DEVELOPMENT OF A NEW METHOD FOR EXAMINING INTERFERON ACTIVITY IN BIOLOGICAL SAMPLES

By

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I dedicate this paper to my family and friends who have helped me along the way to finishing my thesis. My parents have been an amazing source of support in knowing just how much to push me. Also, to my friends who sat with me in the library for hours at a time. Finally, to Dr.Ealy who without his guidance this paper would never have been completed.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

B cells-	Lymphocytes
Bak1-	Bcl-2-Homologous/Antagonist Killer Proapoptotic Gene
Bax-	Bcl-2-associated X Protein, Proapoptotic GeneFIV
BCG-	Bacillus Calmette-Guerin
BLV-	Bovine Leukemia Virus
BRG1-	Brahma-Related Gene 1
CBP-	CREB binding protein
CRK-	cdc2 related kinase
DAP Kinase 2-	Death Associated Protein Kinase 2
dsRNA-	Double-Stranded Ribonucleic Acid
eIF-2-	Eukaryotic Initiation Factor 2
ERK-	Extracellular Signal-Regulated Kinase
FIV-	Feline Immunodeficiency Virus
GAS-	Interferon Gamma Activated Sequence
GCN5-	General Control Non-Depressible 5
IDDM-	Insulin Dependent Diabetes Mellitus
IFN-	Interferon
IFNA-	Interferon Alpha
IFNB-	Interferon Beta
IFNAR 1-	Interferon alpha (Type I) receptor
IFNAR 2-	Interferon alpha (Type I) receptor 2
IFNGR 1-	Interferon gamma (Type II) receptor
IFNGR 2-	Interferon gamma (Type II) receptor 2
IFN-λR1-	Interferon Lambda Receptor 1

IFNT-	Interferon Tau
IL-10R2-	Interleukin-10 Receptor 2 Subunit
IL-28Rα-	Interleukin-28 Receptor Alpha Subunit
IRF-	Interferon Regulatory Factor
IRF3-	Interferon Regulatory Factor 3
IRF9-	Interferon Response Factor 9
ISG-	Interferon Stimulated Gene
ISG3-	Interferon Stimulated Gene 3
ISG15-	Interferon Stimulated Gene 15
ISGF3-	Interferon Stimulated Gene Factor 3
ISRE-	Interferon Stimulated Response Element
JAK-	Janus Activated Kinase
MAPK-	Mitogen Activated Protein Kinase
MAPKAPK2-	Mitogen Activated Protein Kinase-Activated Protein Kinase 2
MDBK cells-	Madin Darby Bovine Kidney Cells
MEK-	Mitogen-Activated Protein Kinase Kinase
MCM5-	Mini-Chromosome Maintenance Deficient 5
MNK1-	MAPK Interacting Protein Kinase 1
mRNA-	Messenger Ribonucleic Acid
MSK1, MSK2-	Mitogen and Stress Activated Kinase 1 and 2
NfkB-	Nuclear Factor Kappa B
OAS-	2’5’-oligoadenylate synthetase
P300-	Co-activator protein
PI3K-	Phosphatidyl-inositol-3-kinase
PKCδ-	Protein Kinase C-Delta type

RAP1-	Ras-Related Protein 1
rboIFNT-	Recombinant Bovine Interferon-Tau
STAT-	Signal Transducer and Activator of Transcription
T cells-	Lymphocytes
Th1 Response-	Immune response, produces interferon gamma, leads to cell mediated immunity
Th2 Response-	Immune response, produces interleukin 4, leads to cell mediated immunity
TLR-	Toll-Like Receptor
TLR7-	Toll-Like Receptor 7
TYK2-	Tyrosine Kinase 2

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Interferons have the ability to prevent virus induced cell death. This activity has been used for decades as a biological assay to study interferon activity. Traditional antiviral assays have several shortcomings, including potential exposure of humans to biohazards (viruses) and length of time to complete the bioassay. In this new cell-based antiviral assay, we incorporated transcriptional element reporter constructs into Madin-Darby bovine kidney (MDBK) cells to measure interferon signaling. Interferon-responsive elements (ISRE and IRF) were coupled to a firefly luciferase (luc) gene. Three separate studies were carried out. The objective of the first study was to determine if MDBK cells containing ISRE- or IRF1-luc reporters would respond to Interferon-α (IFNA) in a dose-dependent way. Both reporters responded to IFNA in a dose-dependent fashion. Interestingly, ISRE- exhibited a more robust response than IRF. The objective of the second study was to determine if shortening the incubation time to 8 hours was suitable for measuring IFNA responses in MDBK cells containing ISRE-luc. Interferon activity could be measured with the shortened incubation period, however, the sensitivity of this assay was reduced under this condition. In the third study, the objective was to determine if cells that contained the ISRE-luc reporter were

responsive to IFNT. As anticipated, the ISRE-luc construct responded to IFNT. In conclusion, the ISRE- (or IRF)-Luc reporter systems have the potential to serve as alternative antiviral bioassays for quantifying Type I IFN activity in biological samples.

CHAPTER 1
LITERATURE REVIEW

Interferons (IFNs) are proteins typically produced in response to viruses, double stranded RNA, mitogens and antigens in numerous animal species (67). They are one of many hormones referred to as cytokines. Cytokines are small signaling molecules that are secreted by immune cells and glial cells of the nervous system. The abilities of IFNs are numerous and include antiviral, antiproliferative and immunomodulatory activities.

Alick Isaacs and Jean Lindenmann first discovered IFNs. The two were working at Mill Hill in London when they made the discovery in the 1950s. They were studying viral interference, which is the ability of one virus to interfere with the growth of another virus (1). Isaacs was working with influenza virus growth and realized that there was more to the interference of virus growth than just the virus particle itself. In order to investigate further, Isaacs carried out a new experiment. First, he treated chick egg membranes with inactivated influenza virus. Then, the membranes were washed and incubated in medium for several hours. After incubation, he removed the medium and used it to treat membranes. They found that the chick membranes (which had never before been exposed to the influenza virus) became infected by the virus. This interfering ability led them to name their new discovery, IFN.

Once Isaacs and Lindenmann discovered IFN, it became important to produce enough of the protein to be able to study its antiviral activities in humans. There were two early systems for the large-scale production of human IFN. Kari Cantell and colleagues at the National Blood Transfusion Center in Finland pioneered the first in the 1960s. They used a procedure where they carried out large-scale cultures of normal

human leukocytes challenged with Sendai virus (3). This was the main source of material used in clinical trials in Europe and the United States until the 1980s (5). In the 1970s this technique was replaced with a different technique that utilized cultured cell lines from normal human fibroblasts (60).

Initially, the leukocyte and fibroblast IFNs were thought to represent different molecules and be tissue specific (6). This view changed once it was shown that human leukocyte and fibroblast IFNs contained heterospecific antiviral activities. One of the first scientists to show this heterospecificity was Kurt Paucker at the Medical College of Pennsylvania. Paucker was working to produce antibodies to IFNs. He found that the antibodies generated from the immunization of rabbits with leukocyte IFN neutralized the activity of homologous IFN and also neutralized human fibroblast IFN. However, the antibodies produced in rabbits against human fibroblast IFN did not neutralize human leukocyte IFN (7). To determine why there seemed to be unidirectional cross-reactivity, Vilcek, Havell, and Paucker carried out a series of experiments. First, they immunized rabbits with leukocyte IFN. Then, they took serum from the rabbits that showed neutralizing activity to both leukocyte and fibroblast IFNs and passed it through an affinity column of human fibroblast IFN that was bound covalently to Sepharose. They found that there were two separate antibody populations, one that was specific for leukocyte IFN and one that was specific for fibroblast IFN. The major component of human leukocyte IFN was termed Le IFN while the minor component of human leukocyte IFN (and major component of fibroblast IFN) was termed F-IFN (8, 9). Later, Le IFN was renamed IFN Alpha (IFNA) and F IFN was renamed IFN Beta (IFNB) (10).

Interferon Families

There are three families of IFNs, Type I, Type II, and Type III. Multiple Type I

IFNs exist. In mammals, the type I IFN family is composed of IFNA, IFNB, IFN-κ (kappa), IFN-δ (delta), IFN-ε (epsilon), IFN-τ (tau; IFNT), IFN-ω (omega; IFNW), and limitin (11). They all share structural homology, including the lack of introns. Only a single type II IFN exists and is better known as IFN-γ (gamma; IFNG). The type III IFNs are a recent addition to this cytokine grouping and contain IFN-λ (lambda; IFNL) and interleukin 28/29.

In humans, IFNA and IFNB have a history of use in the clinical setting (4). Also within the human species, IFNA, IFNB, and IFNG are all thought to hold antiviral activity, but while IFNG is mitogen induced, it is believed to have a principal job other than antiviral activity (15).

Interferon Receptors and Cell Signaling

Type I and type II IFNs bind distinct receptors. Each class of receptors, referred to as IFNA receptors (IFNARs, for Type I IFNs) and IFNG receptors (IFNGRs), are composed of two primary receptor components (59). The type I receptor is made of IFNAR 1 and IFNAR 2 whereas the type II receptor is made of the subunits IFNGR 1 and IFNGR 2. Each of these subunits will interact with a member of the Janus Kinases (JAK). IFNAR 1 constitutively associates with tyrosine kinase 2 (TYK2) while IFNAR 2 associates with JAK 1 (59). For the type II subunits, IFNGR 1 associates with JAK 1 and IFNGR 2 associates with JAK 2. Mice that are deficient for the type I IFNAR or for different components of the IFN signaling pathway are susceptible to viral infection and are unable to establish an antiviral state (26). Likewise, mice that are deficient for IFNGR have a poor immune response when infected with Bacillus Calmette-Guerin (BCG) and have a nearly one hundred percent mortality rate when exposed to certain bacterial strains (76).

The initial step in activation with both types I and type II signaling is the same; activation of receptor associated JAKs. This activation occurs in response to ligand-dependent re-arrangement and dimerization of receptor subunits. After rearrangement and dimerization, autophosphorylation and activation of associated JAKs will occur. This activation of IFN receptor associated JAKs appear to regulate several other downstream cascades, either directly or indirectly (59). The conventional targets for JAKs are the Signal Transducers and Activators of Transcription (STATs). Once the STATs are phosphorylated by JAKs, they form homodimers or heterodimers and translocate to the nucleus where they initiate transcription by binding specific sites in the promoters of IFN-stimulated genes (ISGs). There are hundreds of (ISGs). Within each promoter region of each ISG is either an Interferon Response Element (ISRE) or Interferon Gamma Activated Site Element (GAS). Once an ISG binds to the promoter region of an ISRE, transcription is induced (71).

Transcription of type II IFN dependent genes is regulated primarily by Interferon Gamma Activated Site Elements (GAS). STAT 1 homodimers bind to this DNA element. One particular ISG, which is induced only by type I IFNs, is ISG3. ISG3 is composed of activated STAT1 and STAT2 and Interferon Response Factor 9 (IRF9) and is the protein that recognizes ISREs (71, 72). There are hundreds of known ISGs. Some of these ISGs have both ISREs and GAS elements, while some ISGs have only one of the two. This seems to show that different combinations of STAT-containing complexes are necessary in order to achieve optimum transcription of a specific gene (59).

Similar to type I and II IFNs, antiviral activity of type III IFNs is activated by JAKs, which then induce ISGs. This leads to formation of the ISGF3 complex. However,

instead of using the same receptors as type I or II IFNs, type III IFNs use the receptor subunits IFN-λR1, Interleukin-28Rα (IL-28Rα), or Interleukin-10R2 (IL-10R2) (2).

In the nucleus, STATs also can interact with several co-activator proteins and regulate other transcriptional events. Examples of these co-activating proteins are p300, cAMP-responsive-element-binding protein (CREB) -binding protein (CBP) and mini-chromosome maintenance deficient 5 (MCM5). Other proteins that interact with IFN activated STATs to increase transcriptional capacities are transcriptional co-activator general control non-depressible 5 (GCN 5) and brahma-related gene (BRG 1). These proteins are STAT 1 or STAT 2 specific. Also involved with IFN mediated signaling are CRK proteins. There are 3 members of the CRK family, CRKL, CRKI and CRKII. These proteins function as adapters and facilitate the formation of numerous signaling complexes in response to various stimuli (59).

Another important interaction is the activation of RAP 1 by type I and type II IFNs. Activation of RAP 1 is CRK-L dependent. This activation downstream of CRK-L implies that this is a method by which IFNs can create growth inhibitory responses. Anti-proliferative actions of IFNs are also involved with the activation of RAP 1. RAP 1 regulates the activation of mitogen-activated protein kinase signaling cascades. One of these cascades is the p-38 signaling pathway which transmits signals that are crucial for the anti-proliferative acts of IFNs. In addition, formation of CRKL-STAT5 complexes appears to be essential for gene transcription regulated by GAS elements.

The role of mitogen activated protein kinases (MAPKs) is interesting. The p38 pathway seems to be the most important of the MAPKs for IFN signaling. P38 is associated with the anti-proliferative, anti-leukemic and antiviral properties of type I

IFNs. This pathway is essential for each of these abilities of IFNs. P38 is phosphorylated and activated in a type I IFN dependent manner. There are several downstream effectors of the type I IFN activated p38 pathway. These include MAPK-activated protein kinase 2 (MAPKAPK2), mitogen and stress activated kinase 1 and 2 (MSK 1 and MSK 2) and MAPK interacting protein kinase 1 (MNK 1). MAPKAPK2 is required for IFN dependent transcription of Isg15 and for the induction of the antiviral properties of type I IFNs. In addition, the MEK-ERK pathway (induced by IFNA/B/G) is activated in response to viral infection. This pathway controls the activation of IRF 3 and the production of type I IFNs, in addition to other signals.

Another pathway involved in IFN mediated signaling is the phosphatidyl-inositol- 3-kinase (PI3K) pathway. Activation of this pathway occurs downstream of JAKs in an IRS dependent but STAT-independent manner. Activation of this pathway leads to the induction of serine kinase activity. There appears to be an important role for IFNG and the PI3K pathway in regards to IFNG inducible transcriptional regulation. One of the downstream effectors of IFNG is protein kinase c-delta type (PKC-δ). Activation of PKC-δ is PI3K dependent. PKC-δ is activated by treatment of cells with type I or type II IFNs and then associates with STAT 1. If the kinase activity of PKC-δ is inhibited, both phosphorylation of STAT 1 on Serine 727 and STAT 1 mediated gene transcription through ISREs or GAS elements are blocked. This points to the essential role of PKC-δ in IFN dependent gene transcription and the ability of PKC-δ to act as a serine kinase toward STAT 1.

Interferon Actions

IFNs have a vast number of activities. These include cell growth and differentiation and antiproliferative and immunomodulatory activities. IFNs are also

important for antiviral activities, effects on cell-produced substances within the body, and cell surface alterations. Furthermore, IFNs are used in the treatment of human cancer, hepatitis B, and autoimmune diseases. Specific IFNs also contain other selective activities, and one of special interest in our laboratory is the action of IFNT as a pregnancy hormone. To follow is an overview of these various activities.

Virus-Induced Interferon Induction

Type I IFNs are produced in response to viral infection in most nucleated cells. There are two classes of cellular receptors that recognize viral nucleic acids, toll-like receptors and RNA helicases (29). Toll-like receptors are located in the endosomal compartments of immune cells and detect viral RNA or DNA (27, 61). The RNA helicases detect dsRNA, which is produced in infected cells. There are several toll-like receptors that are involved in IFN activation. Toll-like receptor 3 (TLR3) detects dsRNA which is a common replication intermediate of both DNA and RNA viruses. TLR3 is also important in detecting viral dsRNA, which is released by apoptotic cells (28, 62). TLR7 and 8 detect single stranded viral genomic RNA, while TLR9 detects DNA viruses (63, 64).

The IFN response family consists of 9 genes: IRF1 to IRF 7, IRF8/ICSBP and IRF9/p48/ISGF3 (30). Cellular IRFs share homology in the N-terminal domain, which consists of a helix-turn-helix DNA binding domain. The C-terminal domain has an IRF associated domain that is very important for both homodimeric and heterodimeric interactions. In addition, the C-terminal domain has a gene activation domain (31). The discovery of IRF 3 and IRF 7 was a crucial part in understanding how IFNs are induced and expressed. IRF 3 homodimerizes or heterodimerizes with IRF 7 and then translocates to the nucleus. Here, it associates with CREB binding protein (CBP/p300).

Activated IRF 3 stimulates transcription of several interferon stimulated genes (ISG) including IFNB (32, 65, 66). Meanwhile, IRF 7 is critical for the induction of IFNA (33). The level of IRF 3 and IRF 7 in cells determines the relative level of expression of different individual IFNA subtypes (26). IRF 5 also has important roles including tumor suppression activity and regulation of early inflammatory proteins. If IRF 5 is overexpressed, there is an up-regulation of early inflammatory proteins. This seems to point to a role in the expression of early inflammatory cytokines and chemokines (34). IRF 5 stimulates the cyclin dependent kinase inhibitor, p21. At the same time, it represses cyclin B1 and stimulates the expression of Bak1, Bax, caspase 8 and DAP kinase 2, which are all proapoptotic genes. These abilities of IRF 5 indicate its ability to promote cell cycle arrest and also promote apoptosis independently of the p53 pathway (34, 35).

Response to Viral Infection

IFNs do not act directly on intact viruses. Instead, they act on the cells that are infected by a virus. The antiviral state produced by IFNs is directed against a wide variety of viruses (1). The production of IFNs is actually the first reaction to viral infections, while substantial amounts of antibodies are produced later.

Antiviral activity of type I IFNs involves two different mechanisms: 2’5’-oligoadenylate synthetase (OAS) and dsRNA-dependent protein kinase R (PKR) (40). Both of these mechanisms are enzymatic and they are up-regulated in response to a viral infection and IFN induction (40). Upon activation of OAS, ATP is converted to 2’5’-oligoadenylate (2-5(A)), which activates a ribonuclease that degrades both host and viral mRNA (41). Once PKR is activated, it prevents protein synthesis by phosphorylating the a-subunit of eIF-2 (a translation initiation factor) (42, 43). OAS is

important in that it is up-regulated following treatment with IFNs, which causes the resistance of cells to viral infection (44, 45, 46).

There are two types of viral disease, those that are localized to the site of the primary infection (ex. upper respiratory tract infections) and those in which the primary site of infection is not where the disease is located (ex. Encephalitis). IFNs play important roles in each type of viral disease.

Viral infection in which the disease is localized at the site of primary infection is initiated by multiplication of the virus and the ensuing damage to the cell. In order to effectively fight these types of viral infections, response by the body must be fast. Upon infection, both IFNs and antibodies are produced. Generally, the production of antibodies by the body is too slow to provide an acute response to viral infection. However, there is a correlation between the fall of the virus titer and a rise of the IFN titer and recovery from viral infection. This seems to show that IFNs play a role in recovery from viral infections in which the site of the disease is the site of viral replication and infection (1).

The other type of viral infection is the one in which the primary site of infection is not where the disease is located (this is the most common type of viral infection). Encephalitis is a good example of this. A mosquito will pick up the virus by biting an infected person. Once inside the gut of the mosquito, the virus replicates and the mosquito passes the infection on to the next person by biting them. However, the site of the mosquito bite is not where the disease becomes localized. Instead, the virus will be released into the blood. During this type of infection, IFN production will be induced at several points. IFNs will be produced both at the site of the infection as well as released

into the blood, which will often coincide with viremia (1). This double attack and early release of IFNs helps to decrease virus production, as well as helping to decrease the spread of the virus. However, IFN must be present in adequate quantities. According to Friedman, there is quite a bit of evidence that points to the fact that IFNs contribute to recovery from primary virus infections. This evidence includes; IFNs are produced at the site of primary virus infections early in the infection and they are produced in sufficient quantity to account for the recovery from primary infections. Additionally, inhibiting IFN production increases the severity of viral infections. Furthermore, administering exogenous IFN inhibits the development of viral infections (1).

Interferons and Autoimmune Disease

The relationship between IFNs and autoimmune disease differs based on specific circumstances. Constitutive production of IFN has been associated with the pathogenesis of some autoimmune diseases. However, in other cases IFN therapy is effective in treatment in certain autoimmune disorders.

Two examples of the varying effects of treatment with INFs are the autoimmune diseases insulin dependent-diabetes mellitus (IDDM) and multiple sclerosis. In IDDM there has been an association with IFN treatment and pathogenesis. Namely, the disease has been shown to appear in patients who were treated with IFN for another issue (16). However, the role of IFN on IDDM seems to be dependent on the stage of the disease. In early stages of the disease, IFN may be responsible for an abnormal autoimmune response to a viral inducer. At this point, such an inducer has yet to be found. However, local production of IFN may well be induced by the pro-inflammatory products of damaged cells in addition to secretion from other cytokines. In late stages of IDDM, the proliferation and survival of reactive T-cells seems to be suppressed by IFNs.

Conversely, IFNB is a well-established treatment for multiple sclerosis. However, the mechanism by which beta works is largely unknown. The positive effects of IFNB in preventing relapses in MS may be due to the combination of anti- inflammatory, anti-proliferative and pro-apoptotic abilities of IFN (17).

Response to Bacterial Infection

Type I IFNs are also induced as an immediate innate response to bacterial infections and have also been shown to modulate the innate antibacterial response (19). Bacteria can be recognized both by membrane-bound receptors and also cytoplasmic receptors. The binding of a ligand to a toll-like receptor recruits certain adaptors, which then initiate cellular signaling pathways. These pathways lead to the activation of IRFs and the NFkB family of transcription factors. Both Gram-positive and Gram-negative bacteria can induce the production of type I IFNs. Gram-negative bacteria like salmonella typhimurium, Shigella flexneri and Escherichia stimulate type I IFN synthesis after invasion of the cell (19). Gram-positive bacteria that have a cytoplasmic life cycle phase (such as Listeria monocytogenes) can also activate type I IFNs and probably do so by the cytosolic DNA recognition pathway (20).

Antiproliferative Effects

Treatment of cells with IFNs usually inhibits their growth. This could have numerous clinical applications, including use in treatment of cancers and rapidly dividing tumor cells. Even though IFNs have the ability to inhibit growth, they do not have a cytopathic effect on cells (1). That is, they do not cause degeneration to the actual cell. There has been a lot of work done using mouse tumors to evaluate the efficiency of IFNs in decreasing cell growth. Evidence points to a direct inhibitory ability of IFNs on cell growth. Based on experiments with various types of cancer, it seems that well

differentiated and slowly proliferating cancers are most responsive to IFN treatment (67).

Clinical Applications of Interferons

Possible clinical uses for IFN are in the treatment of hepatitis B and C, respiratory viruses and rabies. In patients with chronic hepatitis B, there have been mixed results in using IFN as a treatment. Generally, women seem to respond better than men. During infection with hepatitis C, the virus triggers the induction of IRF 3 and NKfB. TLR 7 confers immunity against the hepatitis C virus via IFN. Currently, polyethylene glycol modified IFNA 2a and ribavirin are the treatments of choice for chronic hepatitis C infections. IFNA is shown to suppress hepatitis C replication. Although there is a current treatment for HCV, chronic infection with the disease can be established in the liver due to the mechanisms the disease has developed to evade treatment with IFNA 2a and ribavirin. High doses of IFN to treat respiratory viruses must be used repeatedly because of the barrier from mucus and nasal cilia. Finally, rabies vaccines seem to have varying levels of effectiveness and it seems that the more effective vaccines correlate with the ability to induce IFN rather than the ability to induce anti-rabies antibodies (1). Although the use of IFNs as therapeutic devices is promising, there does seem to be a high level of toxicity and side effects. Therefore, more work needs to be done with a new system of delivery or with the development of IFN analogs. This would allow for a more tolerated reaction to treatment with IFNs (26).

Interferons and Pregnancy

IFNs are produced during pregnancy in various mammals. In humans, both the placenta and decidual cells produce Type I and Type II IFNs. The presence of these IFNs is significant for several reasons.

Perhaps the most important IFN related to pregnancy is IFNT. The antiluteolytic effects of IFNT are responsible for maternal recognition of pregnancy. IFNT is critical for extending the lifespan of the corpus luteum (CL). The CL is responsible for producing progesterone, which provides an appropriate environment in which a conceptus can grow. In addition, IFNs exert an immunosuppressive effect on the production of T and B cells, which are important in both the humoral and cell-mediated immune response. This likely is important in preventing the immune system from acting against the embryo. Also, type I and type II IFNs protect the conceptus from viral infections. In addition to this, they regulate cellular differentiation and expression of cell surface antigens (1, 79).

Interferon Tau

IFNT is a type I IFN with a length of 172 amino acids and appears to be the most recently evolved mammalian type I gene family (36). It binds the same receptor as the other type I IFNs (37). INFT is produced by ruminant ungulate species, like cows, sheep and goats. Like other type I IFNs, IFNT has antiproliferative and antiviral properties, but in addition to these abilities IFNT has a very important role in the maternal response to pregnancy (36). There seem to be three main characteristics of IFNT that make it different from the other type I IFNs; its lack of viral inducability, its localization to the embryonic trophectoderm and its ability to have high levels of sustained synthesis over several days (37).

IFNT has been found in cattle, sheep, goats, musk oxen, gazelle, giraffe and deer (37). Each of these species, except for the giraffe, has been shown to have several IFNT genes. IFNT is most closely related to IFNW sharing about 75% identity. IFNW evolved from IFNA around 130 million years ago, coinciding with the time of mammalian origin. However, the IFNT and IFNW split happened only 36 million years ago, around

the same time as the origin of ruminant artiodactyls (37). IFNT probably arose from a duplication event from IFNW. This event seems to have given IFNT its unique characteristics, such as the acquisition of trophoblast expression and its loss of viral responsiveness (37).

IFNT is the major product of the conceptus of ungulate ruminants in the period before the trophoblast makes a firm attachment to the uterine wall and starts to form the placenta (37). It is responsible for extending the lifespan of the corpus luteum (CL). The corpus luteum is a structure that develops on the ovary following the release of the oocyte. If pregnancy is not achieved, the structure regresses. The main function of the corpus luteum is to produce progesterone, which works to provide an environment in which a conceptus could potentially grow and be nurtured. In order for pregnancy to be successful, the CL must not regress. This prevention of regression is dependent upon the biochemical signal of IFNT that originates from the conceptus. IFNT works to prevent the release of prostaglandin F2α, which would cause the corpus luteum to regress and the animal to regain cyclicity (37). In experiments in which IFNT is introduced into the uterus of non-pregnant animals, it has been shown to be able to increase the length of the estrous cycle for days and sometimes even weeks which will maintain progesterone output from the corpus luteum.

Much comparison has been made between IFNT and other type I IFNs in their ability to produce an antiviral state. The main area of comparison has been between IFNT and IFNA. According to Chon et al., IFNA is the current “gold standard,” for the treatment of viral infections (48). IFNA has been used in the treatment of several cancers, including Hairy Cell Leukemia, Chronic Myelongenous Leukemia, Renal

Carcinoma, and Kaposi Sarcoma, which is associated with AIDS (49). However, IFNA is not without its drawbacks. It has a high level of toxicity, which leads to negative side effects that make it less attractive for use in the clinical setting. In a study by Stewart et al, IFNT was more than 30-fold less toxic than IFNA on MDBK cells, while still maintaining similar anti-viral activity. They found that IFNA has a much higher binding affinity than IFNT for the MDBK receptors (50). Subramaniam found that IFNA's binding affinity is more than ten times that of IFNT (39). It is thought that this binding affinity is what makes IFNA more toxic. It was shown that maximal antiviral activity is achieved by only partial occupancy of receptors, while toxicity is associated with maximal receptor occupancy. Therefore, IFNA with its high binding affinity also has a high toxicity rate. However, IFNA has shown that it can act as an antagonist for IFNT in binding to receptors on MDBK cells, but IFNT cannot act as an antagonist for IFNA at any concentration on MDBK cells (39). Because of IFNT's inability to act as an antagonist for IFNA, it is believed that the two IFNs bind to different epitopes on the type I receptor (39).

Because of the lessened level of toxicity of IFNT, it may be a safer option for treatment of various diseases. Compared to IFNA, a larger dose of IFNT is required to induce the same amount of toxicity. Interestingly, similar doses of IFNT stimulate comparable levels of anti-viral activity (39, 51, 52, 53, 54, 55). Because of these qualities, higher doses of IFNT can be administered and adjustments can be made to dosage based on its effectiveness on a patient, rather than based on its toxicity level. IFNT has been shown to be effective in the treatment of several diseases, both animal and human.

One area of work with IFNT has been with human immunodeficiency virus. In a comparison study carried out by Pontzer and others, IFNT reduced HIV reverse transcriptase activity in human HUT74 cells by 91.6% versus IFNA, which was able to reduce activity by 92.5% (54). The big difference here was that IFNT was administered at 1000U/mL, while IFNA was administered at 100U/mL. At 100U/mL, IFNA was very toxic versus IFNT at 1000 U/mL with no indication of toxicity (54). Obviously, being able to administer a higher dose and achieve similar antiviral activity without negative side effects is beneficial. However, while IFNs have been shown to act at multiple steps in the HIV life cycle, IFNT is limited to the early steps in the viral replication cycle (56). In addition, there is some controversy in using IFNT in treatment of HIV because it seems to induce either a Th2 response or a combination of Th1/Th2 response versus a Th1 response by other IFNs (57, 58). Normally, the control of viral infections is dependent on a Th1 response, which suggests that IFNT may not be the best treatment for HIV or that additional work needs to be done to determine exact mechanisms.

IFNT has also been explored as a treatment option for autoimmune disorders, such as multiple sclerosis (51, 52). Mice with experimental allergic encephalomyelitis (EAE) are used as a model for MS. Administration of IFNT to these mice either delayed onset or blocked development of the disease. IFNB is the current treatment option for MS, but it has similar negative side effects to the use of IFNA in other diseases. IFNT is as effective as IFNB in the treatment of mouse EAE, implying that IFNT may be able to be used as a new treatment option.

In addition to human viruses, IFNT has been explored as an option for the treatment of animal viruses such as feline immunodeficiency virus (FIV) and bovine

leukemia virus (BLV). FIV is a retrovirus that weakens a cat's immune system, which can eventually lead to secondary infections and death. Treatment consisting of administration of 5000 U/mL of IFNT at three-day intervals led to a decrease in reverse transcriptase activity in feline lymphoid cells infected with FIV by 98% by day 14. This decrease in reverse transcriptase activity leads to prevention of FIV replication (54). In addition, IFNT is effective in the treatment of bovine leukemia virus both in vitro and in vivo. In vitro, treatment of cells infected with BLV using recombinant bovine IFNT decreases viral titers as well as cytopathology associated with BLV. In vivo, treatment with tau led to antiviral activity similar to that shown in vitro. Furthermore, there were no side effects that are typically shown with IFN treatment.

CHAPTER 2
DEVELOPMENT OF A NEW METHOD TO EXAMINE INTERFERON ACTIVITY IN BIOLOGICAL SAMPLES

The hallmark activity of IFNs is their ability to prevent virus-induced cell death. This activity has been utilized for decades as a biological assay for examining IFN activity in plasma, lymph fluid, cell lysates and cell-conditioned medium. These cytopathic assays are incredibly sensitive, and generally are able to detect IFNs within the pM range. In recent years additional assays have emerged as alternatives to antiviral activities. Most notably, various IFN-specific radioimmunoassay's and enzyme-linked immunosorbant assays have been developed. The ability of these assays to discriminate between IFNs is advantageous. Some but certainly not all of these assays also are able to reach the sensitivity of detection of traditional antiviral assay systems. However, these immunoassays cannot quantify the biological activity of samples. When this information is needed, a bioassay like the antiviral assay is needed.

The conventional antiviral assay contains several shortcomings. Firstly, most assays take several days to complete (2-3 days in most cases). Shortening this time period is challenging given that sufficient incubation time is needed for suitable viral challenge and cellular viral propagation and lysis events. Also, the conventional assay utilizes live virus (usually vesicular stomatitis virus, or an equivalent virus). Although most virus preparations are lab-attenuated, they still may infect humans and other cell lines and usually require handling under biosafety level 2 conditions. Lastly, although most antiviral assays are very sensitive, they generally are not highly accurate and assay-to-assay variation can be substantial.

We propose that a new cell-based biological assay that does not require virus challenge can be developed to examine IFN activity in biological samples. Our

approach was to incorporate a reporter system into cells that can measure IFN signaling. The system contained IFN-responsive DNA elements (ISRE and IRF) coupled to a Firefly Luciferase (Luc) gene. The premise for this work was that cells containing either of these reporters would respond to IFN exposure by producing luciferase, and lysing these cells and measuring luciferase activity would provide a means for quantifying IFN activity. Materials and Methods

Materials and Methods

Madin-Darby bovine kidney cells (MDBK; ATCC#CCL-22) were propagated and passaged as described previously (77) in Dulbecco's modified eagle medium (DMEM; Invitrogen Corp.) containing 10% [v/v] fetal bovine serum (FBS) and antibiotics (50 IU Penicillin G and 50 µg/ml Streptomycin sulfate). For transduction, cells at 50% confluence were incubated in DMEM containing 3 µg/mL Polybrene (Santa Cruz Biotechnology). Lentiviral particles (MOI~2) containing CignalTM Lenti ISRE- or IRF1-luciferase (luc) reporters (SA Biosciences) were added for 4 h and cells were allowed to recover in growth medium for 3 days before selection with 10 µg/mL Puromycin (Invitrogen Corp.) for 14 days.

Interferon Alpha Challenge

In the first study, cells containing ISRE- or IRF1-luc reporter activity were exposed to 1:3 serial dilutions of recombinant human IFNA (3.84 x 108 IU/mg; EMD Biosciences). Dilutions (33,333 to 0.56 IU IFNA) were completed within 96-well culture plates in 100 µL growth medium containing or lacking 10% FBS. Then MDBK cells were trypsinized and added to cultures at ~5 x 104 cells/well (~50% confluency). After 24 h, medium was removed and 30 µL of Dulbecco's Phosphate Buffered Saline (DPBS) and 30 µL One-Glo Luciferase Assay Substrate (Promega Corp.) was added to each well.

After 5 minutes of mixing (100 rpms) firefly luciferase activity was determined using a 20/20 Luminometer (Turner Biosystems). All samples were assayed within 30 minutes of adding the luciferase reagent.

In a second study, MDBK cells containing the ISRE-luc reporter were challenged with 1:5 serial dilutions of IFNA (26,667 to 0.003 IU) for 8 h in medium containing 10% FBS, then cells were processed as described previously to determine luciferase activity.

Interferon Tau Challenge

MDBK cells containing ISRE-luc reporter activity were challenged with 1:5 serial dilutions of recombinant bovine IFNT (1.3 x 108 IU/mg) in medium containing 10% FBS. The antiviral activity of this protein was not completed at the time of the study but contained substantial antiviral activity in previous work (78). After 24 hours exposure to IFNT, MDBK cells were processed as described previously to determine luciferase activity.

Results and Discussion

The first study was conceived to determine if MDBK cells containing ISRE- or IRF1-luc reporters responded to IFNA supplementation in a dose-dependent fashion. Studies were completed using medium containing or lacking 10 % FBS (+/- serum). Both reporters responded to IFNA challenge after 24 hours of incubation (Figures 2-1 and 2-2). The greatest amounts of luciferase activity were detected in MDBK cells containing the ISRE-luc reporter. Also, greater amounts of luciferase activity were detected when cells were exposed to serum. In Figure 2-2, data are presented as fold response to IFNA relative to non-treated cells. The presence or absence of serum did not appear to influence the magnitude of ISRE- and IRF1-luc responses to IFNA except

when MDBK cells containing IRF1-luc were exposed to large quantities of IFNA in medium containing serum.

A biphasic dose response was observed in cells harboring the ISRE- or IRF1-luc reporters. An apparent saturation in luciferase activity was observed when cells were exposed to greater amounts of IFNA (*e.g.* 33,000 to 1234 IU), but lower amounts of IFNA produced a dose-dependent changes in luciferase activity. Linear regression curves were generated from cells containing ISRE-luc when using data between 1234 and 0.56 IU IFNA (y=1,000,000-17,4493x, R2=0.96) and cells containing IRF1-luc when using data between 1234 and 1.7 IU IFNA (424,149-54,708x, R2=0.93). Both analyses were completed in cells exposed to medium containing serum.

A second study examined whether shortening the incubation period to 8 hours was suitable for measuring IFNA responses in MDBK cells containing ISRE-luc. Cells containing the IRF1-luc construct were not examined because they were slightly less sensitive to IFNA challenge than the other cell line. A biphasic outcome was detected in this study (Fig. 2-3). Lower luciferase activity was observed when cells were exposed to high IFNA concentrations (*i.e*. 26,667 and 5,333 IU), and luciferase activity peaked for cells exposed to 1,067 IU IFNA then decreased thereafter. The magnitude of luciferase response to IFNA was less than it was when cells were exposed to IFNA for 24 hours in previous work.

A final study examined whether cells containing the ISRE-luc reporter were responsive to IFNT (Fig.2- 4). Increases in luciferase activity were detected, and a dose- dependent effect was observed between 0.9 and 4.3 pg/mL IFNT. This equates to

between 0.12 and 2.4 IU of antiviral activity based on a test completed on this recombinant preparation 32 months prior to this study.

Summary and Interpretations

The following conclusions can be made. In the first study ISRE- or IRF-luc reporters responded to IFNA in a dose dependent fashion and the greatest luciferase activity was detected in cells with the ISRE-luc reporter. Also, the magnitude of the ISRE- and IRF1-luc response to IFNA did not appear to be greatly influenced by the presence or absence of serum. In the second study, shortening the incubation period to 8 hours was sufficient for detecting IFN activity, but the sensitivity to this assay was less than when incubations occurred for 24 hours. In the third study we verified that the ISRE-luc reporter was responsive to IFNT in MDBK cells.

These outcomes provide convincing evidence that an ISRE (or IRF)-Luc system can substitute for conventional, virus-dependent bioassays for determining IFN bioactivity. One notable benefit is the time savings, this new method being complete in 8 to 24 hours depending on the amount of IFN activity in the sample. Also, the new method does not require the use of a virus and therefore does not need to be completed under highly regulated conditions. The new method also appears to be very sensitive, and contains approximately the same sensitivity of an antiviral assay.

In conclusion, the work completed in this thesis supports the contention that a virus-free bioassay can be used to examine IFN activity in biological samples.

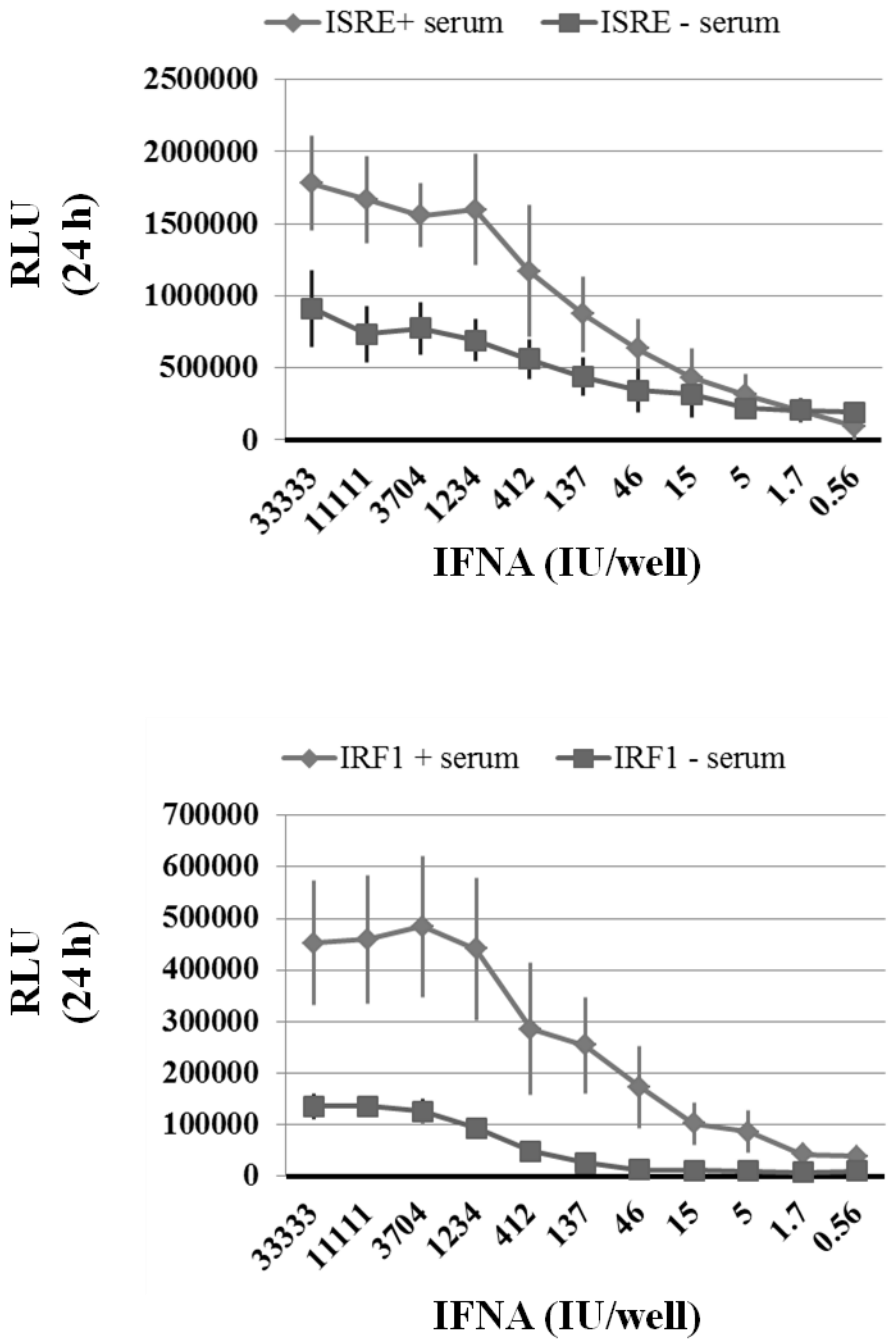

Figure 2-1. Exposure of MDBK cells containing ISRE-Luc with IFNA stimulated luciferase activity in a dose-dependent fashion. MDBK cells that had been transduced with an ISRE reporter construct were cultured in the presence of varying concentrations of recombinant human IFNA for 24 h, then cells were lysed and RLUs were determined. The study was completed on four separate occasions.

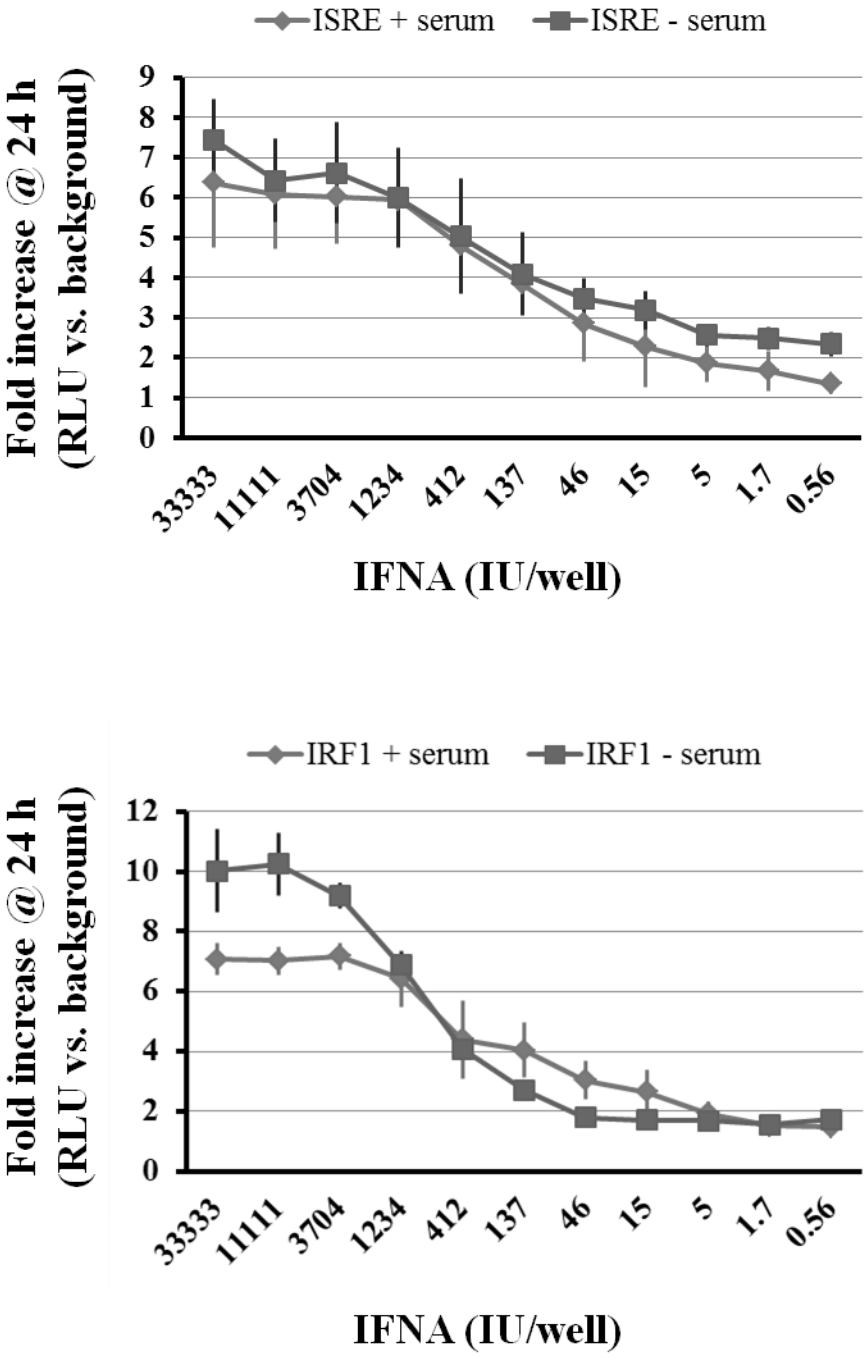

Figure 2-2. Exposure of MDBK cells containing IRF1-Luc with IFNA stimulated luciferase activity in a dose-dependent fashion. MDBK cells that had been transduced with an IRF1 reporter construct were cultured in the presence of varying concentrations of recombinant human IFNA for 24 h, then cells were lysed and RLUs were determined. The study was completed on four separate occasions.

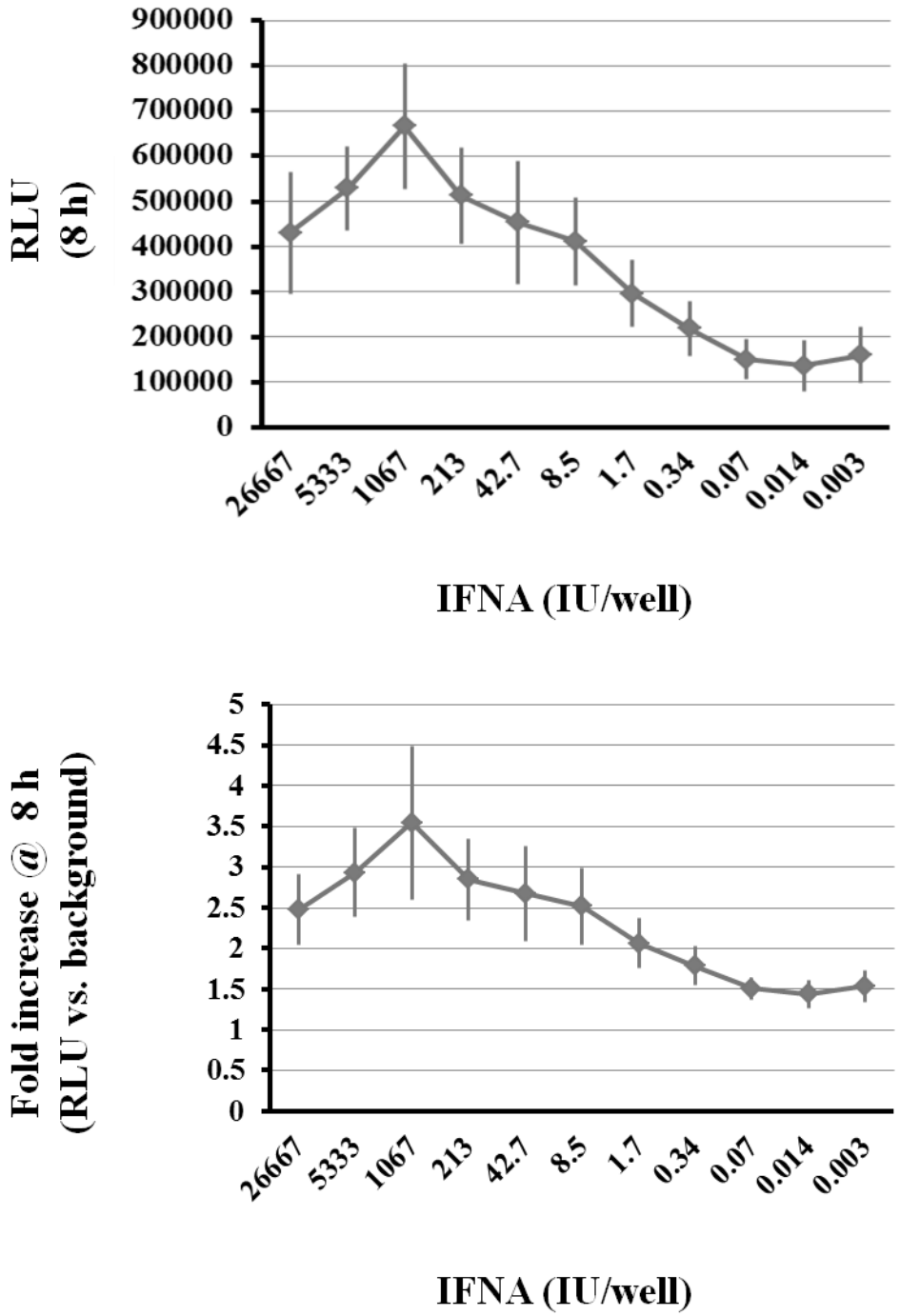

Figure 2-3. Exposure to IFNA for 8 h stimulated luciferase activity of ISRE-Luc MDBK cells in a dose-dependent fashion. MDBK cells were cultured in the presence of varying concentrations of recombinant human IFNA 8 h, then cells were lysed and RLUs were determined. The study was completed on three separate occasions.

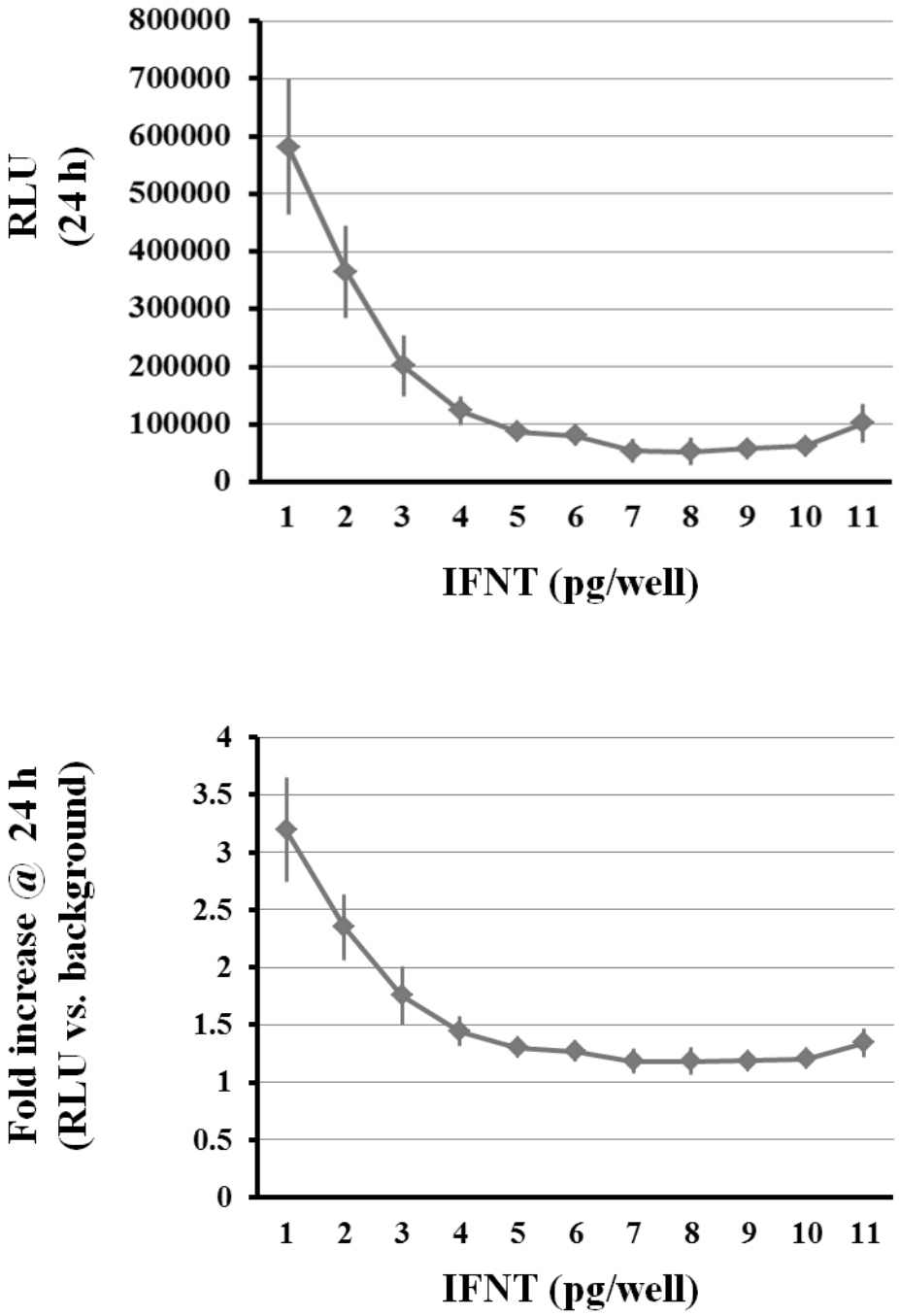

Figure 2-4. Exposure to IFNT stimulated luciferase activity of ISRE-Luc MDBK cells in a dose-dependent fashion. MDBK cells were cultured in the presence of varying concentrations of recombinant bovine IFNT for 24 h, then cells were lysed and RLUs were determined. The study was completed on four separate occasions.

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BIOGRAPHICAL SKETCH

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