfamily of functional genes.

Unlike *INFW*, *IFNT* have highly conserved regions upstream and downstream of the ORF, a conservation previously noted for both intra- and inter-species comparisons of *IFNT* for sheep, cattle, musk oxen, goats, and giraffes (Hansen et al., 1991; Leaman and Roberts, 1992; Liu et al., 1996). The promoter region of *GLEAN 09993* and the two pseudogenes, show a much closer resemblance to *IFNT* promoters rather than to any of the *IFNW*. Specifically, the promoter region of the two pseudogenes have approximately ~78% identity to *IFNT* promoters (based on a region 900 bp upstream of the transcriptional start) but only ~50% identity to *IFNW* promoters. *GLEAN 09993* has an

even higher identity (93%) to *IFNT* promoters. The 3′ UTR plus approximately 1200 bp region downstream from the polyadenylation site also resemble *IFNT* more than *IFNW*.

All *IFNT*, *GLEAN 09993*, and the two pseudogenes contain an L2b/LINE element and two tandem Bov-tA2/SINE downstream from the ORF region (Figure 13). These elements are absent from all *IFNW* (with the exception of *GLEAN 09993* itself). All three *IFNT* contain a sequence with 87.4 % identity to ART2A/SINE approximately 660 basepairs 5′ of the transcriptional start site. A sequence with 95.1% identity to BovB/LINE is present immediately upstream of the ART2a insertion. *GLEAN 09993* also possesses similarly positioned sequences with close identity to ART2A (89.6% identity) and to BovB/LINE (95.9% identity), emphasizing the similarity of the two genes. The two pseudogenes, on the other hand, do not have these conserved repeat elements. These data suggest the two pseudogenes are the present day representatives of early precursors of the *IFNT* while *GLEAN 0993* embodies a later stage in this process.

The examination of *IFNW* described earlier in the chapter suggests that the defining characteristic of the gene family is the promoter elements. We therefore attempted a closer examination of the *IFNT* promoters to determine whether inferences could be made about their evolution from the homologous regions of *IFNW* and the creation of a new gene family. As discussed earlier, the definition of a promoter is tricky because there is a seldom a clear delimitation on how far 5′ transcriptional control of a gene extends. For the purpose of this work, the 5′ extent of the *IFNT* promoter has been arbitrarily defined as the distal end of BovB/LINE element, because currently no empirical evidence suggests transcriptional regulation extends beyond this point (Imakawa et al., 1989; Leaman and Roberts, 1992). By this definition, the promoters of

the three *IFNT*, *GLEAN 0993*, and the two pseudogenes can be broken into three domains (Figure 14). Domain 1 extends 220 nucleotides upstream from the transcriptional start point and has greater than 65% nucleotide identity across all five genes. Such a degree of identity allows construction of a reasonable nucleotide alignment. The data reflect a marked divergence from both *IFNW* class 1, represented by *GLEAN 09988*, and class 2, represented by *GLEAN 09997*, to provide a unique *IFNT* promoter. Domain 1 represents a well defined regulatory region of *IFNT* and includes characterized transcription factor binding sites for ETS2, AP1, and DLX3 (Ezashi and Roberts, 2004; Das et al., 2008; Ezashi et al., 2008). These sites, particularly the one that binds ETS2, are known to be critical for the unique transcriptional regulation of *IFNT* during early pregnancy. Domain 2 is highly conserved between the three *IFNT* and the two pseudogenes and GLEAN 09993, but has retained less than 30% nucleotide conservation with the *IFNW*. Making evolutionary inferences about the origin of this region of the *IFNT* is therefore not feasible. This domain of the promoter could either have undergone unusually rapid divergence, which seems unlikely, or has acquired this part of its gene through a transposition or homologous recombination event. Extensive searches were performed through porcine, bovine, equine, human, mouse, and bacterial genome databases and the nr/nt GenBank database. No sequences resembling Domain 2 could be found. While highly conserved sequences that could potentially bind CDX homeobox domains are present in Domain 2 of the *IFNT* promoter in both cattle and sheep (Leaman et al., 1994; Imakawa et al., 2006), the importance of these or other elements to gene expression have not been examined. Domain 3 extends upstream from position 743 and contains the repetitive elements described above. The importance of these repeats is unknown, but

such sequences have been implicated in the rapid duplication of genes within multiple gene families (Kulski et al., 2004; Nagao et al., 2005; Wallis and Wallis, 2006) and it could, hypothetically, have provided a similar role for *IFNT*.

These findings, combined with visual examination of the locus and the information garnered from the selective pressure analysis on the *IFNW* family, creates a plausible theory for the evolution of *IFNT*. A single *IFNW* is hypothesized to have become deposited into a region outside of the three *IFNA/INFW* clusters. The "escaped" *IFNW* is then itself presumed to have duplicated, with one copy becoming inactive, i.e. became transformed into a pseudogene, thereby permitting the rapid, unconstrained divergence of its promoter region and possible loss of viral response elements. Around the time the gene acquired the upstream repeat elements, a recombination event placed the new "promoter" ahead of a functional *IFNW* OPR. Alternatively, one of the pseudogenes experienced a second mutation reversing the early stop codon. This now functional gene is presumed to be the direct ancestor of *GLEAN 09993*, which subsequently duplicated to form the primordial *IFNT*. The presence of the repeat elements in the 5′ and 3′ regions may have aided expansion of the new family through homologous recombination. Fine tuning of transcription factor binding sites eventually provided the highly specialized expression features of the modern day *IFNT*.

None of the three genes identified in the *IFNT* assembly correspond exactly to cDNA or gene sequences deposited in GenBank. *GLEAN 10006* and *GLEAN 10009* have 99.8% identity to *IFNT-3a*, while GLEAN 10007 has 99.7% identity to *IFNT-1a*. These three genes do represent two of the three previously described IFNT classes (Ealy et al., 2001); however, another IFNT class (class 2) is not represented at all in the genome. The

presence of only three *IFNT* and the complete absence of any class 2 genes in the current assembly was surprising in view of the fact that previous studies have indicated as many as 18 different *IFNT* transcripts (identified as cDNA) or genes may exist in Bos taurus (Walker et. al, unpublished data). Allelic variation may explain some of these additional sequences but seem unlikely to account for all the unique *IFNT* sequences deposited in GenBank, the majority of which have been confirmed as independent isolates (Ealy et al., 2001). Extensive searches within the BAC libraries through the "traces-WGS" database indicate an additional three *IFNT* may be present in the genome, which could have become "lost" during the assembly process. The presence of six genes is more consistent with earlier studies that utilized fluorescent in situ hybridization and restriction fragment length polymorphisms and defined approximately 6 *IFNT* and 20 *IFNW* on chromosome 8 (Iannuzzi et al., 1993; Ryan et al., 1993). Another possibility is that the number of *IFNT* genes varies among individual animals and breeds. Copy number variation within multigene families has been well documented in humans, and an extensive study of African, European, and Asian populations identified 1,447 copy number variable regions in the human genome (Khaja et al., 2006; Redon et al., 2006). A multigene family within one of these variable regions, olfactory receptor genes, which number over 300, displayed a mean of copy number of variation of 10 functional genes per individual (Nozawa et al., 2007). The likelihood of a copy number variable region increases in loci with segmental duplications and high sequence identity among genes. The Type I IFN locus has both of these characteristics suggesting these genes are good candidates to have variable copy numbers.

IFNX: Evidence for a novel subfamily and its expression

Based on the current assembly, the IFNX subfamily consists of three potential genes and one probable pseudogene. The origin of these genes is currently unclear, but they appear to constitute a unique IFN subfamily, whose closest relatives are the *IFNA* based on a Maximum Composite Likelihood divergence analysis, with *IFNX* sharing over 65% nucleotide identity to *IFNA* and only about 45% nucleotide identity to *IFNB*. They are clearly defined on phylogenetic trees based on their ORF as a distinct clade that is most closely related to *IFNA*; however, phylogenetic analyses are conflicting with regard to when *IFNX* first appeared. Phylogenetic trees calculated on the assumption that all nucleotide sites within the coding sequence change at the same rate indicate *IFNA* emerged from *IFNX* prior to or corresponding with the radiation of eutherian mammals, i.e. more than 150 MYA (Roberts et al., 1998) (Figure 15). Conversely, phylogenetic trees that take into account substitute rate variation from site to site indicate that bovine *IFNX* and bovine *IFNA* emerged from a common ancestor after the radiation of the major mammalian orders (Figure 16). In other words, this model recognizes that certain amino acids and, by corollary certain nucleotides, are more highly conserved than others across subfamilies. This second model most closely matches the amino acid differences among IFN subfamilies since amino acids critical to preserving the tertiary structures of *IFNB, IFNA, IFNT*, and *IFNL* are much more highly conserved than ones in less critical regions of the proteins (Oritani et al., 2001; Kumaran et al., 2007). Preliminary examination of IFNX indicate some of the more highly conserved amino acids for other Type I IFN, such as the cysteines 1 and 99 (discussed below), are also conserved in IFNX. In addition *IFNX* and the *IFNX* pseudogene are absent in human, mouse, porcine, feline, or canine

genomic databases, yet some remnants of their presence might be expected if this family emerged early in the evolution of mammals and prior to the main radiation events.

Two of the three potential IFNX genes contain the four conserved cysteine residues required for the disulfide bonds (1-99; 29-139) encountered in *IFNA* and those Type I subfamilies that emerged from *IFNA*, namely *IFNW*, *IFND*, and *IFNT*. The third potential gene, GLEAN 24316, contains an early termination codon at codon 125, which eliminates the second disulfide bond (29-139) (Figure 17), raising the possibility that it is a second pseudogene. The "trace-WGS" database was visually examined to verify the presence of this early stop codon. Five BACs, all of which were in the minus orientation, contained sequences that exactly matched the complementary sequence to Glean 24316, i.e. all contained the early termination codon.

Previous studies of IFNA have identified three regions that are strongly associated with IFN-receptor interaction and are termed interferon receptor recognition peptides (IRRP)1-3 (Piehler and Schreiber, 1999; Chill et al., 2002; Kumaran et al., 2007). IRRP1 (27-35) and IRRP2 (78-105) control the initial binding of IFN to the Type I receptor and are highly conserved among IFNA. IRRP3 (123-140) modulates the downstream signaling pathway rather than preventing IFN-receptor interaction, so that amino acid changes in this region can explain some differences in biological activity among different IFNA. The protein products of the three *IFNX* do not possess identical IRRP1 and IRRP2 motifs as IFNA, but these two regions are highly conserved within the subfamily, emphasizing first the importance of this motif and second that the IFNX family is unique and distinct from IFNA. IRRP3 was absent in GLEAN 24316 again raising concern that it may be a pseudogene. The two remaining IFNX members differed in their IRRP3

sequences, a not unexpected finding as changes in this region may provide subtle differences in biologic activity between the two family members.

No evidence for IFNX expression could be found in any EST databases, although, genes with high identity to IFNX exist in the equine genomic database. The conservation of this gene family in species that diverged at least 80 million years ago suggests that the family may have an important function in ungulates. However, the apparent absence of *IFNX* genes in pigs, also an ungulate, is puzzling. Possibly, *IFNX* has a specific function in herbivores that is not required in omnivores, most likely in immune defense against particular viruses or other pathogenic organisms affecting such species.

Equally puzzling is why an *IFNX* pseudogene was not found the porcine genome in pigs since cattle and pigs diverged more recently (65 MYA) than horses and cattle (83 MYA). One possible explanation is that the *IFNX* was removed from the porcine genome after becoming a pseudogene. This idea does have some support based on other studies in Type I IFN. Previous analysis of the porcine *IFNA* family found that pseudogenes retain a higher degree of homology in critical residues necessary for protein tertiary structure and receptor interaction than was expected in an inactive gene (Cheng et al., 2006). Studies on other multigene families suggest that homologous recombination events between pseudogenes and functional genes often allow the creation of novel family members. Cheng's observations can be explained through this mechanism since pseudogenes would need to retain many characteristics of the functional gene if a recombination event occurred. Active policing of "incorrect" pseudogenes with deleterious changes in the coding sequence is necessary based on this assumption. One

possible explanation for the absence of an *IFNX* in the porcine genome is such a policing mechanism.

In order to determine whether IFNX genes are functional and respond to virus, MDBK cells were challenged with VSV and analyzed for expression of IFNX by using RT-PCR with primers for conserved regions of all three potential genes. An RT-PCR product of the correct size was amplified (Figure 18) and subcloned. Sequencing of two clones revealed a match to GLEAN 24316, the potential pseudogene, in both cases. Although the early termination codon eliminating the second disulfide bond in GLEAN 24316 might be expected to relax the structure of the protein product of this gene, it might potentially still interact with the Type I IFN receptor. It is also possible that a fourth gene with high identity to GLEAN 24316 but lacking the early stop codon exists.

In conclusion, the identification of a novel Type I IFN, the IFNX, is an unexpected and possibly important finding. The proteins encoded by this family of genes differ sufficiently in primary sequence from related Type I IFN to justify a separate designation from the related IFNA and IFNB. The presence of a distinct cluster of *IFNX* within the Type I IFN locus, the phylogenetic position of *IFNX* as a separate clade within the IFN tree, and the conservation of critical amino acid residues, are totally consistent with classifying the *IFNX* as a distinct Type I IFN subfamily. Whether IFNX are able to interact with the Type I IFN receptor and elicit a typical Type I response in its target cells has yet to be verified, but the fact that the expression of at least one of the IFNX genes can be detected in virally challenged cells suggests that these genes have a role in innate immune defense analogous to most other Type I IFN subfamilies. Substantial work is

obviously necessary to characterize this gene subfamily fully, but its place within the Type I IFN family would appear to be assured.

Final Comments

Type I *IFN* locus has undergone substantial transformation in ruminants compared to humans and mice. The conserved locus structure has been transformed, subfamilies have expanded, and two novel subfamilies exist. The division of the locus into two sub-loci may have reduced physical constraints inherent in the chromosomal structure on gene duplication, providing a window of opportunity for gene duplication which contributed to expanded function of the Type I IFN. For example, the expansion of the successful pecoran ruminant sub-order and its geographic spread might have required improved protection against unique ruminant pathogens. The *IFNX* sub-family and the greatly expanded ruminant specific IFNW are likely candidates for providing increased pathogenic protection. Radically new functions for Type I might also have been gained, such as the one exemplified by the *IFNT*, whose appearance appears to have coincided with, and possibly permitted, the acquisition of the unique, synepitheliochorial placentation that characterizes the Ruminantia sub-order and requires powerful conceptus signaling before the trophoblast has even attached to the uterine wall. The ancient Type III IFN (*IFNL/IL28-29*) may have become a casualty of the expansion and broadened role of the Type I locus. Only an inactive *IFNL* remains in the bovine genome. It is tempting to speculate that the function of *IFNL* has been replaced as the Type I *IFN* locus inflated.

The authors concede that the bovine genome assembly is a work in progress and that the predicted arrangement of individual IFN genes will change as data are

reanalyzed. As a perspective, the first review of the draft human genome was released in 2001, the "finished" human genome in 2003, and the "complete" human genome in 2006 (Lander et al., 2001; Dhand, 2006). Small updates to individual genes are still being made within the human genome as better information becomes available. In addition, it is clear that individuals possess their own unique, genomic attributes, including inversions, duplications, and presence and absence of specific genes. Jim Watson is not Craig Ventner. It would be over-confident at this stage to assume that the present assembly of the bovine genome, whose sequence coverage was in any case less "deep" than that of the human could be perfected without a similar review. However, the major findings that we have made concerning the organization and sequence of the bovine Type I IFN have remained relatively constant through the last two versions 2.1 and 3.1 of the assembly with one exception, namely the size of the "gap" between the two sub-loci. The unique features, which include the presence of the gap itself, the arrangements of *IFNW/IFNA* clusters, the dramatic expansion of the *IFNW*, the presence of the *IFNX*, and the separation of *IFNT* from the *IFNW/IFNA* clusters are consistent observations and are unlikely to alter drastically in future versions of the assembly.

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Species	Gene	Accession No.	Species	Gene	Accession No.
Bovine	IFNA	AY325272	Bovine	IFNT	AF196324
Bovine	IFNA	M10954	Porcine	IFND	Z22707
Bovine	IFNA	AY523531	Porcine	IFND	Z22706
Bovine	IFNA	DQ396807	Human	IFNK	NM_020124
Bovine	IFNA	Z46508	Human	IFNE	NM_176891
Bovine	IFNB	M15478	Human	IFNL	AY184374
Bovine	IFNW	M11002	Human	IFNL	AY184373
Bovine	IFNT	M31557	Human	IFNL	AY184372
Bovine	IFNT	AF196320	Murine	IFNZ	NM_197889
Bovine	IFNT	AF196322			

Table 1. *Query Sequences*. The query sequences used for the genomic searches megaBLAST and TBLASTN searches are listed.

	Gene Number		
Subfamily	Human	Mouse	Cow
IFNK	1	1	1
IFNE	1	1	1
IFNB	1	1	6
IFND	0	0	0
IFNZ	0	2	0
IFNA	13	13	13
IFNW	1	0	24
IFNT	0	0	3
IFNX	0	0	3

Table 2. *Cross-species comparison of IFN subfamilies*. The number of predicted IFN genes in each subfamily based on genomic analysis of the mouse, human, and bovine are shown. Predicted pseudogenes based on frameshift mutations or stop codons within the first 100 aa of the coding sequence have been excluded from the table. The bovine Type I IFN locus has an expansion of both the IFNB and IFNW subfamilies. Cattle have also acquired two novel IFN subfamilies, IFNT and a previously unidentified IFN family, termed IFNX, discovered during this analysis.

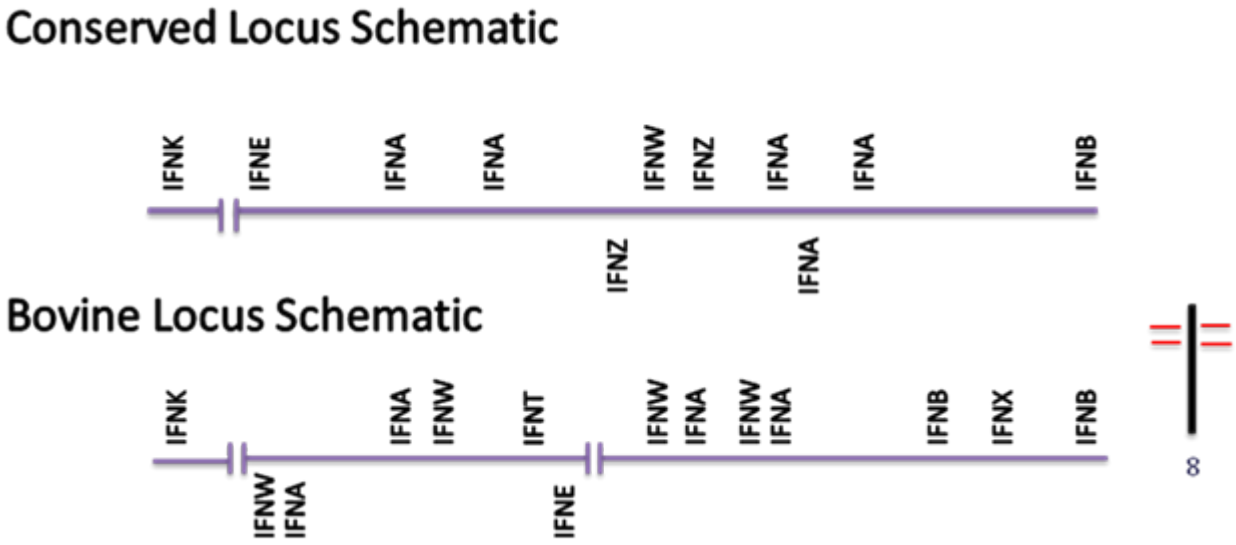


Figure 1. *Type I IFN Locus Schematics* These schematics, while not drawn to scale, illustrate the basic characteristics of the locus in mouse and human, the Conserved Locus Schematic, and the bovine locus. Each IFN gene is represented by its abbreviated name and its position above and below the schematic line represents the direction of its transcription. A gene map of chromosome 8 is present to the right of the bovine locus schematic.

Conserved Locus: Certain features of the Type I IFN locus are consistent in species previously examined and these features reflect the evolution of Type I IFN. *IFNK*, the most ancient Type I IFN, is slightly separated from the rest of the locus. *IFNE* is located at one extreme of the locus and *IFNB* is located at the opposite extreme. These two IFN are the oldest in the main Type I IFN locus and are believed to have evolved at approximately the same time. The remaining IFN families are present between *IFNE* and *IFNB* and are transcribed in parallel to these genes. They duplicated and evolved into their respective families as time progressed resulting in spreading of the locus.

Bovine IFN Locus: The bovine Type I IFN genes have lost the conserved arrangement present in human and mouse. *IFNK* is still separated from the rest of the Type I IFN genes in bovine. The main locus, however, is split into two subloci illustrated by the two red lines on the gene map of bovine chromosome 8 to the right of the schematic of the bovine locus. These subloci are separated by approximately 1100 kb in assembly 3.1 and 750 kb in assembly 4.0. *IFNB* is present at the extreme of one of these subloci, but *IFNE* is not located at the opposite extreme of the other subloci. The majority of the genes are still transcribed in parallel to *IFNB*, but one cluster of *IFNW* and *IFNA* and the solitary *IFNE* are transcribed in the opposite direction.

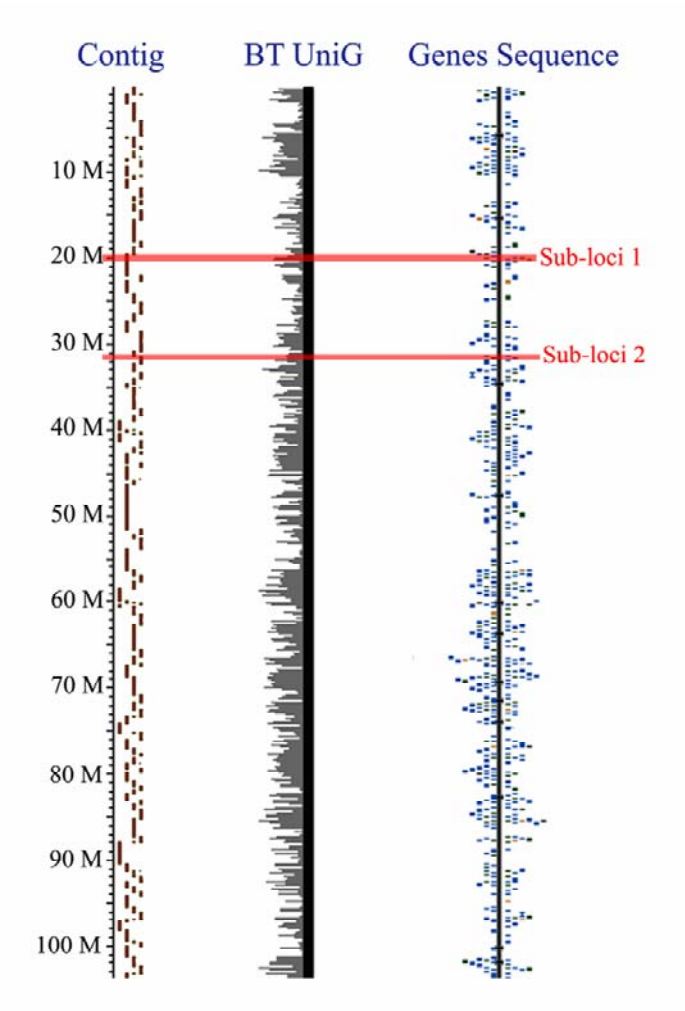


Figure 2. *Bovine chromosome 8 maps.* The chromosome maps reproduced here with slight alterations are from the NCBI bovine genome resource. The contig map used to construct chromosome 8 is shown on the far left. The scale bar corresponds to the arbitrarily assigned start and end points used by the bovine consortium for chromosome assembly. The BT UniG map, which represents all matches between bovine ESTs and genomic sequence, and the Genes Sequence map, which represents putative gene annotations, illustrate gene density of chromosome 8. The location of the Type I IFN sub-loci is shown by a red bar crossing all three chromosome maps.

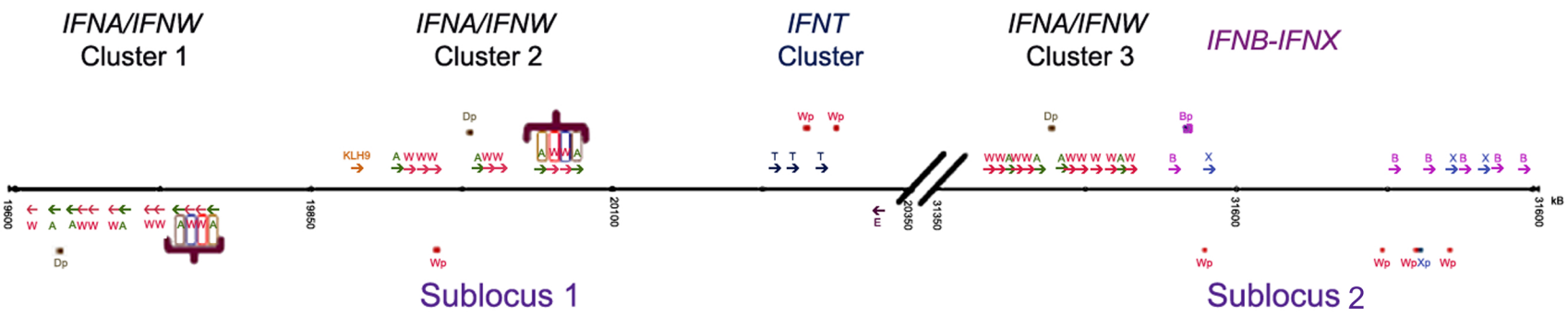

IFNA/IFNW
Cluster 1
IFNA/IFNW
Cluster 2
IFNT
Cluster
IFNA/IFNW
Cluster 3
IFNB-IFNX
19600
19850
20100
20350
31350
31600
kB
KLH9
Dp
Wp
Bp
Xp
Sublocus 1
Sublocus 2

Figure 3. *Genomic map of the bovine Type I IFN locus*. Blast searches of the bovine genome database revealed all matches to known IFN genes, except *IFNK*, reside on two sub-loci. These sub-loci are illustrated in the gene map. Both the position of each gene relative to the line and the direction of the arrow on the map denote the direction of transcription. The family for each gene is designated by the final letter of the abbreviated name. Pseudogenes are indicated by a box instead of an arrow and the letter "p" after the family designation. Specific gene clusters have been labeled according to their family. A recent example of gene conversion is illustrated in the IFNW/IFNA cluster 1 and 2. The bracketed gene set in IFNA/IFNW cluster 1 is a palindrome to the bracketed gene set in the *IFNA/IFNW* cluster 2 with identical coding sequences for genes specified by correspondingly colored boxes.

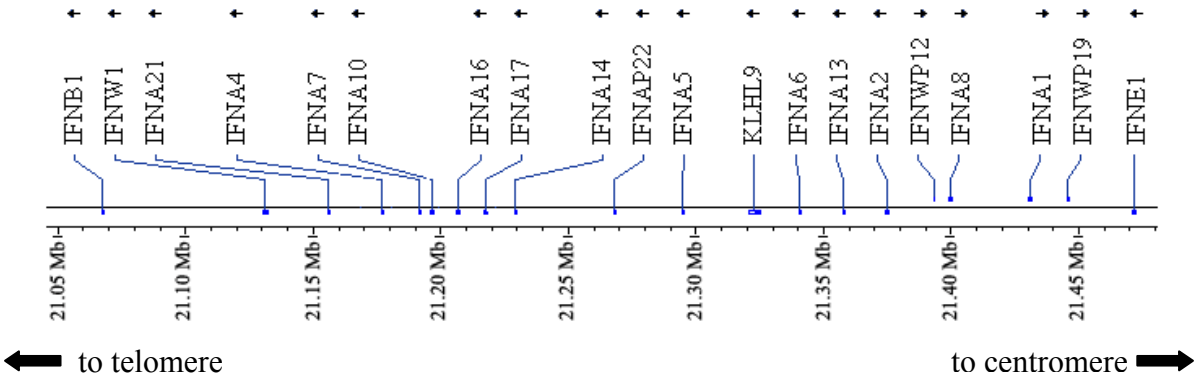


Figure 4. *Human Type I IFN Locus* The Type I IFN locus from the NCBI Human Gene Sequence Map is reproduced here with minor modifications. Genes are listed by their HUGO name and pseudogenes have a "P" after the gene name. The arrow above each gene indicates the direction of transcription.

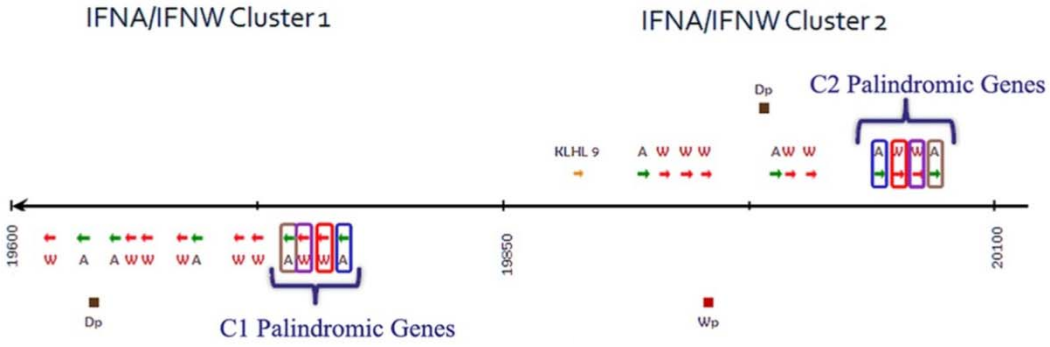
	IFNA/IFNW Clusters		
Subfamily	1	2	3
IFNW	0.086 ± 0.007	0.071 ± 0.007	0.063 ± 0.006
IFNA	0.053 ± 0.006	0.04 ± 0.006	0.045 ± 0.006

Table 3. *Divergence within IFNA/IFNW clusters*. Divergence time ± standard error for *IFNA* and *IFNW* within clusters was calculated using the Maximum Composite Likelihood method. Cluster 1 genes have a slightly higher divergence rate than cluster 1 and 2, but genes within all three clusters are relatively equal in their rate of change.

	Sub-locus 1	Sub-locus 2
SINEs:	15.29%	15.68%
Alu/B1	0.00%	0.00%
MIRs	0.51%	0.81%
LINEs:	17.74%	11.23%
LINE1	10.33%	2.20%
LINE2	0.44%	0.29%
L3/CR1	0.04%	0.00%
RTE	6.93%	8.73%
LTR elements:	4.01%	2.75%
MaLRs	0.89%	0.98%
ERVL	0.12%	0.03%
ERV classI	0.79%	0.22%
ERV classII	0.00%	0.00%
DNA elements:	2.36%	2.68%
MER1_type	1.72%	2.45%
MER2_type	0.45%	0.00%
Unclassified:	0.00%	0.00%
Total interspersed repeats:	39.40%	32.35%
Small RNA:	0.24%	0.40%

Table 4. *Repetitive elements within the bovine Type I IFN sub-loci.* The percentage of repetitive elements for the Type I IFN sub-loci was calculated in RepeatMasker 3.1.9. Sub-locus 1 has a higher proportion of LINE1 and LTR elements than sub-loci 2 resulting in a higher total number of interspersed repeats within sub-locus

.A



B

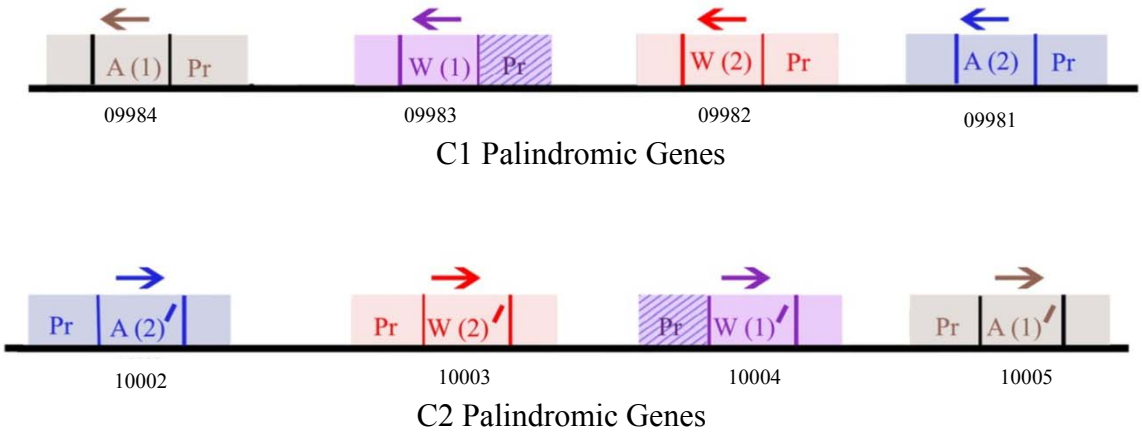


Figure 5. *Palindromic gene sets within IFNA/IFNW clusters 1 and 2.* An enlarged image of *IFNA/IFNW* clusters 1 and 2 from Figure 3 is shown (A). The promoter (Pr), ORF, and 3′ UTR for each gene has been depicted in a schematic of both gene sets (not drawn to scale). The direction of gene transcription is indicated by an arrow above each gene and the GLEAN number is written below each gene. Genes with 100% nucleotide identity within their promoters, ORFs, and 3′ UTRs are shown in matching solid colors. The only gene pair that does not have 100% nucleotide identity in the promoter, W(1)/W(1)′, is indicated by diagonal stripes through the promoter box.

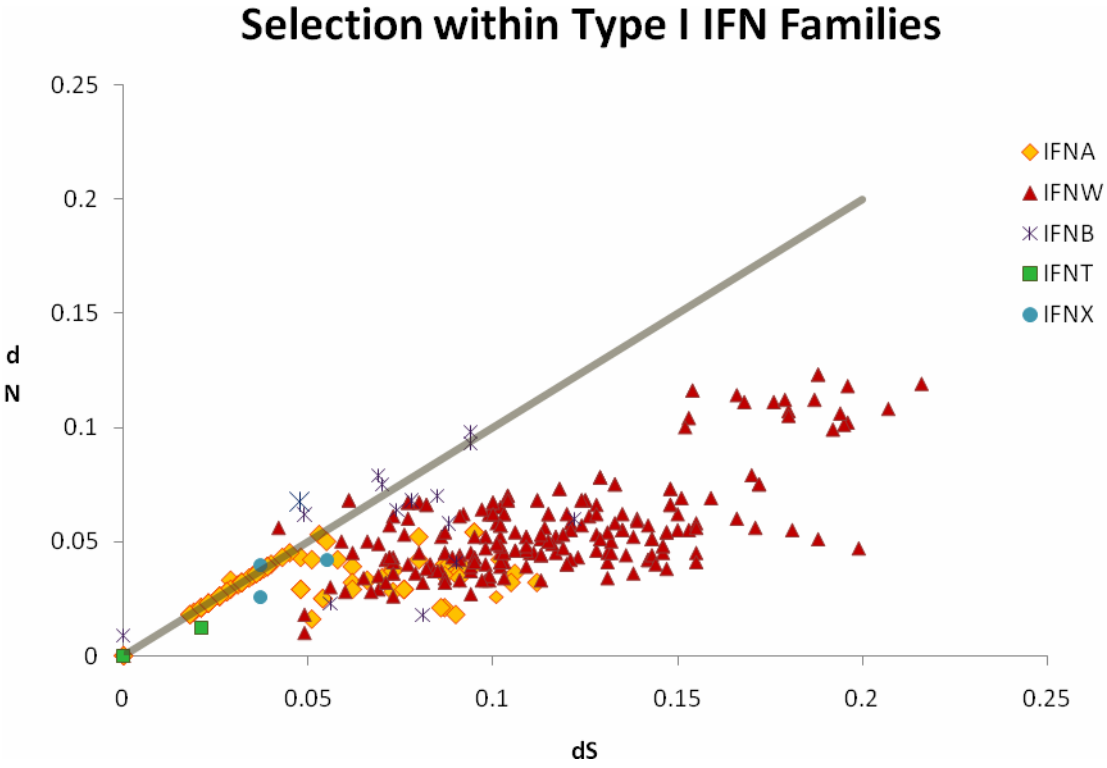


Figure 6. *Selective Pressure on the coding regions of bovine Type I IFN* The above graph illustrates the selective pressure on Type I IFN subfamilies within their coding regions. Pairwise analysis of the coding region for each Type I IFN family was used to calculate dS and dN and the two values were plotted against one another. The grey diagonal line in the graph represent neutral selection rate where dS = dN. Gene pairs undergoing positive selection would appear above the diagonal and gene pairs undergoing purifying selection would appear below the diagonal. The graph clearly shows that no positive selection is occurring within the coding regions of any Type I IFN family. Furthermore, *IFNW* and *IFNA* subfamilies appear to be undergoing purifying selection.

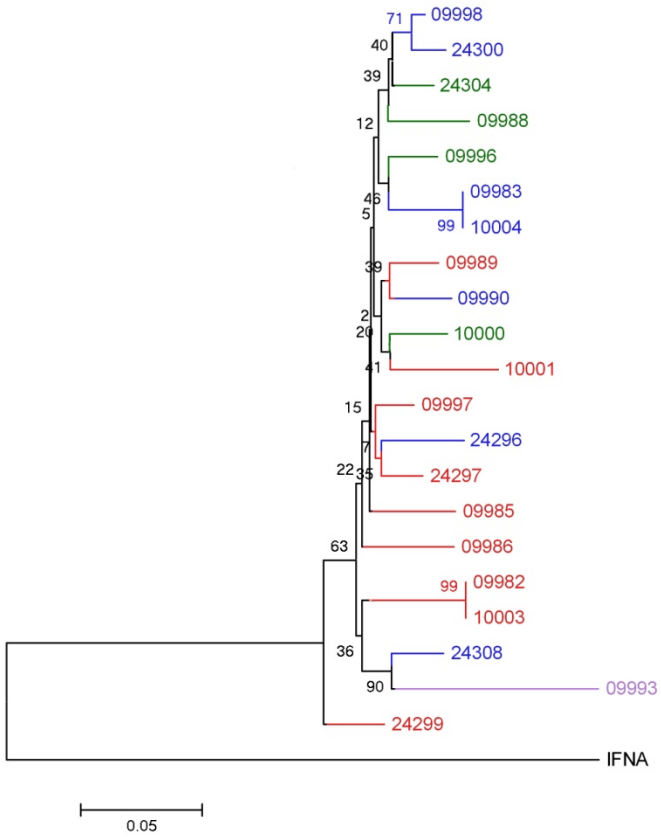
Subfamily	p-Value	dS-dN
IFNA	0.0	3.824
IFNW	0.0	4.013

Table 5. *Purifying selection within IFNW and IFNA coding regions*. The significance of purifying selection in the coding regions of *IFNW* and *IFNA* subfamilies was verified through a codon based Z-test analyzing the overall average for the subfamilies.

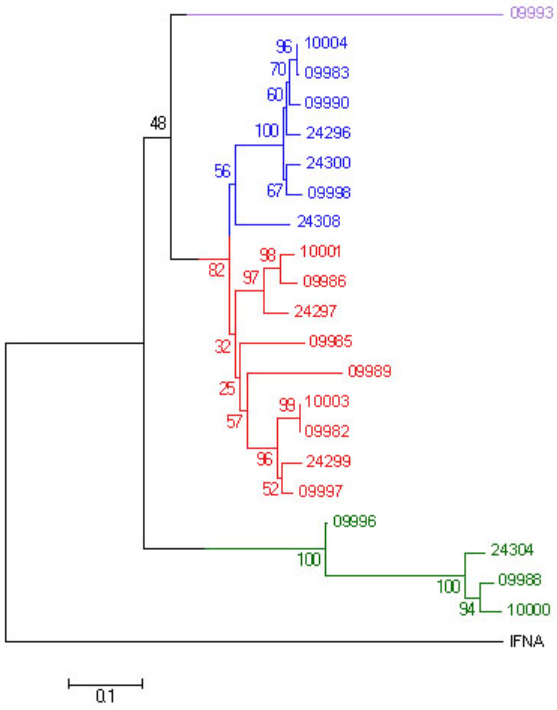
	24309	24311	24313	24315	24317	24318
IFNB (24309)						
IFNB (24311)	0.486					
IFNB (24313)	1.000	1.000				
IFNB (24315)	0.032	0.339	0.342			
IFNB (24317)	0.303	1.000	1.000	0.036		
IFNB (24318)	0.150	1.000	1.000	0.008	0.050	

Table 6. *Selection in IFNB*. Pairwise comparisons between *IFNB* through the codon based Z-test are shown in the above table with the statistically significant comparisons highlighted in yellow.

A


71
09998
40
24300
39
24304
12
09988
09996
46
5
09983
99
10004
39
09989
09990
2
20
10000
41
10001
15
09997
7
24296
22
35
24297
09985
63
09986
99
09982
10003
36
24308
90
09993
24299
IFNA
0.05

B


09993
96
10004
70
09983
60
09990
48
100
24296
24300
56
67
09998
24308
98
10001
82
97
09986
24297
32
09985
09989
25
99
10003
57
09982
96
24299
52
09997
09996
100
24304
100
09988
94
10000
IFNA
0.1

C

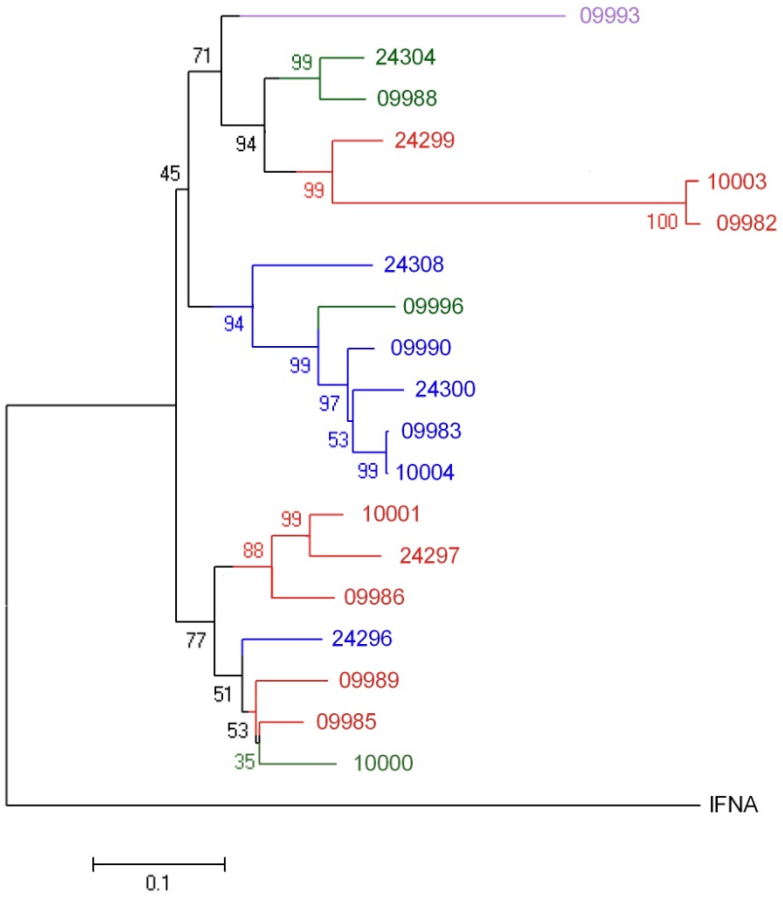
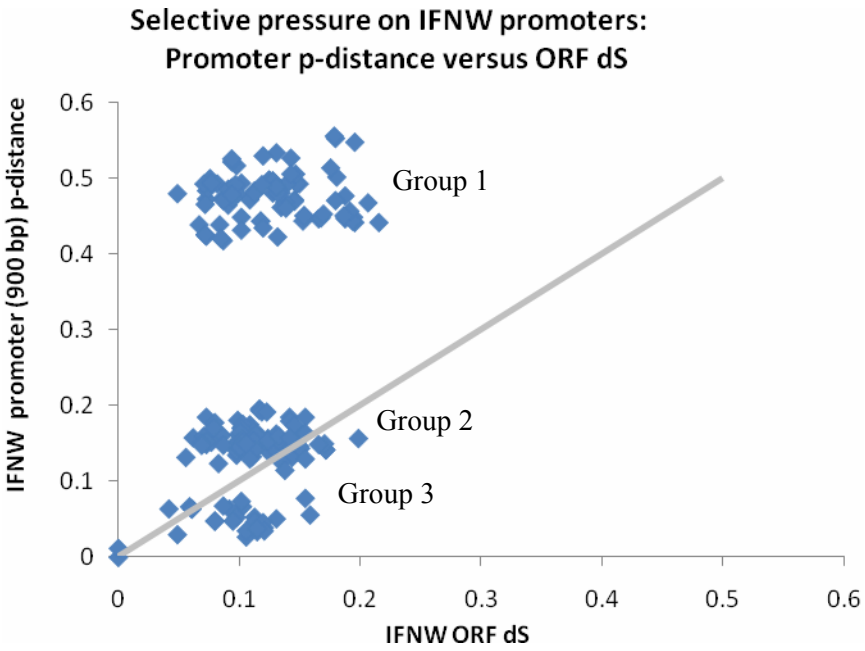


Figure 7. *Phylogenetic reconstruction of IFNW* The evolutionary history of bovine *IFNW* was inferred using the Neighbor-Joining method with bootstrap test (1000 replicates). Three trees were reconstructed based on the ORF (A), promoter (B), and 3′ UTR (C). The phylogenetic reconstruction based on the promoter analysis divide *IFNW* into class 1 (green), class 2 (red and blue), and the IFNW-T intermediate (purple). IFNW class 2 can be additionally partitioned into subclass 1 (blue) and subclass 2 (red). These genes were color coded in the phylogenies of the ORF and 3′ UTR to correspond to their classification based on the promoter analysis. *IFNA* was included as an outgroup for all phylogenies and calculations were based on uniform rates of change for all sites.

A


Selective pressure on IFNW promoters:
Promoter p-distance versus ORF dS
IFNW promoter (900 bp) p-distance
0.6
0.5
0.4
0.3
0.2
0.1
0
Group 1
Group 2
Group 3
0
0.1
0.2
0.3
0.4
0.5
0.6
IFNW ORF dS

B

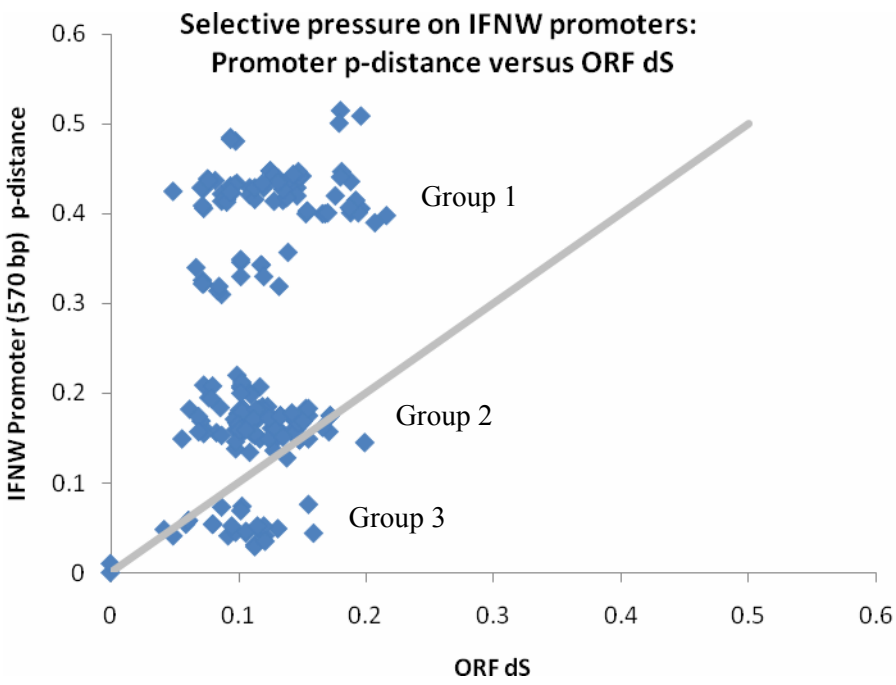

Selective pressure on IFNW promoters:
Promoter p-distance versus ORF dS
IFNW Promoter (570 bp) p-distance
0.6
0.5
0.4
0.3
0.2
0.1
0
Group 1
Group 2
Group 3
0
0.1
0.2
0.3
0.4
0.5
0.6
ORF dS

Figure 8. *Selective pressure on the IFNW promoters*. The number of base differences per site (p-distance) for the *IFNW* promoters plotted against the dS of the coding region for the corresponding gene pairs. The gray diagonal indicates neutral mutation rate where promoter p-distance:ORF dS = 1. Promoter p-distance values based on a 900 bp promoter (A) and a 570 bp promoter (B) were analyzed. The results in both cases indicate the promoters are diverging faster than the coding region. Three statistically different ($p < 0.0001$) groups are formed based on the nucleotide diversity between promoter pairs.

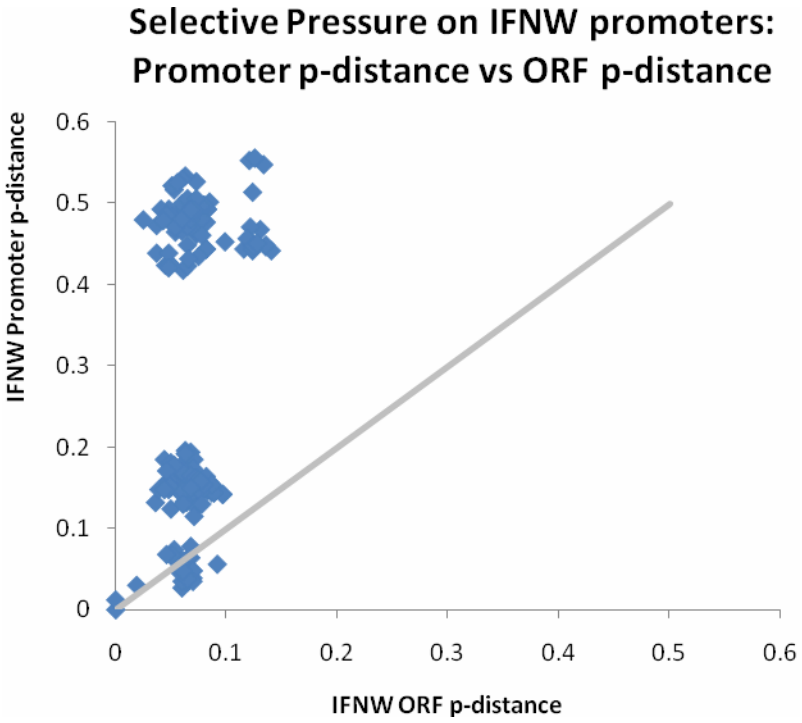


Figure 9. *Comparison of p-distance in open reading frame (ORF) to the promoter.* The total nucleotide diversity in the promoter region was plotted against the total nucleotide diversity in promoter. A very similar pattern was observed to the p-distance versus dS, but the graph is shifted the increased divergence in the promoter compared to the ORF is more pronounced.

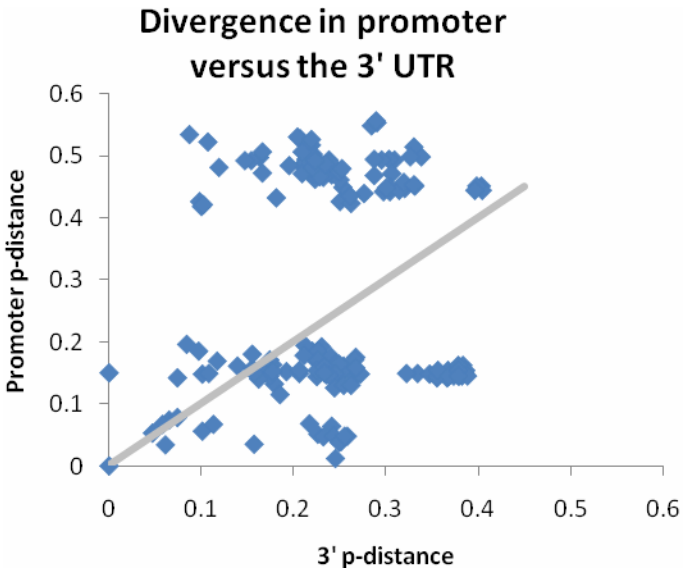


Figure 10. *Comparison of p-distance in the promoter to the 3' UTR.* The total nucleotide diversity in the promoter was plotted against the total nucleotide diversity in the 3' UTR. The results support the previous two tests suggesting a subset of promoters are diverging faster than the other regions of the gene under, assumedly, neutral selective pressure.

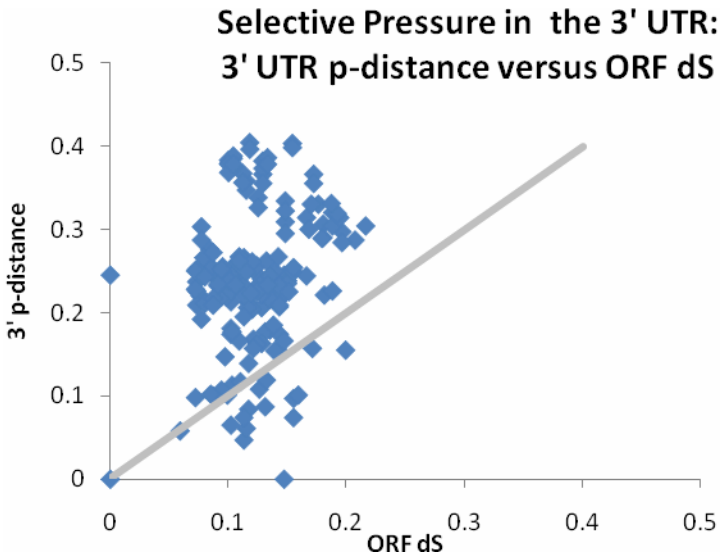


Figure 11. *Selective pressure on the 3' UTR.* A comparison of ORF dS to the 3'UTR reveals that the 3'UTR has an very different divergence pattern than dS and portion of the 3'UTR are altering faster than the assumed neutral mutation rate. The 3'UTR has a continuous range of divergence patterns rather than the clustering observed in the promoter analysis.

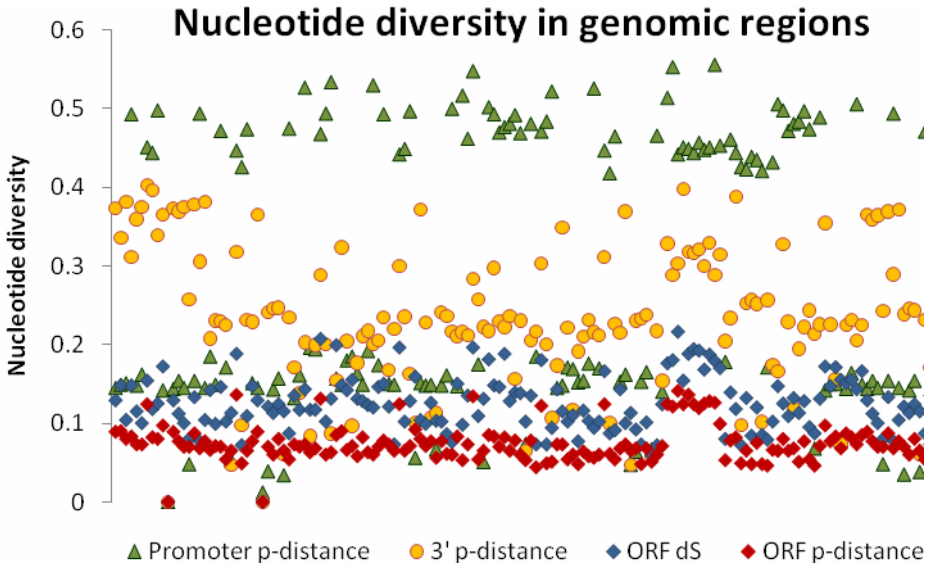
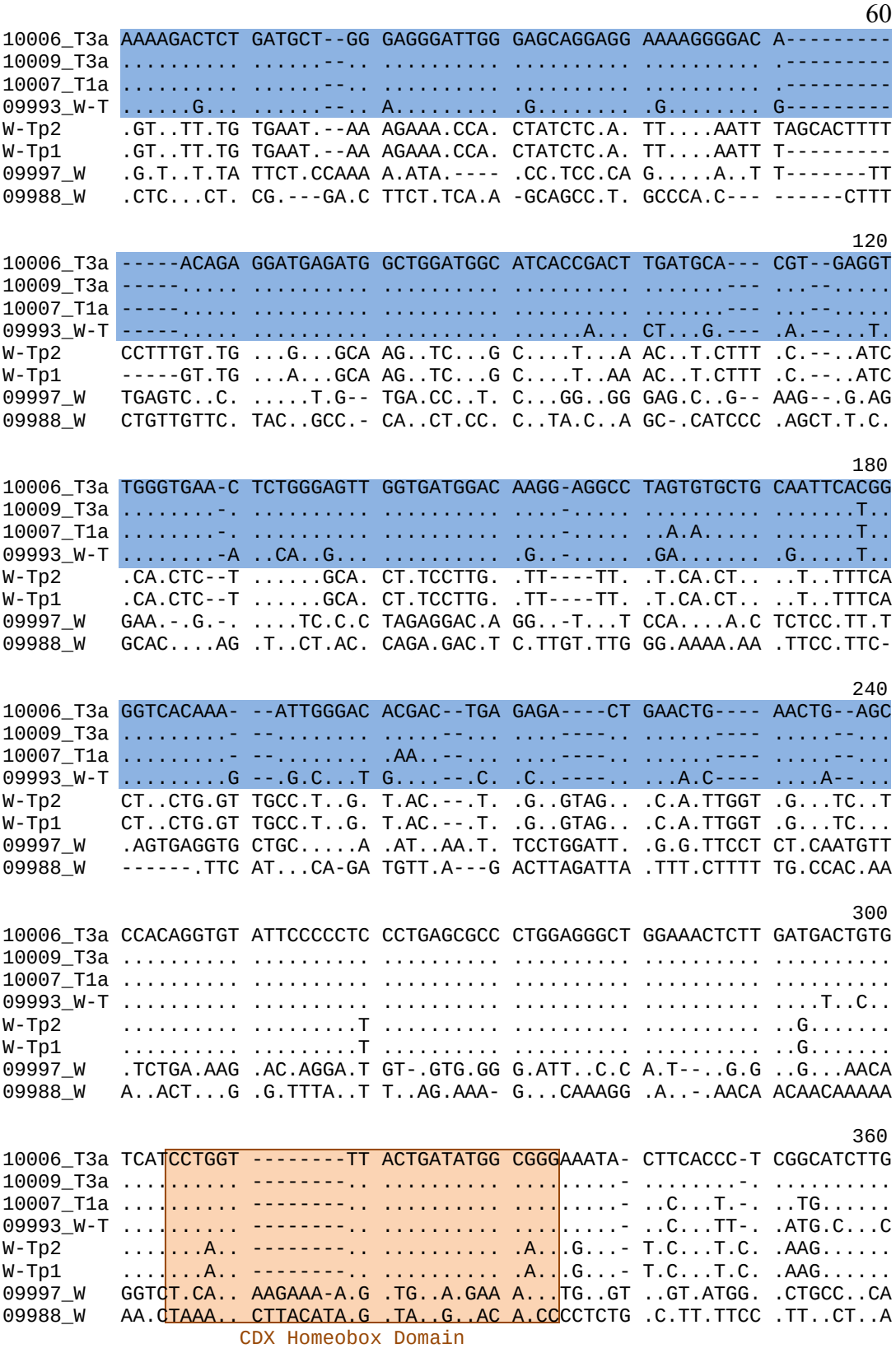


Figure 12. *Nucleotide diversity in genomic regions.* The graph illustrates stratification of nucleotide diversity within the IFNW family based on gene region. The ORF p-distance, ORF dS, 3/ UTR p-distance, and promoter p-distance for each gene pair is graphed with each gene pair plotted as one point along the horizontal axis. The coding region is highly conserved and dS, the best estimate for the neutral mutation rate, is only slightly higher than the total nucleotide diversity in that ORF. The 3' UTR has a wide range in nucleotide diversity and is evenly distributed between the ORF p-distance to slightly below the most divergent promoter p-distances. The promoters are either clustered slightly higher than the dS, or neutral mutation rate, or are the most highly divergent genomic region in the highest stratum.

Figure 13. *Schematic of repetitive elements associated with IFNT*. LINE/L2b and tandem Sine/Bov-tA2 are conserved 3/ of the ORF in GLEAN 09993, *W-Tp1*, *W-Tp2*, and all examined IFNT. A SINE/ART-2a and tandem LINE/BovB are upstream of all *IFNT* and GLEAN 09993. These repetitive elements are absent in *W-Tp1* and *W-Tp2*. *Cis*-elements are shown above the line (with respect to the ORF) and trans-elements are shown below the line.


60
10006_T3a AAAAGACTCT GATGCT--GG GAGGGATTGG GAGCAGGAGG AAAAGGGGAC A---------
10009_T3a ....-. ....---------
10007_T1a ....-. ....---------
09993_W-T ......G... ....- A......... .G........ .G........ G---------
W-Tp2 .GT..TT.TG TGAAT.--AA AGAAA.CCA. CTATCTC.A. TT....AATT TAGCACTTTT
W-Tp1 .GT..TT.TG TGAAT.--AA AGAAA.CCA. CTATCTC.A. TT....AATT T---------
09997_W .G.T..T.TA TTCT.CCAAA A.ATA.---- .CC.TCC.CA G.....A..T T-------TT
09988_W .CTC...CT. CG.---GA.C TTCT.TCA.A -GCAGCC.T. GCCCA.C--- ------CTTT
120
10006_T3a -----ACAGA GGATGAGATG GCTGGATGGC ATCACCGACT TGATGCA--- CGT--GAGGT
10009_T3a -----..... ....--- ...--.....
10007_T1a -----..... ....--- ...--.....
09993_W-T -----..... ....A... CT...G.--- .A.-...T.
W-Tp2 CCTTTGT.TG ...G...GCA AG..TC...G C....T...A AC..T.CTTT .C.--..ATC
W-Tp1 -----GT.TG ...A...GCA AG..TC...G C....T..AA AC..T.CTTT .C.--..ATC
09997_W TGAGTC..C. .....T.G-- TGA.CC..T. C...GG..GG GAG.C..G-- AAG--.G.AG
09988_W CTGTTGTTC. TAC..GCC.- CA..CT.CC. C..TA.C..A GC-.CATCCC .AGCT.T.C.
180
10006_T3a TGGGTGAA-C TCTGGGAGTT GGTGATGGAC AAGG-AGGCC TAGTGTGCTG CAATTCACGG
10009_T3a ....-. ....-..... ....T..
10007_T1a ....-. ....-..... ..A.A..... ....T..
09993_W-T ....A ..CA..G... .... .G..-..... .GA....... .G.....T..
W-Tp2 .CA.CTC--T ......GCA. CT.TCCTTG. .TT----TT. .T.CA.CT.. ..T..TTTCA
W-Tp1 .CA.CTC--T ......GCA. CT.TCCTTG. .TT----TT. .T.CA.CT.. ..T..TTTCA
09997_W GAA.-.G.-. ....TC.C.C TAGAGGAC.A GG..-T...T CCA....A.C TCTCC.TT.T
09988_W GCAC....AG .T..CT.AC. CAGA.GAC.T C.TTGT.TTG GG.AAAA.AA .TTCC.TTC-
240
10006_T3a GGTCACAAA- --ATTGGGAC ACGAC--TGA GAGA----CT GAACTG---- AACTG--AGC
10009_T3a ....- ....--... ....----.. ....--- .....--...
10007_T1a ....- .... .AA..--... ....----.. ....--- .....--...
09993_W-T ....G --.G.C...T G......C. .C.-----.. ...A.C---- ....A--...
W-Tp2 CT..CTG.GT TGCC.T..G. T.AC.--.T. .G..GTAG.. .C.A.TTGGT .G...TC..T
W-Tp1 CT..CTG.GT TGCC.T..G. T.AC.--.T. .G..GTAG.. .C.A.TTGGT .G...TC...
09997_W .AGTGAGGTG CTGC.....A .AT..AA.T. TCCTGGATT. .G.G.TTCCT CT.CAATGTT
09988_W ------.TTC AT...CA-GA TGTT.A---G ACTTAGATTA .TTT.CTTTT TG.CCAC.AA
300
10006_T3a CCACAGGTGT ATTCCCCCTC CCTGAGCGCC CTGGAGGGCT GGAAACTCTT GATGACTGTG
10009_T3a ....
10007_T1a ....
09993_W-T ....T..C..
W-Tp2 ....T ....G.......
W-Tp1 ....T ....G.......
09997_W .TCTGA.AAG .AC.AGGA.T GT-.GTG.GG G.ATT..C.C A.T--..G.G ..G...AACA
09988_W A..ACT...G .G.TTTA..T T..AG.AAA- G...CAAAGG .A..-.AACA ACAACAAAAA
360
10006_T3a TCATCCTGGT --------TT ACTGATATGG CGGGAAATA- CTTCACCC-T CGGCATCTTG
10009_T3a .... --------.. ....- ....
10007_T1a .... --------.. ....C...T.-. ..TG......
09993_W-T .... --------.. ....C...TT-. .ATG.C...C
W-Tp2 .....A.. --------.. .... .A...G...- T.C...T.C. .AAG......
W-Tp1 .....A.. --------.. .... .A...G...- T.C...T.C. .AAG......
09997_W GGTCT.CA.. AAGAAA-A.G .TG..A.GAA A...TG..GT ..GT.ATGG. .CTGCC..CA
09988_W AA.CTAAA.. CTTACATA.G .TA..G..AC A.CCCTCTG .C.TT.TTCC .TT..CT..A
CDX Homeobox Domain

```
                                                                                                                  420
10006_T3a TGGCCCCAG- TGAAATGTAA TCTCAGGAAC CTGAATGAA- -TGTTCTCAA A-AGAACAGA
10009_T3a .........- .......... .......... .........- -.......... .-........
10007_T1a .........- .......... .......... .........- -.......... .-........
09993_W-T .........- .......... .......... .........- -.......... .-........
W-Tp2     .........- .......... .......... .........- -.......... .-........
W-Tp1     .........- .......... .......... .........- -.......... .-........
09997_W   C..TT..C-- ....GG.C.G ------.G.T T..C.G...T G......AG. .-G...G...
09988_W   .T.TT.TTAT .TC..G..G. AT.AGAA--- ..AG.GC.G- GA.CAGCA.G .CCA.T.CTG

                                                                                                                  480
10006_T3a AACAACTGAG TAGTTCATTC ACCCTTAATT GCAAGCTTAT CCTCT--GTG AGACAGAAAA
10009_T3a .......... .......... .......... .......... .....--... ..........
10007_T1a .......... .......... .......... A......... .....--... ..........
09993_W-T .......... .......... .......... A......... .....--... ..........
W-Tp2     .-........ .......... ..T....... A.....C.C. G....--... .A........
W-Tp1     .-........ .......... ..T....... A.....C.C. G....--... .A........
09997_W   ..TGTGG... G......AGG ..TGA....A ATC...C.C. T....AAA.C .A...CT...
09988_W   GT....AAGT C.C.AA.AGA .-TG.AG.AA CT...TGA.A AA.TATAC.C .AGT.A.GG.

                                                                                                                  540
10006_T3a GAAAA----- -------TAC AAACATCAAT ATGGCCTGAG TGACTC--TG CATACCT--A
10009_T3a ..........- -------... .......... .......... ........-. .......--.
10007_T1a ..........- -------... .......... .......... ........-. ...T...--.
09993_W-T ..........- -------..T .G.A.....C .......T.. ........-. ...T...--.
W-Tp2     C...C----- -------... .G.A.C.... .......... .........- A..T...--.
W-Tp1     C...C----- -------... .G.A.C.... .......... .........- A..T...--.
09997_W   .....CTCAT CATTTGT.TT G..G.ATTTA ..T.A.A... ...TCTGG.A A..C...GA.
09988_W   A.G..GGCTA TTTCTCCCTG ..C..GT.GC C...TTCTCA CC.TC.--.T ...T.TCAG.

                                                                                                                  600
10006_T3a TGTGTAAGAT AA-------- -----GGAGG GAAAAATGCA GTTAAGAATC AACGGAAAAT
10009_T3a .......... ..-------- -----..... .......... .......... ..........
10007_T1a .......... ..-------- -----..... .......... .......... ..T.......
09993_W-T .......... ..-------- -----..... .......... .......... ..T......C
W-Tp2     ..A....... ..-------- ------..G.. .........T C.......... ..G.A.....
W-Tp1     ..A....... ..-------- ------..G.. .G........ C.......... ..G.A.....
09997_W   .AAA..C.GA ..CCACAGAG TTGAG....C CTCT.T.C.C A.GT.A.G.A .GA..G...A
09988_W   G.AAG..AGA G.T------- ---AGA.GAA C.G.C..ATC CAGTG..... ..T..TCTGA
                                                                  CDX Homeobox

                                                                                                                  660
10006_T3a TATAT-TCCT GAGATA---- AGATAAACAA AAGGAATGTT TATATATATT ATACCTAAAT
10009_T3a .....-.... .......... .......... .......... .......... ..........
10007_T1a .....-.... ..C....... .......... .......... .......... ..........
09993_W-T ...G.-.... ..C....... .......... .......... ..C....... ..........
W-Tp2     .T.G.-.T-. ..C..----- GC.CG..... .......... .......... ....T.....
W-Tp1     .T.G.-.T-A ..C.C.---- GC.C.C.A.. .......... .......... ....T..C..
09997_W   A.GGA-G... A.T..C---- ..GGCTC.CG .T.TGT.CA. GGATA.G.GG .G.G.A.C..
09988_W   ATG.ATGA.. ..A.CTCTCT ....TCTGTG .CAT..GC.. CCAG.GAGAG .C.A.ACC..
          Domain

                                                                                                                  720
10006_T3a TTGTACATAA TAACTATGTA CACATC-TAT AA-GTCTTTG CATACTTACA TAACTTCAGC
10009_T3a .......... .......... ......-... ..-....... .......... ..........
10007_T1a .......... .......... ......-... ..-....... .......... ..........
09993_W-T .......... .......... ......-... ..-....... .......... ..........
W-Tp2     G.A....... ---G...... T..C..-... ..-.......A .......... ...T......
W-Tp1     G......... ...G...... T..C..-... ..-.......A .......... ...T......
09997_W   ..A.G...G. G.GAC.AAG. GCA..AA.G. .T-A.AA..A ..A.TG.C.. .T.A...T.A
09988_W   CAC.GA..CT CCTTCC.CC. GCTCAGAA.C TCT.AGCAGA A.AGTG.TG. A..G..T.AG
```

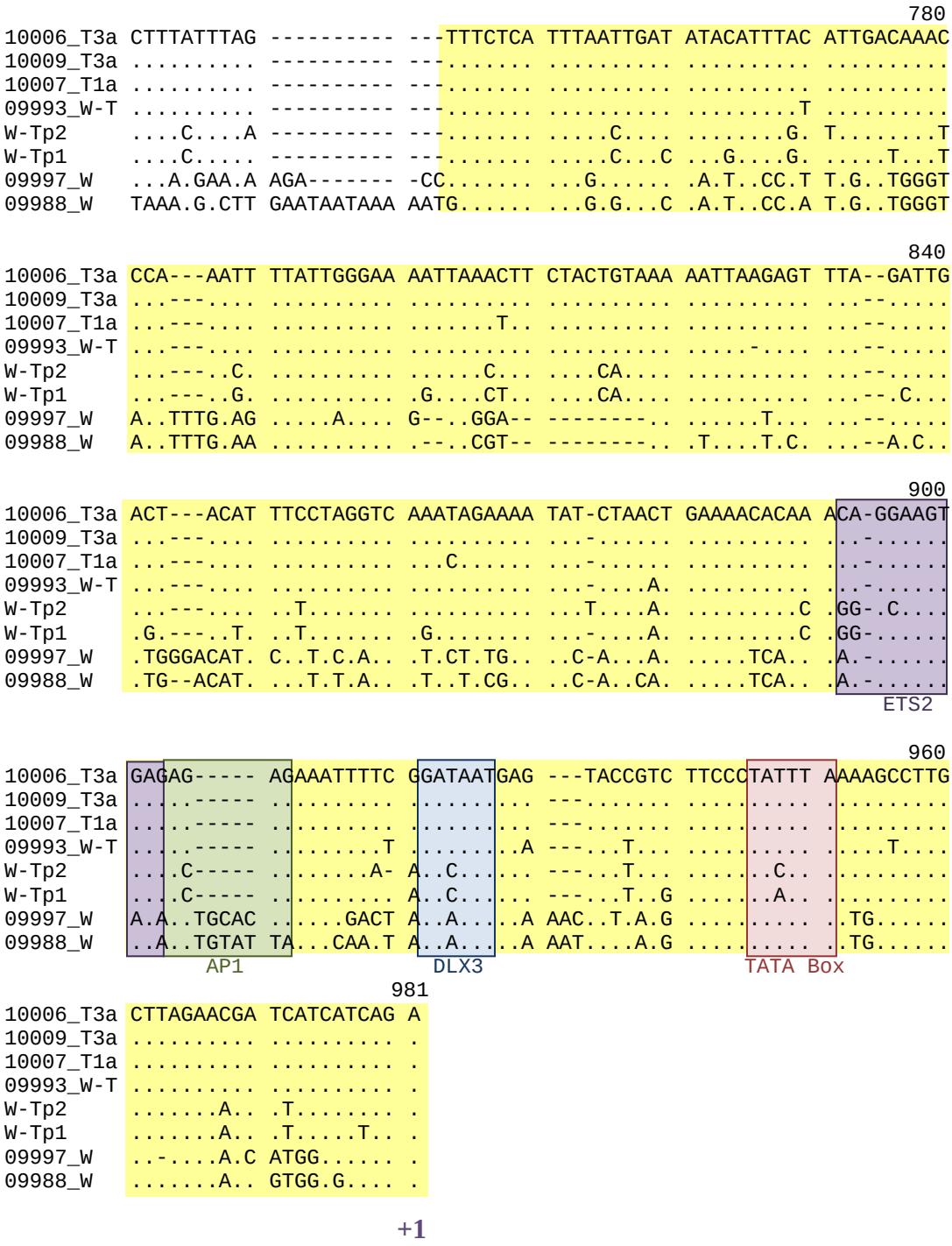

780
10006_T3a CTTTATTTAG ---------- ---TTTCTCA TTTAATTGAT ATACATTTAC ATTGACAAAC
10009_T3a ..... ---------- ---..... ....
10007_T1a ..... ---------- ---..... ....
09993_W-T ..... ---------- ---..... ....T .....
W-Tp2 ....C....A ---------- ---..... .....C.... .....G. T.....T
W-Tp1 ....C..... ---------- ---..... .....C...C ...G....G. .....T...T
09997_W ...A.GAA.A AGA------- -CC..... ...G..... .A.T..CC.T T.G..TGGGT
09988_W TAAA.G.CTT GAATAATAAA AATG..... ...G.G...C .A.T..CC.A T.G..TGGGT
840
10006_T3a CCA---AATT TTATTGGGAA AATTAAACTT CTACTGTAAA AATTAAGAGT TTA--GATTG
10009_T3a ...---.... ....--.....
10007_T1a ...---.... ....T.. ....--.....
09993_W-T ...---.... ....-.... ...--.....
W-Tp2 ...---..C. ....C... ....CA.... ....--.....
W-Tp1 ...---..G. ..... .G....CT.. ....CA.... ....--.C...
09997_W A..TTTG.AG .....A.... G---..GGA-- --------.. .....T... ...--.....
09988_W A..TTTG.AA ..... .---..CGT-- --------.. .T....T.C. ...--A.C..
900
10006_T3a ACT---ACAT TTCCTAGGTC AAATAGAAAA TAT-CTAACT GAAAACACAA ACA-GGAAGT
10009_T3a ...---.... ....-..... ....-.....
10007_T1a ...---.... ....C..... ...-..... ....-.....
09993_W-T ...---.... ....-....A. ....-.....
W-Tp2 ...---.... ..T..... ....T....A. .....C .GG-.C....
W-Tp1 .G.---..T. ..T..... .G..... ...-....A. .....C .GG-.....
09997_W .TGGGACAT. C..T.C.A.. .T.CT.TG.. ..C-A...A. .....TCA.. .A.-.....
09988_W .TG--ACAT. ...T.T.A.. .T..T.CG.. ..C-A..CA. .....TCA.. .A.-.....
ETS2
960
10006_T3a GAGAG----- AGAAATTTTC GGATAATGAG ---TACCGTC TTCCCTATTT AAAAGCCTTG
10009_T3a ....
10007_T1a ....
09993_W-T ....T ....A ---...T... ....T....
W-Tp2 ....C----- .....A- A..C..... ---...T... .....C.. .....
W-Tp1 ....C----- ..... A..C..... ---...T..G .....A.. .....
09997_W A.A..TGCAC .....GACT A..A.....A AAC..T.A.G ....TG.....
09988_W ..A..TGTAT TA...CAA.T A..A.....A AAT....A.G ....TG.....
AP1
DLX3
TATA Box
981
10006_T3a CTTAGAACGA TCATCATCAG A
10009_T3a ....
10007_T1a ....
09993_W-T ....
W-Tp2 .....A.. .T..... .
W-Tp1 .....A.. .T.....T.. .
09997_W ..-....A.C ATGG..... .
09988_W .....A.. GTGG.G.... .
+1

Figure 14. *Promoter region alignments.* The region 980 bp upstream of the predicted transcriptional start site is aligned for the three *IFNT*, GLEAN 09993, two *IFNW-T* intermediate pseudogenes, and two representative *IFNW*. The sequences are indicated by their GLEAN model numbers and subfamily abbreviation where W is *IFNW*, T is *IFNT*, and W-T is *IFNW-IFNT* intermediates. Pseudogenes lack a GLEAN number and have a "p" beside their subfamily designation. The transcriptional start site is represented by a +1 at position 980 and the TATA Box is denoted by a red box 36 bp upstream of +1. Transcription factor binding sites known to be important in the regulation of *IFNT* are labeled. The promoter alignment can be divided into three sections. The first section illustrated in yellow (744-980) has sufficient agreement for nucleotide alignment for all genes and pseudogenes indicating that rapid divergence of a previous *IFNW* promoter resulted in the formation of this portion of the *IFNT* promoter. The first section contains the best characterized transcription factor binding sites and is known to be critical for the unique transcriptional regulation of *IFNT*. The second section (240-743) does not contains virtually no sequence conservation between the *IFNT* or the *IFNW-T* intermediates making it difficult to determine its evolutionary history. This region of the promoter could either have undergone incredibly rapid divergence to remove all similarity between *IFNW* and *IFNT* or it could have been acquired through a transposition or homologous recombination event. There is not sufficient evidence to differentiate the two options at this time. While CDX homeobox domains are present in this section of the IFNT promoter, the importance of these or other elements in this section of the promoter have not been well characterized. The third section of the promoter extends upstream from position 743 beyond the extent of this figure. The *IFNT* promoters and GLEAN 09993 have acquired tandem repeat elements shown in blue that are absent in the *IFNW-T* pseudogenes and *IFNW* in this section of the alignment.

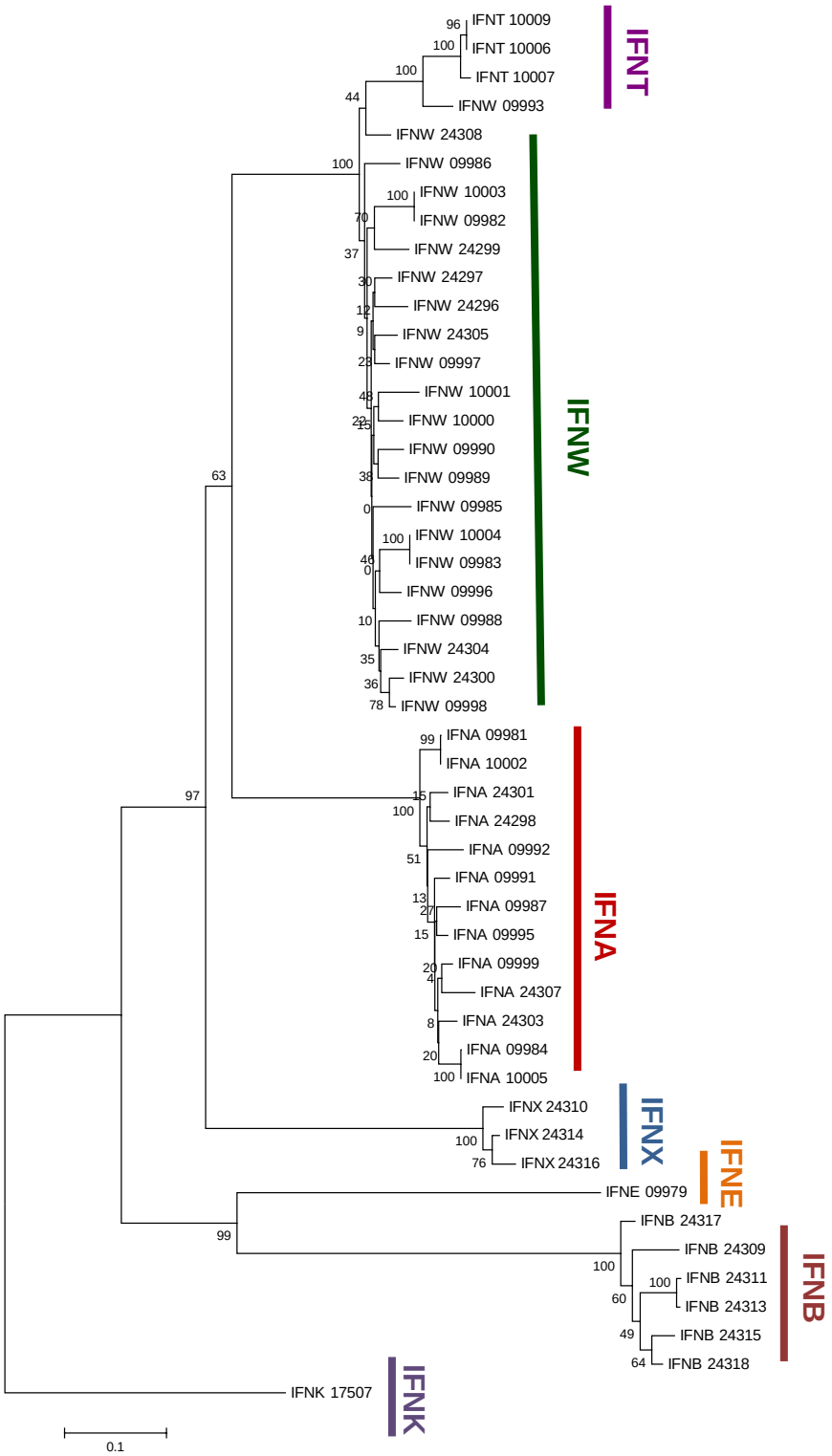


Figure 15. *Bovine Type I IFN Phylogenetic Tree without uniform rate of change*. The evolutionary history of bovine Type I IFN was inferred using the Neighbor-Joining method with bootstrap test (1000 replicates). The tree was rooted to IFNK and calculations were based on uniform rates of change for all sites. *IFNX* emerged prior to *IFNA* in this analysis.

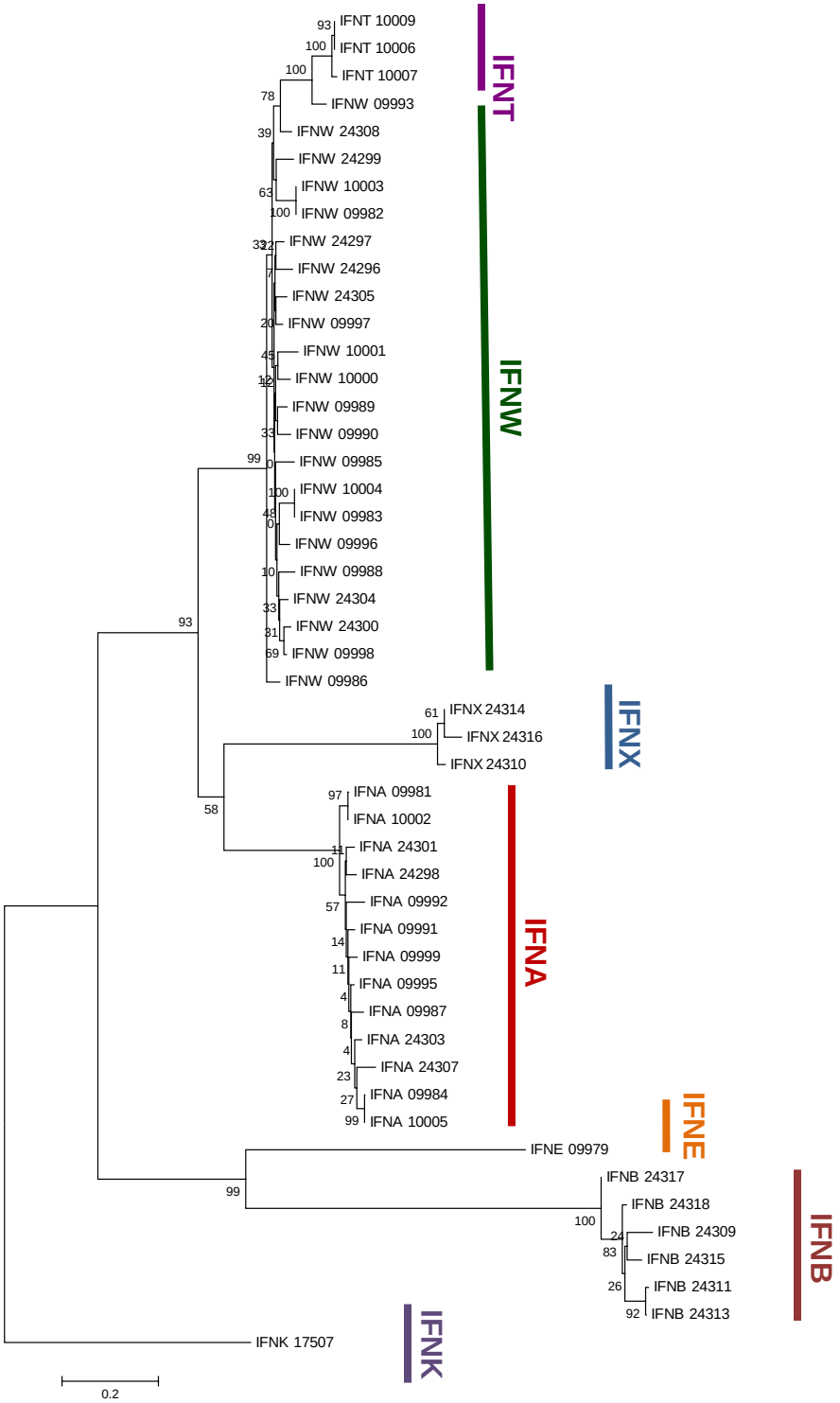


Figure 16. *Bovine Type I IFN Phylogenetic Tree with consideration of differing rates of change among sites*. The tree illustrates the evolutionary history of bovine IFN based on different rates of change between sites (gamma = 1). The tree was again based on the Neighbor-Joining method with bootstrap test (1000 replicates) and rooted to *IFNK*. *IFNX* and *IFNA* branched from common ancestor in this analysis.

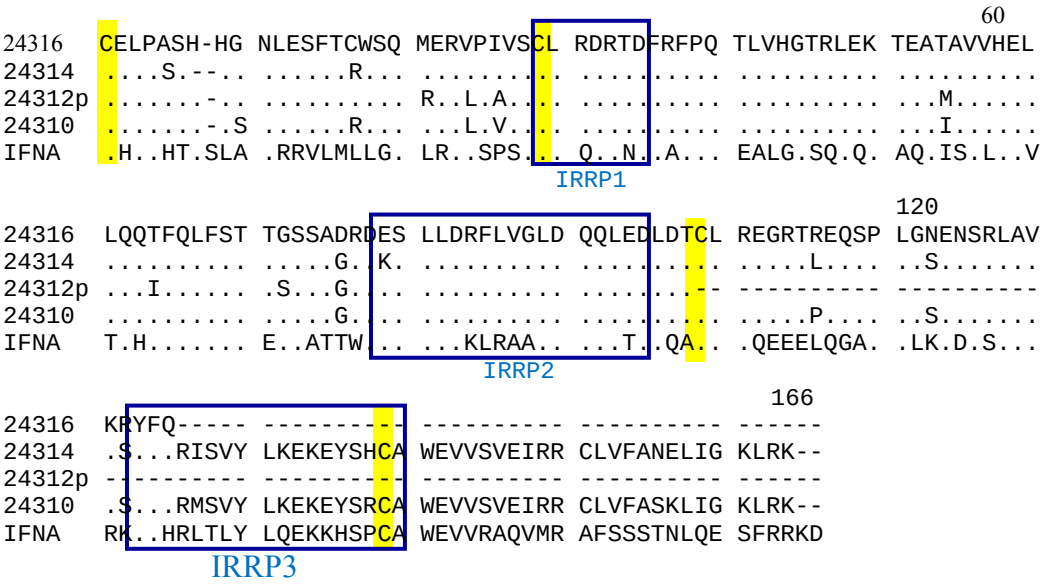


Figure 17. *IFNX alignment.* The coding region minus the predicted signal peptide for the three predicted IFNX genes denoted by their GLEAN numbers (24314, 24310, and 24316) are aligned with bovine *IFNA* (DQ396807). The mature coding sequence of the three potentially expressed IFNX genes differs as follows 164 amino acids (aa) for Glean 24310, 163 aa for Glean 24314, and 124 aa for 24316. Conserved cysteine residues form disulfide bonds in IFNA between positions 1→99 and 29→139 and are shown in yellow. Glean 24316 has an early stop codon that eliminates the second disulfide bond, but both Glean 24314 and Glean 24310 contain the conserved residues. Regions strongly associated with receptor binding in IFNA, or interferon receptor recognition peptides (IRRP), are boxed in blue.

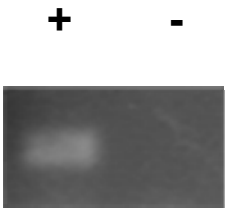


Figure 18. *Expression of IFNX.* A 210 bp fragment (+) was amplified from virally challenged bovine kidney cells through RT-PCR with *IFNX* specific primers. A control RNA sample (-) that was not incubated with reverse transcriptase verified the product was not amplified from genomic DNA. The product was sequenced and matched Glean 24316.

Appendix A

The development of an ELISA for IFNT

Abstract

The first identified signal for maternal recognition of pregnancy in ruminants is IFNT. Both infusion of IFNT into the uterus and high-dose intramuscular injection of the protein result in extension of the luteal lifespan in cattle and sheep. IFNT has classically been measured in experimental studies through biological assays, particularly antiviral assays (AVA). Biological assays have several shortcomings including the inability to differentiate IFNT from other Type I IFN. IFN variants when used at comparable concentrations may also differ in their relative biological activities. Finally, the AVA, despite its sensitivity, lacks precision so that small differences in IFN concentration cannot be detected. An improved means to quantify IFNT would facilitate studies of the cytokine and our understanding of early pregnancy in ruminants. The purpose of this work has been to develop an enzyme-linked immunosorbent assay (ELISA) that could accurately and specifically detect IFNT at low concentrations. An antibody trap or "sandwich" ELISA was developed that utilized a mouse monoclonal antibody to trap both glycosylated and non-glycosylated forms of IFNT, and a rabbit polyclonal antiserum to detect the bound protein. The sensitivity limit of the developed ELISA is 1.3 ng for ovine IFNT and 0.2 ng for bovine IFNT.

Introduction

Conceptus loss in the first trimester of pregnancy is a major cause of financial hardship in livestock operations and is a major hurdle in repopulating endangered species. Improving the tools to study the earliest steps in establishing pregnancy is critical to reducing these problems. A type I interferon (IFN), termed IFN-tau (IFNT), with high structural similarity to IFN-alpha (IFNA) and IFN-beta (IFNB), is the first known signal from the conceptus to the dam preventing pregnancy termination (Roberts et al., 1992; Bazer et al., 1996). Both infusion of IFNT into the uterus and high-dose intramuscular injections of IFNT result in extension of the luteal lifespan (Godkin et al., 1984; Martal et al., 1990; L'Haridon et al., 1995; Meyer et al., 1995; Chen et al., 2006). IFNT causes this luteal extension through its ability to modulate prostaglandin $F_{2\alpha}$ release, which is probably accomplished through decreased expression of estrogen and oxytocin receptors in uterine epithelial cells and possibly reduced expression of COX-2 (Helmer et al., 1989; Lamming et al., 1995; Meyer et al., 1995; Emond et al., 2004; Chen et al., 2006).

IFNT has classically been measured through the same sensitive biological assays employed for other Type I IFN. The most well established assay for IFN is the antiviral assay (AVA); however, cell proliferation and immunomodulation assays have also been used (Familletti et al., 1981; Pontzer et al., 1988; Fray et al., 2001; Meager, 2002). There are drawbacks to these assay systems, however. They cannot differentiate IFNT from other Type I IFN. Furthermore, human IFNA variants have also been shown to have different antiproliferative, immunomodulatory, and antiviral potencies (Li et al., 1990; Pfeffer et al., 1998). Specifically, IFNA7 cannot augment natural killer cell activity, even

at concentrations 10,000 times higher than the active concentration of IFNA2 (Ortaldo et al., 1984). Similarly, human IFNA8 had at least 100 times higher antiviral activity than human IFNA1, IFNA2, IFNA4, and IFNA10 tested at the same concentration (Yanai et al., 2001). Different IFNT variants, like human IFNA variants, also differ in their biological activities at the same concentrations (Niswender et al., 1997; Alexenko et al., 1999). Thus an AVA may not provide an accurate assessment of the quantity of IFN present when examining different IFN subtypes or variants. The AVA also cannot detect small differences in IFN concentrations. The AVA requires serial dilutions, usually 3-fold, to be performed (Meager, 2002). Accordingly, values that differ by less than 3-fold cannot readily be differentiated. Finally, AVA requires the maintenance of a cell line that is permissive to the virus, biohazard conditions to conduct the assay, and a virus that is a biohazard (Meager, 2002). For example, vesicular stomatitis virus, which is the virus most commonly used in routine laboratory assays for IFNT (Pontzer et al., 1988; Alexenko et al., 1999), is a pathogen to horses, cattle, swine, and potentially humans (Rodriguez, 2002), although the laboratory strains employed are attenuated.

A unique biological assay that determines IFN concentration by measuring an IFN-inducible gene, Mx, has also been developed (Fray et al., 2001). Madin-Darby bovine kidney (MDBK) cells that are transfected with a plasmid containing a human MxA promoter driving a chloramphenicol acetyltransferase (CAT) cDNA are the basis for this assay. CAT expression is quantified in a commercially available enzyme linked immunosorbent assay after the MDBK cells are treated with IFN. This assay has similar disadvantages to the previously discussed biological assays in that it cannot differentiate

between IFN subtypes and it is unclear if IFN subtypes and variants induce Mx with equal potency.

Two early papers describe radioimmunoassays (RIAs) that utilize a monoclonal antibody directed towards ovine IFNT (Vallet et al., 1988; Ashworth and Bazer, 1989). However, the antibodies used react poorly with bovine IFNT (Roberts et al., unpublished data) and cross-react with IFNA (Vallet et al., 1988). Two enzyme-linked immunosorbent assays (ELISAs) designed to measure ovine IFNT have also been developed (Zhu 1996; Lo 2002). These ovine IFNT ELISAs are not recommended for the measurement of bovine IFNT, though, because the first could not detect bovine IFNT (Zhu et al., 1996), and the second was never tested on bovine IFNT. Finally, an RIA for bovine IFNT, which uses an anti-bovine IFNT polyclonal antibody, has been described (Takahashi 2005). The bovine RIA is the only assay that can detect bovine IFNT at concentrations as low as 7.8 ng/ml without cross-reacting with other Type I IFN. This RIA still has serious drawbacks though. As with all RIAs, including those for ovine IFNT, the use of ^{125}I is a disadvantage, in part because the labeled reagent must be regularly regenerated but also because ^{125}I constitutes a biohazard. An assay that does not involve radioactive reagents is, therefore, desirable for bovine IFNT quantification. The purpose of the present work has been to develop an ELISA that could detect bovine IFNT in its different subtype forms.

Materials and methods

Polyclonal Antiserum Production and Purification

Polyclonal antiserum against IFNT was produced in a rabbit by immunizing with 200 µg purified recombinant bovine IFNT 2b mixed with Freund's complete adjuvant (Harlow 1988). The rabbit was boosted with 100 µg antigen combined with Freund's incomplete adjuvant approximately every four weeks after the initial immunization. Blood was collected two weeks after the second and third boost and allowed to clot at 4 C. Antiserum was separated from the clot and stored at -20 C until use. All basic rabbit care, immunizations, and blood collection were performed through the Office of Animal Resources (OAR) at the University of Missouri-Columbia in accordance with procedures approved by the Animal Care and Use Committee.

SDS-polyacrylamide (SDS-PAGE) and Western blotting to detect IFNT

Bovine recombinant IFNT-2b and ovine IFNT-4 (1 µg) were subjected to electrophoresis in 12.5% polacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (Ealy 2001, Chen 2006). The proteins were then electrophoretically transferred to PVDF membranes, which were then blocked with 4% nonfat dry milk and 1% bovine serum albumin in Tris buffered saline (pH 8.2). Membranes were incubated in the bovine IFNT antiserum (1:2500) or pre-immune rabbit antiserum (1:1000), washed, and incubated with horse radish peroxidase-conjugated anti-rabbit IgG (1:7500) (Cell Signaling Technology, Danvers, MA). The blots were again washed and developed with the Phototrope-HRP Detection System (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions. The monoclonal antibody produced by the

MK G10-7 hybridoma line discussed below replaced the polyclonal rabbit IFNT antiserum in an identical procedure.

Polyclonal Antibody Purification

Bovine recombinant IFNT-2b was dialyzed against 100mM sodium bicarbonate pH 8.2, containing 0.02% sodium azide, 1mM phenylmethylsulphonyl fluoride (PMSF), and 1mM ethylene diamine tetraacetic acid (EDTA) in 12,000 molecular weight cut-off (MWCO) Spectra/Por membrane. IFNT was then incubated with biotin ester [0.015% mass:volume (m:v)] for 3 hours at room temperature. Free biotin was removed through dialysis of the IFNT in phosphate buffered saline, pH 7.2, (PBS) containing 0.02% sodium azide, 1mM PMSF, and 1mM EDTA. Biotinylated IFNT was crosslinked to Agarose Avidin D (Vector Laboratories, Burlingame, CA) for a final concentration of 1 mg IFN/ ml agarose.

Polyclonal rabbit IFNT antiserum was dialyzed in PBS with 0.02% sodium azide, 1mM PMSF, and 1mM EDTA in 50,000 MWCO Spectra/Por membrane. Antibodies specific to IFNT were purified on the IFNT-agarose affinity column discussed above. The column was washed with PBS pH 7.2 for five column volumes and antibody eluted with 3M $MgCl_2$. The eluted fractions were then dialyzed in 1 X PBS, 0.02% sodium azide, 1mM PMSF, and 1mM EDTA and their protein content assessed on a Nanodrop N-1000 (Wilmington, DE) at 280 nm. Antibody was aliquoted and either stored at -20 C or 4 C until use.

Hybridoma Production and Screening

Hybridomas were produced through a standard protocol by the Cell and Immunology Core (CIC) at the University of Missouri-Columbia (http://biotech.missouri.edu/cic/index.html). In brief, two mice were immunized with a mixture of recombinant bovine IFN (IFNT-1a, 1c, 2b) and an ovine IFNT (IFNT-6) at a total concentration of 250 μg mixed with Freund's complete adjuvant. Mice were boosted approximately 4 weeks later with 100 μg of the mixture in Freund's incomplete adjuvant and sacrificed two weeks after the boost. Spleen cells were fused with murine myeloma cells in polyethylene glycol (PEG) prior to plating on 96-well plates. Fused cells were cultured in RPMI-1640 with 10% FBS, 1% Hybridoma Cloning Factor (Bioveris, Gaithersburg, MD), and HAT supplement (Invitrogen, Carlsbad, CA). Two additional hybridoma lines were also created by CIC through another project by Michael Kubisch. These lines were produced through the same protocol with the bovine IFNT-1a replacing the IFNT mixture as the immunization agent.

Direct ELISAs and indirect or "sandwich" ELISAs for the IFNT immunization mixture were used to determine if reactive antibody to IFNT was being produced by each hybridoma line. The antigen mixture (25 ng) was coated on a 96-well plate, and hybridoma culture medium added for the direct assay. In the indirect assay, an orientating anti-mouse antibody was first coated on the plate prior to addition of hybridoma culture medium. IFNT was then incubated on the indirect assay overnight. Bound antigen was detected by a rabbit polyclonal antiserum. Culture medium conditioned on STO fibroblasts was used to eliminate false positives. Clones that tested positive in either assay were then tested on an indirect assay for several IFNT variants: bovine IFNT-1a,

bovine IFNT-1c, bovine IFNT-3b, ovine IFNT-4, ovine IFNT-6, ovine IFNT-11, ovine truncated IFNT-11, modified bovine IFNT-11, and native glycosylated IFNT produced by CT-1 cells.

Clones that tested positive on the initial ELISAs were weaned into medium that replaced the HAT supplement with HT supplement. The only clone that that tested positive for the native glycosylated IFNT, termed MK 1G10-7, was then transferred to a standard hybridoma medium without HT supplement. A single cell was selected from MK 1G10-7 by flow cytometry at CIC and then propagated to obtain a pure cell line.

Monoclonal Antibody Production and Isolation

MK 1G10-7 was cultured in RPMI 1640, 10% fetal bovine serum, 5% penicillin/streptomycin, and 5% Hybridoma Cloning Factor. Five liters of medium were collected and concentrated via PEG to 100 ml in 50,000 MWCO Spectra/Por membrane. The concentrated medium was centrifuged at approximately 500 g to remove particulate material. The supernatant was dialyzed in 3.2M NaCl, 1.6M glycine, 0.02% sodium azide, 1mM PMSF, and 1mM EDTA. Immunoglubulins were purified from culture supernatant on a protein A-agarose column (BioRad, Hercules, CA) with a BioLogic fast protein liquid chromatography (FPLC) (Bio-Rad, Hercules, CA). The protein A column was washed with 3.2M NaCl and 1.6M glycine pH 9.0, and antibodies were eluted with 100mM sodium citrate pH 3.0. The eluted fractions were neutralized with 100mM Tris buffer, pH 9.0, and dialyzed in 1 X PBS, 0.02% sodium azide, 1mM PMSF, and 1mM EDTA. Monoclonal antibody concentration was assessed on a Nanodrop N-1000

(Wilmington, DE) at 280 nm. Antibody was aliquoted and either stored at -20 C or 4 C until use.

Antiviral Assays

Mardin Darby bovine kidney cells (MDBK) were used to determine antiviral activity through previously described procedures (Roberts et al., 1989). Cells were exposed to a series of 3-fold dilutions of recombinant of bovine IFNT-1a and a human IFNA standard (Inteferonsource, Piscataway, NJ) of known activity (1 X 10^6 IU). Two rows per plate of IFNT-1a were untreated and two rows were treated with one μg of monoclonal antibody (MK 1G10-7) per well. Cells were incubated with the IFNT for 24 h at 37 C with 5% CO_2 and then challenged with vesicular stomatitis virus (VSV) in serum free medium for 1 h. Cells were then cultured for a further 19 h on medium containing serum. At this time, cells were fixed in methanol and stained with 0.5% gentian violet. The human IFNA concentration providing 50% protection against VSV was used to define and compare antiviral activity. Triplicate wells of cells not exposed to virus and triplicate wells of cells exposed to VSV without prior IFN treatment served as the controls. All samples were run in duplicate on each plate, and a total of three plates were used for each assay. An unpaired Students t-test was used to analyze the assay results.

Indirect ELISA for detection of IFNT

The outline of the indirect ELISA is illustrated diagrammatically in Figure 1. Monoclonal antibodies were oriented in the wells by the use of donkey anti-mouse IgG,

which has minimal cross-reactivity to bovine and rabbit antibody (Jackson ImmunoResearch, West Grove, PA). The wells were successively exposed to 100 µl of the orienting antibody (1 µg in100 µl) in 0.1M sodium bicarbonate pH 9.5 at 4 °C overnight, a blocking solution (0.1% bovine serum albumin and 5% nonfat dry milk for 1 h at room temperature), and MK 1G10-7 (10 µg/ml in Tris buffered saline with tween (TBST) for a minimum of 6 h at 4 C). After removal of the monoclonal antibody solution, wells were washed (x 3) with 0.15M NaCl, 0.05% Tween-20 by using a 96-well plate washer (ELx405, BioTek, Winooski, VT). Samples containing IFN were then added to the wells. Recombinant bovine IFNT 2b, bovine IFNT 1a, and ovine IFNT 4 diluted in PBS or conditioned STO medium were used to generate standard curves. Medium conditioned on STO cells was used as a negative control. The plates were incubated overnight at 4 C. After washing, each well was exposed successively to rabbit anti-IFNT polyclonal IgG (100 µl of a 15 µg/ml solution; 3 h at room temperature) and 100 µl of alkaline phosphatase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA; diluted 1:7000) with an intervening washing step. After removal of the detecting antibody solution and three washes, bound alkaline phosphatase was detected by addition of 100 µl of a 1 mg/ml solution of para-nitrophenyl phosphate (PNPP) (Sigma, St. Louis, MO, USA) for 30 minutes. Absorbance at 405 nM was measured on an EL808 plate reader (BioTek, Winooski, VT).

The standard curves were generated by non-linear regression of LOG (ng IFN) versus absorbance plot by using Graphpad Prism software (version 3.02 for Windows; San Diego, CA, USA). Samples representing a range of IFN concentrations were assayed four times in quadruplicate to calculate intra- and inter-assay variation.

Results

Antibody Characteristics

The polyclonal rabbit IFNT antiserum recognized both ovine and bovine IFNT on Western blots (Figure 2). Nonspecific binding was minimal at the concentrations used for the Western blotting and, later, for the ELISA (Figure 2 and 3). Medium from hybridoma cell lines were tested for the presence of anti-IFNT on direct and indirect ELISAs. A total of 23 hybridoma lines produced antibodies that recognized the mixture of IFNT. Supernatants from these cell lines were tested against specific IFN subfamilies and IFNT variants as shown in Table 1. Antibodies produced by all hybridoma lines except MK 1G10-7 provided almost identical recognition patterns, characteristically binding only the recombinant, non-glycosylated bovine IFNT, indicating that the recognized epitope was the uncovered glycosylation site for those antibodies. MK 1G10-7 recognized all ovine and bovine IFNT variants when they were tested at 25 ng/well, while bovine IFNA, IFNB, or IFNW (at 1 μg) were not detected. MK 1G10-7 did not alter biological activity of IFNT when placed in excess in antiviral assays (Figure 3).

ELISA Validation

Typical standard curves obtained from serial dilutions of bovine IFNT-2b and ovine IFNT-4 are illustrated in Figure 4. As expected they were sigmoidal, as seen for most common antigen-antibody interactions. Intra-assay coefficients of variance (CV) were 5.98% and 4.87% for IFNT-2b and IFNT-4, respectively, at their sensitivity limits (1.3 ng for ovine IFNT-4 and 0.2 ng for bovine IFNT-2b). Samples at the lower

concentrations had increased variance, as would be expected due to the shallow curve in these ranges, which create increased sensitivity to small changes in absorbance.

Standard curves were also generated for bovine IFNT-1a. It provided statistically identical values to those observed with bovine IFNT-2b (Figure 5). Thus, distinct IFNT variants from different bovine family members can be quantified with one bovine IFNT standard. As seen in Figure 5, the ovine IFNT standard curve was distinct from that of the two bovine IFNT variants. Species specific IFNT standards will be necessary.

Discussion

IFNT is released by bovine trophoblast prior to implantation, with maximal production between days 16 and 22 of pregnancy (Bartol 1985). Its unique role as the primary and, possibly, earliest signal for maternal recognition of pregnancy has made it a prime target in the mission to improve successful pregnancy rates in livestock operations (Abecia et al., 1999; Kubisch et al., 2004). Obviously, the ability to quantify IFNT is essential for the success of many experiments, so that several groups have pursued various means of measuring the protein. An ELISA specific for IFNT that has several advantages over other IFNT assays has been described in this work.

Hybridoma lines were first created to produce monoclonal antibodies specific for IFNT. While 23 hybridoma lines produced antibodies that recognized recombinant bovine IFNT, only one of the monoclonal antibodies recognized native glycosylated bovine IFNT (Figure 2). The 22 cell lines that did not recognize native bovine IFNT did not recognize ovine IFNT-4 either. Bovine IFNT has an N-linked glycosylation site (Asn^{78}-Thr-Thr), but ovine IFNT-4 lacks the predicted site for N-glycosylation (Imakawa

et al., 1989). This antibody reactivity pattern suggests the bovine Asn^{78} glycosylation site is highly antigenic when it is not covered by sugar residues. All native bovine IFNT are glycosylated in either a 22 kDa high mannose form or a 24 kDa complex form (Anthony et al., 1988) but most animal studies have utilized recombinant non-glycosylated IFNT produced in bacteria (Ealy et al., 1998; Tuo et al., 1998; Kim et al., 2000; Chen et al., 2006). The increased antigenicity of this site poses interesting questions about using recombinant bovine IFNT in cattle. A humoral immune response against injected non-glycosylated recombinant IFNT is likely.

The only monoclonal antibody that recognized native glycosylated IFNT also recognized ovine IFNT, albeit with much less avidity. The epitope recognized by this monoclonal antibody, termed MK 1G10-7, was not mapped but some speculation can be made about its location. Antiviral assays were performed in the presence of excess MK 1G10-7 and the biological activity was not altered (Figure 4). Therefore, the epitope is likely not associated with the location of binding to its two receptor subunits, interferon alpha receptor (IFNAR)1 and IFNAR2. Helix E, Helix A, and the AB loop are strongly associated with IFNAR2 binding (Runkel 2000; Piehler 2000). Failure to bind to IFNAR2 eliminates antiviral activity and mutations in these regions are known to greatly reduce antiviral activity (Piehler 2000; Webber 1987); therefore, the epitope recognized by MK 1G10-7 is unlikely to be in these regions. Portions of helices B, C, and D and loops BC and DE are predicted to be involved in IFNAR-1 binding (Runkel 2000; Piehler 2000). The glycosylation site is close to the junction between helix C and the BC loop, eliminating this site as the recognized epitope (Sommereyns 2006). However, the remaining external regions of these latter loops and helices could potentially be bound by

an antibody without interfering with receptor binding either directly or through steric hindrance. These remaining regions are the most probable targets for MK 1G10-7 binding.

The ELISA described here provides an accurate, sensitive method for measurement of IFNT that overcomes the disadvantages associated with previously developed assays. Although it can only detect IFNT in the nanomolar range instead of the picomolar range like the antiviral assay (Meager, 2002), it can detect much smaller differences in IFNT concentrations than most of the bioassays and does not require working with hazardous virus. Similarly, the ELISA avoids the hazardous nature of RIAs, and unlike the existing RIAs, is able to detect both bovine and ovine IFNT. Finally, unlike the bioassays, it measures bovine IFNT variants with equal affinity, and can distinguish between IFNT and other type I IFN.

Bovine blastocysts and CT-1 cells express a mixture of IFNT variants with *IFNT-1c* and *3a* being the most prevalent based on cDNA sequencing (Ealy et al., 2001). Bovine IFNT variants have different biological activities from each other at the same concentration. IFNT-1a and 2b have over twice as high antiviral activity on MDBK cells as IFNT-3b, therefore, antiviral assays cannot be expected to provide an accurate measure of their relative amounts (Ealy et al., 2001). The previous ELISAs and RIAs did not test different variants to determine if the standard curves were identical for all. Members of two distinct bovine IFNT families had highly similar standard curves in this ELISA. This is probably a reflection of bovine IFNT's high amino acid identity (> 98%) (Ealy et al., 2001) . Consequently all bovine IFNT proteins should be very similar in their tertiary structure and have many of the same epitopes for antibody recognition. Ovine IFNT did

not provide a standard curve parallel to that noted for bovine IFNT. The assay was not expected to be optimal for ovine IFNT since both the trapping polyclonal immunoglobulin and the detecting monoclonal were raised against bovine IFNT. A specific standard set of reagents will probably be needed in order to cross the species barrier between these ruminants. Additionally, ovine IFNT variants can vary by as much as 13% from one another at the amino acid level (Winkelman et al., 1999). Consequently, ovine IFNT variants are unlikely to have the identical standard curves observed across bovine variants, and individual standards for each ovine IFNT class would be needed.

In conclusion, a sensitive and specific assay for bovine IFNT was developed. The assay utilizes a monoclonal antibody that does not alter biological activity of IFNT and does not recognize the, apparently, highly antigenic naked glycosylation site of bacterially produced recombinant IFNT. The assay successfully measured IFNT in a commonly used experimental model system, thereby demonstrating its viability in future studies. The standard curves generated in this ELISA for bovine IFNT variants from two separate families are the same.

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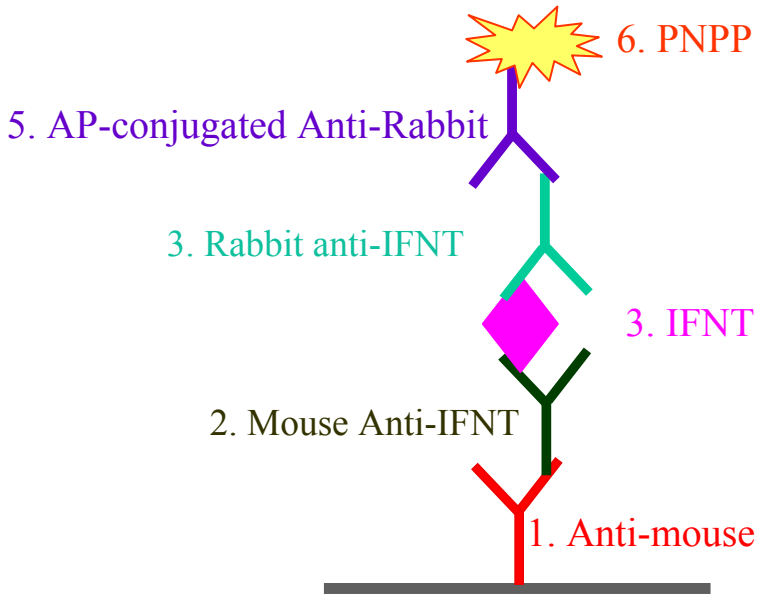

Figure 1. *Model of the indirect or "sandwich" ELISA.* The indirect ELISA utilizes an anti-mouse antibody to orientate the mouse anti-IFNT antibody in a direction to increase substrate binding. This step increases the sensitivity of the ELISA. A polyclonal rabbit IFNT antibody was used to detect the bound IFNT. Absorbance was read at 405 nm after treatment with PNPP to measure IFNT concentration.

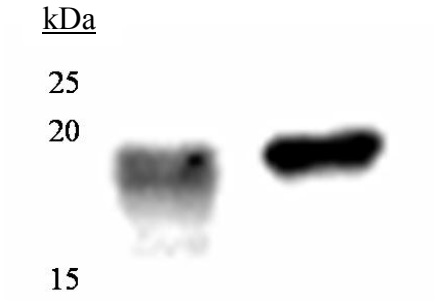

Figure 2. *Western Blot* The rabbit IFNT antibody recognized both ovine and bovine IFNT on Western blot. Lane 1: Bovine recombinant IFNT-2b; Lane 2: ovine recombinant IFNT-4.

	Bov IFNT1a gly	Bov IFNT1a	Bov IFNT1c	Bov IFNT3b	Ov IFNT4	Ov IFNT6	Ov IFNT11	Bazer mod IFNT	Bov IFNA1	Blank
1A4	0.155	**0.422**	**0.498**	**0.956**	0.157	0.149	0.161	0.152	0.159	0.176
MK 1G10	**0.981**	**1.188**	**1.202**	**1.244**	**0.489**	**0.712**	**1.02**	**0.322**	0.159	0.17

Table 1. *Reactivity pattern of two monoclonal antibodies* These data are from a preliminary indirect ELISA to determine the antibody recognition patterns of unpurified antibodies in the hybridoma lines produced. Antibodies produced by all hybridoma lines except MK 1G10-7 had a recognition pattern similar to 1A4. They only recognized non-glycosylated bovine IFNT, indicating the recognized epitope was the uncovered glycosylation site for those antibodies. MK 1G10-7 recognized all ovine and bovine IFNT variants tested, but did not recognize bovine IFNA, IFNB, or IFNW (data not shown for last two IFN subtypes). The blank in these ELISAs was conditioned medium from a fibroblast feeder cell line (STO cells).

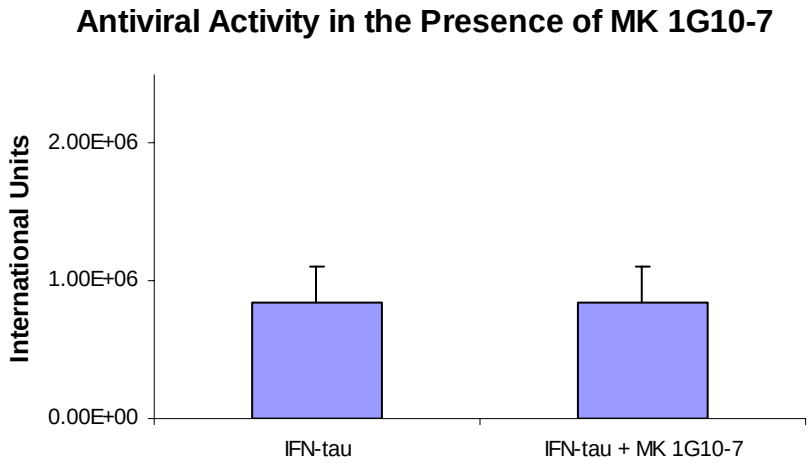

Figure 3. *Antiviral activity of IFNT in the presence of MK 1G10-7* Antiviral activity of IFNT-1a was measured in the presence and absence of MK 1G10-7. No difference in biological activity was seen in this assay due to interactions of the antibody with IFNT (p=1).

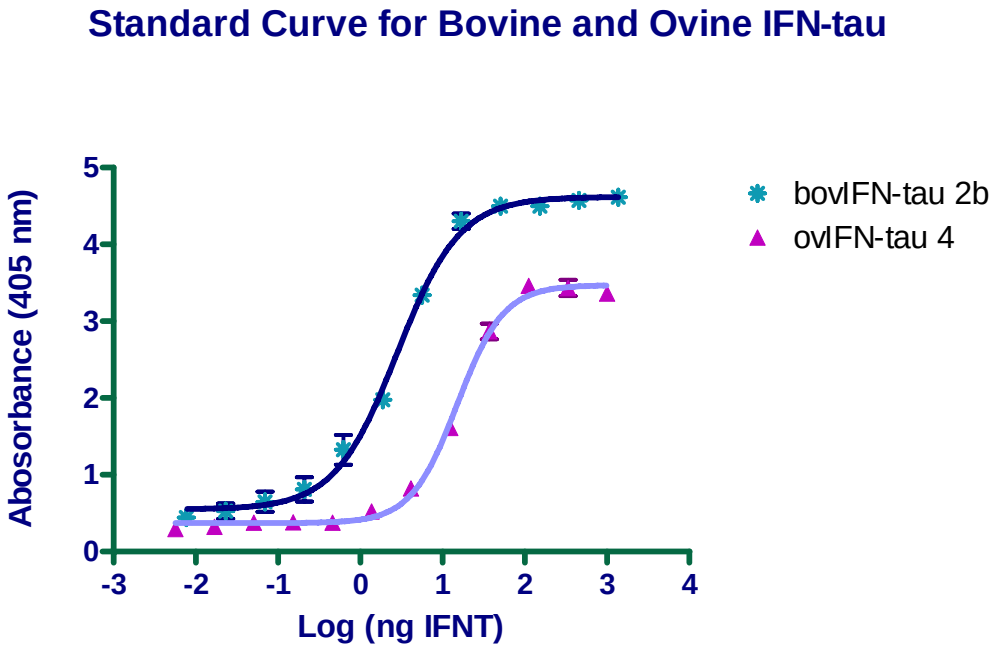

Figure 4. Typical standard curves for bovine (bov) and ovine (ov) IFNT are represented in this graph. Recombinant IFNT was serially diluted and allowed to incubate on the ELISA overnight at 4 C to achieve antibody-antigen equilibrium. The sensitivity limit is 0.15 ng for bovine IFNT and 1.3 ng for ovine IFNT.

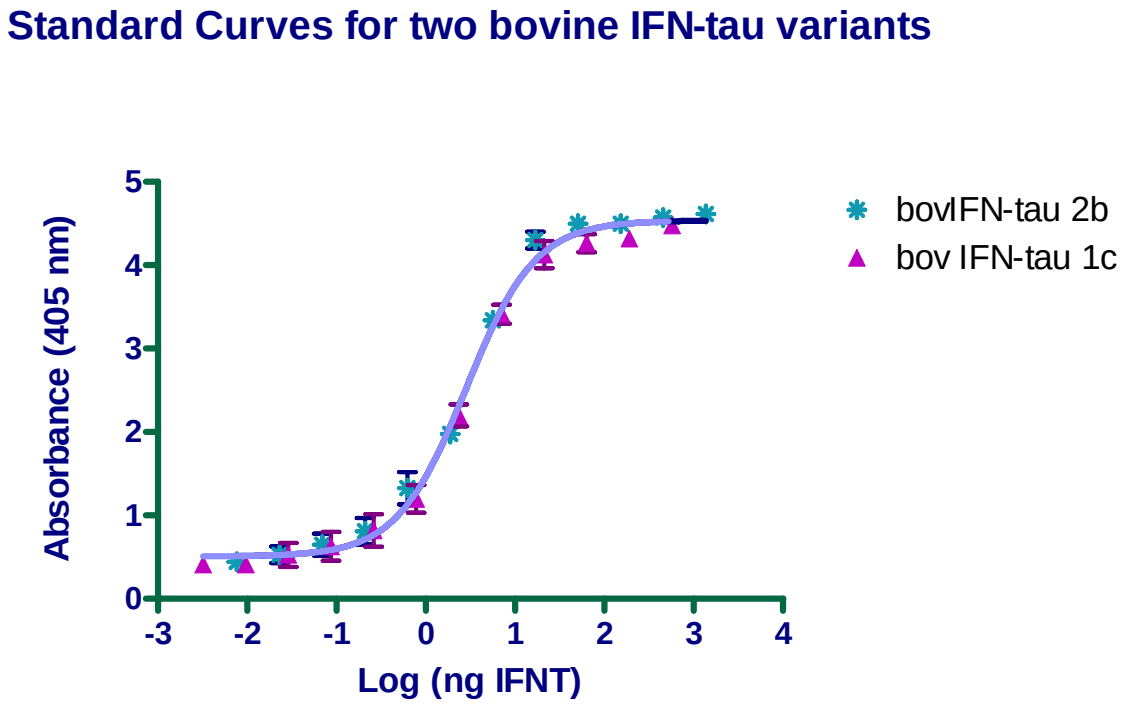

Figure 5. The standard curves are the same (p = 0.0695) for two bovine (bov) IFNT variants belonging to different classes.

VITA

Angela Marie Walker was born in Little Rock, Arkansas on April 10, 1978. She lived close to Little Rock with her parents Rose Marie Brandt and Louis W. Brandt III, and Robert C Walker until 1982 when her family moved to northern Arkansas close to the Buffalo National River. She graduated from St. Joe Public Schools in May 1996 and then attended University of Missouri-Columbia where she received a Bachelor of Science degree in both Biochemistry and Animal Science. She remained in Columbia, Missouri after her undergraduate training to attend the College of Veterinary Medicine and received her Doctorate of Veterinary Medicine in May 2004. While in veterinary school, she dual-enrolled in the graduate program and completed her doctorate of philosophy in the spring of 2008.