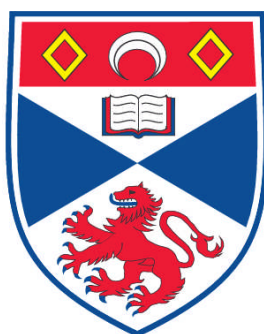# VIRAL INTERFERON ANTAGONISTS AND ANTIVIRAL DRUGS

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A Thesis Submitted for the Degree of MPhil
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Viral Interferon Antagonists and Antiviral drugs

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Abstract

For this project, we developed reporter cell lines that express viral proteins with the potential to be used in cell-based screening assays to select chemical candidates for antiviral drugs. The viral proteins expressed in these reporter cell lines (Hepatitis B core and precore, Hepatitis C core (1a and 4a), and Rabies P (BH and SADL16)) were presented in the literature as responsible for interfering with the IFN signaling pathway, specifically for blocking the expression of its key protein STAT1.

We cloned the viral genes into the pdl'SurvpkIB reporter plasmid and, through a lentiviral delivery system, infected the Hep2Mx1TIPSE cells and the A549Luc cells resulting in the Hep2Mx1TIPSEHBVprecore, Hep2Mx1TIPSEHBVcore, Hep2Mx1TIPSEHCVcore (1a and 4a), and A549lucRabiesP (SADL16 and BH) reporter cell lines.

We assessed the obtained viral cell lines according to their ability to block the IFN signaling pathway by using three different assays: an immunoblot targeting the protein STAT1, a phenotypic assay for survival in the presence of puromycin (in viral Hep2Mx1TIPSE cells), and a quantitative measure of luciferase expression (in viral A549Luc cells).

Concerning the immunoblot targeting STAT1, the results showed that only cell lines expressing the Rabies P protein (namely the A549lucRabiesPSADL16 cell line) were able to decrease the level of expression of STAT1.

The phenotypic assay conducted on the Hep2Mx1TIPSE viral cell lines were intended to show impairment of the IFN signaling pathway through the down-regulation of the IFN stimulated gene Mx1. Normal Hep2Mx1TIPSE cells contain a puromycin resistance gene controlled by the Mx1 promoter. Therefore, when puromycin is added to these cells in the presence of IFN, the signaling pathway is activated and Mx1 as well as the puromycin resistance gene are expressed resulting in cell survival. Results showed that the cell lines expressing the HCV core and HBV precore proteins also survived puromycin addition. However, the Hep2Mx1TIPSEHBVcore cells died in the presence of

puromycin suggesting that in these cells the HBVcore protein affects Mx1 protein expression.

Since it was expected that all viral cell lines would be able to down-regulate Mx1 by impairing the IFN signaling pathway, it was assumed that the level of viral expression may not have been enough to be detected by this kind of assay and therefore a quantitative study would be crucial for the continuation of this project.

The cell lines expressing the Rabies P protein demonstrated their ability to block STAT1 and contained a luciferase gene under the control of an IFN regulated promoter. These cells were therefore considered the best candidates for the quantitative assay. We compared the difference between luciferase expression in the viral cells in the presence and absence of IFN with A549Luc cells (control cells) and verified that in both cases there was an increase in the amount of luciferase expression upon the addition of IFN, which is concordant with the up-regulation of the IFN signaling pathway. However, this increase was considerably less in cells expressing the viral protein. This result confirms a partial blockage of the IFN signaling pathway in these cells. This experiment demonstrates a new alternative step in the creation of cell lines that express the Rabies P protein and that can be applied to the manufacturing process of antiviral drugs. However, in order to achieve the successful production of cell lines, it would be essential to improve viral protein expression.

Declarations

i) I, Rita Luisa Valentim de Avelar Nobre, hereby certify that this thesis, which is approximately 25,000 words in length', has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date........................   Signature of Candidate..................................

ii) I was admitted as a research student in March 2006 and as a candidate for the degree of Master of Philosophy in Molecular Virology in September 2006; the higher study for which this is a record was carried out in the University of St Andrews between 2006 and 2009.

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iii) I hereby certify that the candidate has fulfilled the conditions of the Resolutions and Regulations appropriate for the degree of Master of Philosophy in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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Abbreviations

Units

%	percentage
°C	degrees Celcius
bp	basepair
Da	dalton
g	gram
h	hour
kb	kilobase
l	litre
M	molar concentration
m	meter
min	minutes
ml	mililitre
µl	microlitre
nm	nanometer
pfu	plaque forming unit
sec	seconds
U	unit
V	volt

Viruses

BVDV	Bovine viral diarrhea virus
CMV	Cytomegalovirus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HPIV2	Human parainfluenza virus 2
HSV-1	Herpes Simplex virus 1
MuV	Mumps virus
NDV	Newcastle disease virus
PIV5	Parainfluenza virus 5
Rv	Rabies virus
SFFV	Spleen Focus-Forming Virus

VSV   Vesicular Stomatitis Virus

Nucleic acids

A   Adenine
C   Cytosine
cDNA   Complementary DNA
DNA   Deoxyribonucleic acid
dsRNA Double-stranded RNA
G   Guanine
GTP   Guanosine triphosphate
mRNA Messenger RNA
RNA   Ribonucleic acid
ssRNA Single-stranded RNA
VA-RNA Adenovirus associated
T   Thymidine

Proteins and Complexes

ATF/CREB activating transcription factor/cAMP responsive element binding
AP-1   Activator protein
APOBEC Apolipoprotein B editing complex-1 gene
Cardif   CARD containing adaptor protein
CBP   CREB-binding protein
C/EBP CCAAT-enhancer-binding proteins
CIAP   Calf intestinal alkaline phosphatase
CTL   Cytotoxix T lymphocytes
Cys   Cysteine
DDB1   Damage specific DNA binding protein
FADD   Fas associated death domain
Egr1   Early growth response factor 1
Eif2   Eukaryotic initiation factor 2
GCV   Ganciclovir
HRP   Horseradish peroxidase monoclonal Antibody
IFN   Interferon
IFNAR Interferon alpha receptor
IKK   IκB kinase

IPS-1	Interferon promoter stimulator 1
IRF	Interferon regulatory factor
IRAK-1	Interleukin(IL-1) receptor associated kinase 1
ISRG	Interferon stimulated response gene
ISGF3	Interferon-stimulated gene factor 3
JAK	Janus tyrosine kinase
L	Large protein
M	Matrix protein
ML	Matrix protein Long
MAVS	Mithocondria antiviral signaling protein
MAPK	Mitogen-activated protein kinase
MDA-5	Melanoma differentiation-associated gene 5
MHC	Major histocompatibility complex
MMPs	matrix metalloproteiases
MyD88	Myeloid differentiation primary response protein 88
NF-κB	Nuclear factor kappa-B
NF/AT	Nuclear factor Activated T cell
NP	Nucleocapsid protein
NS1	Non structural 1
NS 5A	Non structural 5A
OAS	2′-5′ oligoadenylate sythetase
P	Phosphoprotein
P53	Protein 53
Pac	Puromycin N-acetyltransferase
PACT	P53-associated cellular protein-testes derived
PKR	dsRNA-dependent protein kinase R
PIAS1	Protein inhibitor of activated Stat1
PML	Promyelocytic leukaemia
PP2A	Protein phosphatase 2A
PRMT1	Protein arginine. methyltransferase 1
PVR	Polyvirus Recceptor
RACK-1	Receptor for activated C-kinase 1
RIG-I	Retinoic acid-inducible gene I
RNase L	Endoribonuclease L
SOCS3	Suppressor of cytokine signalling 3
STAT	Signal transducer and activator of transcription
TAB	Tubulin antisense-binding protein
TAK-1	Thylacoid associated kinase 1

Taq   Thermus Aquaticus
TBK1   TANK-binding kinase 1
Tk   Thymidine kinase
TLR   Toll-like receptor
TNF-α   Tumor necrosis factor-alpha
TRAF6   TNF associated-receptor factor 6
TRIM   Tripartite motif
TRIF   Toll/interleukin 1 receptor domain-containing
         adaptor protein inducing IFN-β
TyK2   Tyrosine kinase 2
Tyr   Tyrosine
V   V protein
VISA   Virus induced signaling adaptor protein
VP 24   Vacuolar Protein 24

Chemicals and Reagents

DAPI   4′,6′-diamino-2-phenylindole
DMEM   Dulbecco's modified eagle's medium
EDTA   ethylenediaminetetraacetic acid
FCS   Fetal calf serum
PBS   Phosphate buffer saline
BSA   Bovine serum albumin

Miscellaneous

2′-5′ A 2′-5′ linked oligomers of adenosine
ATP   Adenosine Triphosphate
β-gal   β-galctosidase
CARD   Caspase recruiting domain
cDC   Conventional dendritic cell
DTT   Dithiothreitol
ECACC   European Collection of Cell cultures
ECL   Enhanced chemiluminescence
GTP   Guanosine Triphosphate
IRES   Internal ribosomal entry site
ISG   Interferon-stimulated gene
ISRE   Interferon-stimulated response element

LB   Luria-Bertani
mDCs   Mature dendritic cells
M.o.i.   Multiplicity of infection
NK   Natural Killer
ORF   Open reading frame
PAMPs   Pathogenic Associated Molecular patterns
PCR   Polymerase chain reaction
pDC   Plasmacytoid dendritic cell
Poly(I:C)Polyinosinic:polycytidylic acid
PMSF   Phenylmethylsulfomylfluoride
SCID   Severe Combined Immunodeficiency
TXRD   Texas Red
UV   Ultraviolet

Contents

1. Introduction

1.1. Interferon System: Induction, Signaling, and Action

The importance of interferon (IFN) in antiviral defense derives from its ability to limit viral growth during a cell's initial infection while simultaneously preventing the virus from spreading to nearby cells. The IFN produced by infected cells signals to neighboring cells by binding to a receptor in those cells. This process activates the expression of hundreds of genes, many of which have direct or indirect antiviral activity. In addition, the IFN response creates a general defense in order to allow the body enough time to generate an adaptive response to an invading virus.

A great deal of progress has been made toward understanding the function of IFN since its discovery in 1957 by Isaacs and Lindemann (Isaacs and Lindemann, 1957). One experiment, for example, revealed the importance of IFN when mice lacking IFN-(α/β) receptors were found to be incapable of mounting an efficient response against viral infection (Müller et al., 1994; Van den Broek et al., 1995).

IFN belongs to a group of proteins called cytokines produced by cells as part of the innate response to a viral infection. The roles of IFN are various. It can induce enzymes that limit viral replication by interfering with cellular processes and it can also induce apoptosis in infected cells or activate cells of the adaptive immune system (Figure 1.1).

IFN can be classified according to the amino acid sequence, the mode of induction, the receptor usage and the biological activity. There are three types of interferon: Types I, II, and III. Type I is primarily composed of IFN-α and IFN-β, Type II of IFN-γ, and Type III of IFNλ1, IFNλ2, and IFNλ3 (Ank et al., 2006). Although different cells can produce different subtypes of IFN, all of the body's cells produce Type I IFNs. For example, plasmacytoid dendritic

Figure 1.1: Interferon Role in Infected Cells and Neighboring Cells

This figure shows a diagram explaining the role of IFN in cells. The cells produce IFN as part of an immune response to a viral infection. The production of IFN induces an antiviral state in neighboring cells where there is an up-regulation of innumerous antiviral genes. These genes code for proteins that can limit viral replication by interfering with various cellular processes inducing growth arrest or even apoptosis in infected cells. Examples of these proteins are the OAS proteins (responsible for mRNA degradation), the PKR protein (responsible for translational arrest), and Caspases (responsible for apoptosis).
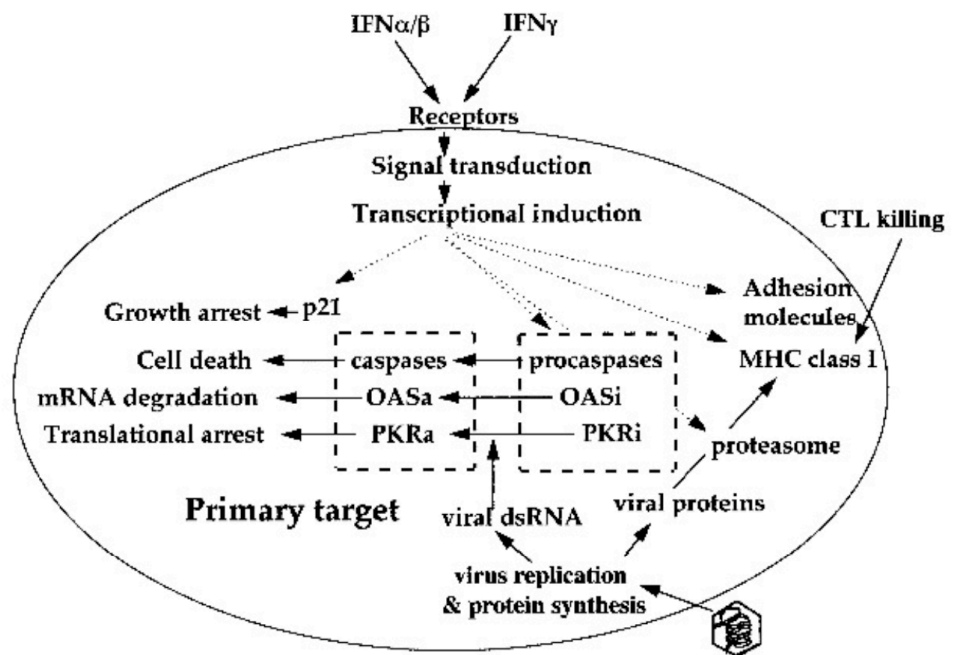
(Goodbourn et al., 2000)

cells (pDCs) secrete IFN-α whereas fibroblasts secrete primarily IFN-β as an initial response to an infection then switch to IFN-α during the amplification of the immune response. Type II IFN is only secreted by cells of the immune system—by Natural Killer (NK) cells during an innate response and by subsets of T-lymphocytes during an adaptive response. The newly researched Type III IFN shows limited tissue distribution and does not appear essential for host survival (Ank et al., 2006; Uze and Monneron, 2007).

Several publications confirm that there are multiple pathways leading to the expression of Type I IFNs: fibroblasts, hepatocytes, and conventional dendritic cells (cDCs) use the "classical" pathway (Figure 1.2) while pDCs use Toll-like receptors (TLRs) expressed on the cell surface or in endosomes to sense extracellular viral material (Haller et al., 2006) (Figures 1.3 and 1.4).

1.1.1. Classical Pathway for Interferon Production

When a virus infects a cell it produces molecular motifs such as dsRNA, which have pathogenic associated molecular patterns (PAMPs). These PAMPs can be recognized by the cells via the intracellular RNA helicases MDA-5 and RIG-I. In the presence of PAMPs, the helicases become activated and induce a cascade that will lead to the transcription of the IFN-β gene. In fact, RIG-I and MDA-5 interact with their downstream partner IPS-1/Cardif/VISA/MAVS leading to the independent activation of two transcription factors, IRF3 and NF-κB. Once activated, both transcription factors IRF3 and NF-κB together with ATF-2cjun assemble cooperatively to form an "enhanceosome" responsible for the activation of the transcription of the IFN-β gene (Kawai et al., 2005; Seth et al., 2005; Xu et al., 2005) (Figure 1.2).

MDA-5 and RIG-I

The helicases MDA-5 and RIG-I serve similar functions. They both recognize viral dsRNA produced during viral replication. However, RIG-I has

Figure 1.2: Classical Pathway for IFN-β Production and IFN Signaling Pathway

When dsRNA invades a cell the two helicases RIG-I and MDA-5 become activated inducing a cascade that leads to the transcription of IFN-β. RIG-I and MDA-5 interact with their downstream partner IPS-1/Cardif/VISA/MAVS leading to the independent activation of two transcription factors, IRF3 and NF-κB. In order to activate IRF3 the adaptor IPS-1/Cardif/VISA/MAVS activates the molecules TBK-1 and IKKε. The activation of NF-κB occurs by the engagement of the IKK(α/β) subunits of the IKB kinase, which are also dependent on the same adaptor as the IRF3 activation. PKR, when activated by dsRNA can induce a signaling cascade that can also activate NF-κB. Once activated, both transcription factors IRF3 and NF-κB together with ATF-2cjun activate the transcription of IFN-β.

The produced IFN-β is then recognized by receptors in neighboring cells designated IFNAR and a signaling cascade is initiated. IFNAR is composed of two subunits, IFNAR1 and IFNAR2. When induced by IFN these subunits heterodimerize and the Jak1 and the Tyk2 proteins transphosphorylate the resulting dimmer. Tyk2 phosphorylates a tyrosine residue Tyr466 on IFNAR1 allowing the SH2 domain of STAT2 to bind to IFNAR1 and also phosphorylates STAT2 at the Tyr690 enabling STAT1 to bind to the complex. The newly formed heterodimer then dissociates from the receptor and forms a heterotrimeric complex with a DNA binding protein of the IRF family. This complex, designated by ISGF-3, then translocates to the nucleus and binds to the IFN-stimulated response elements (ISREs) present in the promoters of certain IFN-stimulated genes (ISGs) initiating their transcription. This process leads to the transcription of several antiviral proteins such as Mx proteins, OAS proteins, or PKR. It also leads to an up-

regulation of the transcritption of proteins that are responsible for the process of production and signaling of IFN such as STATs or IRF3 or even IRF7.
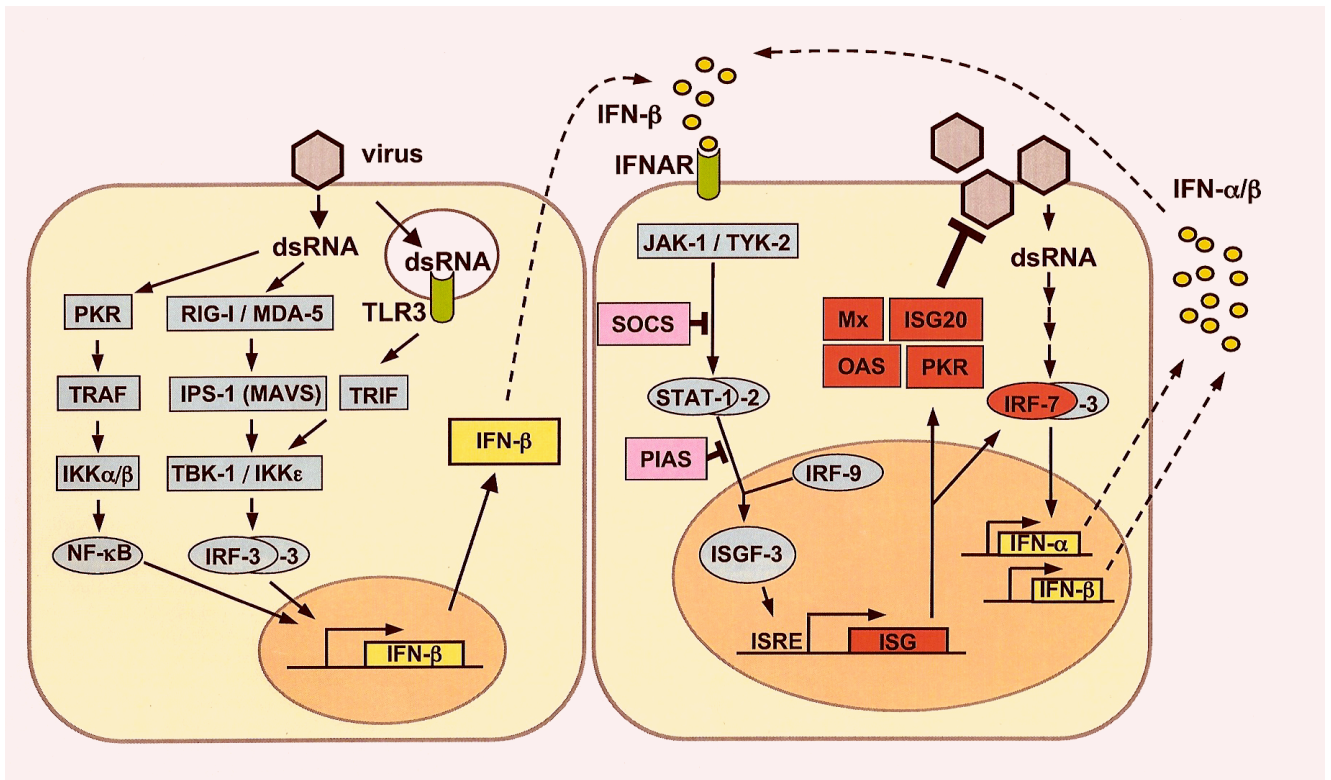
(Haller et al., 2006)

been shown also to recognize ssRNA molecules that contain uncapped and unmodified 5'triphosphate (Hornung et al., 2006).

The activation of each helicase is specific to the type of virus that infects the cell. While RIG-I plays an essential role in the production of IFNs when cells are infected with Paramyxovirus*es*, the influenza virus, or the Japanese encephalitis virus, MDA-5 specifically pertains to infections by picornaviruses (Kato et al., 2006).

In vitro studies revealed that both MDA-5 and RIG-I bind to (poly(I:C))—a synthetic analogue for dsRNA. *In vivo* studies conducted with mice demonstrated that, when subjected to (poly(I:C)), mice lacking MDA-5 did not produce interferon while those lacking RIG-I did (Kato et al., 2006).

Both RIG-I and MDA-5 contain an N-terminal caspase-recruiting domain (CARD)-like region and a C-terminal DExD/Hbox RNA helicase domain. The RNA binds to the helicase domain and induces a conformational change that enables the CARD domain to interact with the CARD domain of its downstream signaling partner IPS-1/Cardif/VISA/MAVS (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005) (Figure 1.2).

Activation of the Transcription Factors NF-kB and IRF3

The downstream partner IPS-1/Cardif/VISA/MAVS—its four names resulting from four different studies—leads to the activation of the transcription factors IRF3 and NF-κB through two independent pathways (Kawai et al., 2005). The activation of IRF3, a member of the interferon regulatory factor family, involves the kinases TBK-1 and IKKε, which are responsible for its phosphorylation (Fitzgerald et al., 2003). The phosphorylated IRF3 homodimerizes and enters the nucleus where it recruits the transcriptional coactivators p300 and the CREB-binding protein in order to initiate the IFN-β mRNA synthesis (Suhara et al., 2002).

The activation of the other transcription factor NF-κB is induced by the IKB-(α/β) subunits of the multicomponent IKB kinase that are recruited by the adaptor IPS-1/Cardif/VISA/MAVS. NF-κB can also be activated via the

protein kinase PKR by dsRNA. The transcription factor is predominantly located in the cytoplasm, in a quiescent state, bound to its inhibitory protein IκB. In the presence of viral dsRNA, the PKR complex activates the IKK-β subunit. This subunit phosphorylates the IκB that is then ubiquinated by an E3 ubiquitin ligase and targeted for degradation. The degradation of IκB results in the release of an active NF-κB protein into the nucleus where it in turn activates the transcription of IFN-β (Zamanian-Daryoush et al., 2000) (Figure 1.2).

NF-κB can be activated in response to various stimuli such as pathogens, stress signals, and pro-inflammatory cytokines (TNF-α and IL-1) and regulates both the innate and adaptive immune responses. It also regulates pro-inflammatory gene expression and induces the transcription of pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteiases (MMPs), cyclooxygenase 2, and nitric oxide synthase (Li et al., 2002).

Formation of the "Enhanceosome"

The two transcription factors IRF3 and NF-κB together with the ATF-2/cjun bind to the IFN-β promoter to form a multiprotein complex known as the "enhanceosome". This complex is responsible for the activation of the transcription of IFN-β. It is generally held that the binding of IRF3 is indispensable for IFN induction whereas the action of NF-κB and ATF-2/cjun may not be essential at all (Panne et al., 2007) (Figure 1.2).

The initial production of IFN-β leads to the expression of the protein IRF7, which does not normally exist in most cells save the notable exception of pDCs in which it helps the expression of IFN-α noted below. In the "enhanceosome", the negative-acting protein IRF2 can compete with IRF3 and IRF7 for limited binding sites down-regulating the transcription process (Harada et al., 1989).

Induction of IFN–α

4

Though the induction of IFN-α genes is not greatly understood, recent research yields potentially useful information. The IFN-α genes show no NF-κB sites in their promoters. Instead, they have a number of binding sites for IRF3 and IRF7. In fibroblasts, IFN-α production occurs only during a second wave of induction. The reason for this is because IRF7 (which preferentially stimulates the production of IFN-α) does not exist in these cells. When IFN-β is first produced it activates a signaling pathway that induces the expression of different genes including the gene that codes for IRF7 (Hata et al., 2001; Sato et al., 2000)

In lymphocytes, however, viral infection activates the production of IFN-α without the need to pre-produce IFN-β (Hata et al., 2001). Therefore, it is tempting to speculate that this is correlated with the pre-existence of IRF7 in these cells. In pCDs, there is clear evidence of constitutive IRF7 expression, allowing them to produce substantial quantities of IFN-α as well as IFN-β (Honda et al., 2005).

1.1.2. Other Pathways for Interferon Production

IFN can also be produced through other pathways that do not involve recognition of nucleic acids by the helicases MDA-5 and RIG-I.

1.1.2.1. dsRNA Delivered through Endosomes

Cells can have dsRNA receptors in the endosomes. These receptors are activated when the cell internalizes dsRNA that is present in the extracellular environment or is produced by the uncoating or the degradation of entire viral particles. One of these receptors is called Toll-like receptor 3 (TLR3). TLR3 is distributed widely through tissues and is expressed in dendritic cells (DCs). In mDCs cells it relocalizes from the endoplasmic reticulum to the endosomes (Johnsen et al., 2006). In bone marrow-derived macrophages it

relocalizes to the lysosome (Lee at al., 2006). With regards to fibroblasts, TLR3 relocalizes from the reticulum to the endosomes and to the cell surface (Matsumoto et al., 2002).

When TLR3 detects dsRNA it dimerizes and phosphorylizes. The dimer then recruits PI3 kinase and an adaptor called TRIF. This adaptor is responsible for inducing two independent cascades that lead to the activation of the transcription factors IRF3 and NF-κB, which are responsible for the induction of the transcription of the IFN-β gene (Figure 1.3).

The IRF3 activation occurs when TRIF recruits TRAF3. This molecule then binds to TANK, which, in turn, binds to TBK1 and IKKε. The last two proteins are then responsible for phosphorylizing IRF3 and thus activating it (Fitzgerald et al., 2003; Hacker et al., 2006).

Meanwhile, TRIF also recruits TRAF6 and RIP1 (Meylan et al., 2004; Sato et al., 2003; reviewed in Randall et al., 2008). The recruitment of TRAF6 leads to its ubiquitination and to RIP ubiquitination as well. The polyubiquitinated chains are then recognized by TAB2 and TAB3, which chaperone the kinase TAK1 to the complex. Then NEMO, a subunit of the IKK complex, recognizes RIP1 thereby recruiting the IKK complex to the TRIF/RIP1/TRAF6/TAK1 complex. Subsequently, TAK1 phosphorylates the IKK-β subunit of the IKK complex inducing a downstream phosphorylation and degradation of IκB and the subsequent nuclear uptake of NF-κB (Kanayama et al., 2004; Wang et al., 2001).

The two transcription factors now activated and in association with the molecule ATF-2/cjun induce the transcription of IFN-β.

1.1.2.2. ssRNA Delivered through Endosomes

This pathway occurs primarily in pDCs since they are one of the few cell lines expressing TLR7.

When exposed to ssRNA, pDCs dependent on TLRs for IFN induction may initiate strong IFN responses. ssRNA activates TLR-7 which recruits the adaptor MyD88 which, in turn, recruits a complex containing the kinases

Figure 1.3: dsRNA Delivered Through Endossomes

This diagram represents the IFN induction pathway when dsRNA is detected by endosomes. In this pathway, upon stimulation by dsRNA, the molecule TLR3 dimerizes and phosphorylizes. It then relocates from the endoplasmic reticulum to endosomes (in monocy DCs) or to lysosomes (in bone marrow derived macrophages) where it recruits PI3 kinase and TRIF. Then two independent cascades, which lead to the activation of the two transcription factors IRF3 and NF-κB, are initiated.

The path that leads to the activation of IRF3 starts when TRAF3 binds to TANK enabling this molecule to bind to TBK-1 and IKKi/IKKε. This will directly activate IRF3.

The NF-κB activation cascade involves the recruitment of TRAF-6 and RIP as well as TAK1 and the molecules belonging to the IKK complex. Next, the IKKβ subunit of the IKK complex is phosphorylated leading to the phosphorylation of IκB, the NF-κB inhibitor. IκB is subsequently ubiquitinated and degraded releasing the active NF-κB. The two transcription factors IRF3 and NF-κB together with ATF-2cJun can then activate the transcription of IFN-β.
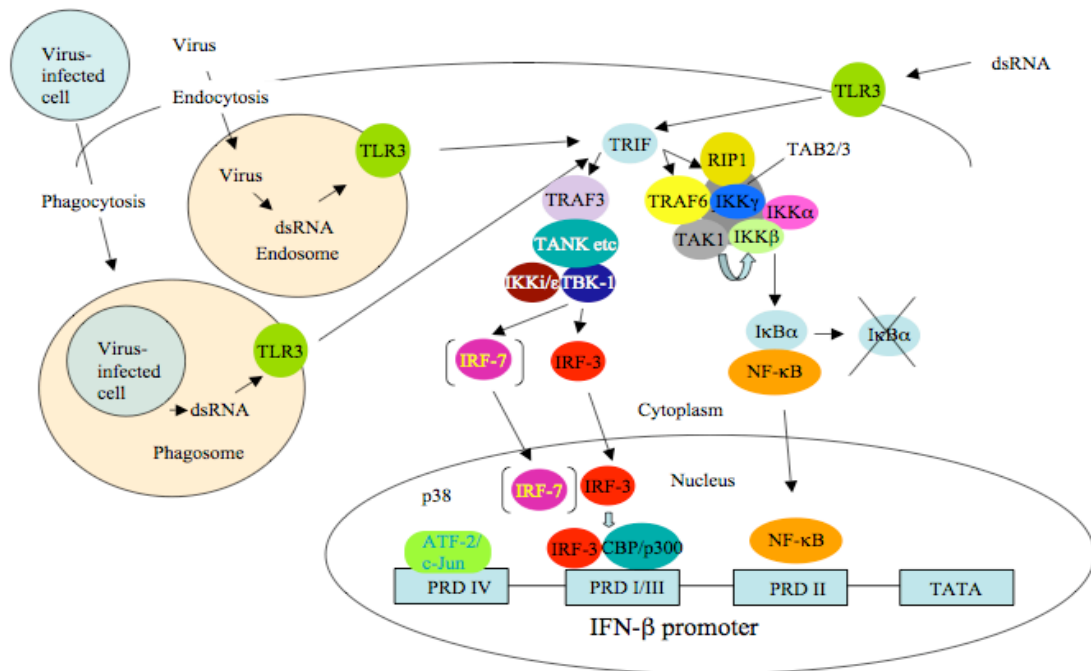
(Randall and Goodbourn, 2008)

IRAK-4, IRAK-1, and TRAF6 (Figure 1.4). The MyD88/IRAK-1/IRAK-4/TRAF6 complex directly binds to IRF7 and TRAF6 utilizes its ubiquitin E3 ligase function to polyubiquitinate IRF7. The complex is then phosphorylated by IRAK-1 and translocated to the nucleus where it binds to the DNA and stimulates IFN-β transcription. Simultaneously, NF-κB is also activated through a TRAF6 induced pathway. In this pathway, TRAF6 recruitment activates the TAK1/TAB2/TAB3 and the IKK complex leading to the release and destruction of IκB, resulting in the nuclear uptake of NF-κB (Kanayama et al., 2004; Kawai et al., 2004) (Figure 1.4).

1.1.2.3. DNA Delivered through Endosomes

Some Peripheral Blood Mononuclear cells (PMBCs) exhibit a response to foreign DNA resulting in IFN-β production. This process is similar to the process described for ssRNA. The receptor, which recognizes the foreign unmethylated DNA, is TLR9 (Figure 1.4).

The pathways by which pDCs and mDCs activate IFN-β gene are different. pDCs use the same cascade as described above for ssRNA delivered by endosomes involving the adaptor MyD88 in order to activate IRF7. NF-κB is also activated in these cells (Honda et al., 2005). For mDCs, however, IRF7 is not activated and instead the molecule IRF1 is used to induce the transcription of the IFN-β gene (Schmitz et al., 2007).

1.1.2.4. Cytoplasmic DNA

The macrophages and DCs appear capable of responding to foreign DNA present in the cytoplasm. Mammalian genomes are relatively G-C rich and therefore DNAs that have a high A-T content are easily recognized as non-self (Ishii et al., 2006: Stetson and Medzhitov 2006). The receptor for the cytoplasmic DNA has been recently identified as DA1/DLM-1/2BP-1 and it is distinct from TLRs and RIG-I/MDA-5. The process by which IFN-β is

Figure 1.4: **ssRNA and DNA Delivered Through Endossomes**

This diagram represents the cascade of IFN induction initiated by ssRNA in endossomes. When stimulated by ssRNA, the molecule TLR-7 is activated and recruits the adaptor MyD88, which in turn recruits a complex containing the kinases IRAK-4, IRAK-1, and TRAF6. The MyD88/IRAK-1/IRAK-4/TRAF6 complex directly binds to IRF7 and TRAF6 utilizes its ubiquitin E3 ligase function to polyubiquitinate IRF7. IRAK-1 then phosphorylates the complex and translocates it to the nucleus where it will bind to the DNA stimulating the IFN-β transcription. In parallel, the transcription factor NF-κB is also induced by TRAF-6. This molecule activates the IKK complex leading to the phosphorylation of the IKKβ subunit of the IKK complex. This results in phosphorylation, ubiquitination, and degradation of IκB the NF-κB inhibitor. Subsequently, NF-κB, IRF3, and ATF-2 c-Jun activate the transcription of IFN-β. In the case of DNA stimuli, instead of TLR-7, TLR-9 recognizes the nucleic acid and the same cascades are activated leading to the transcription of IFN-β.
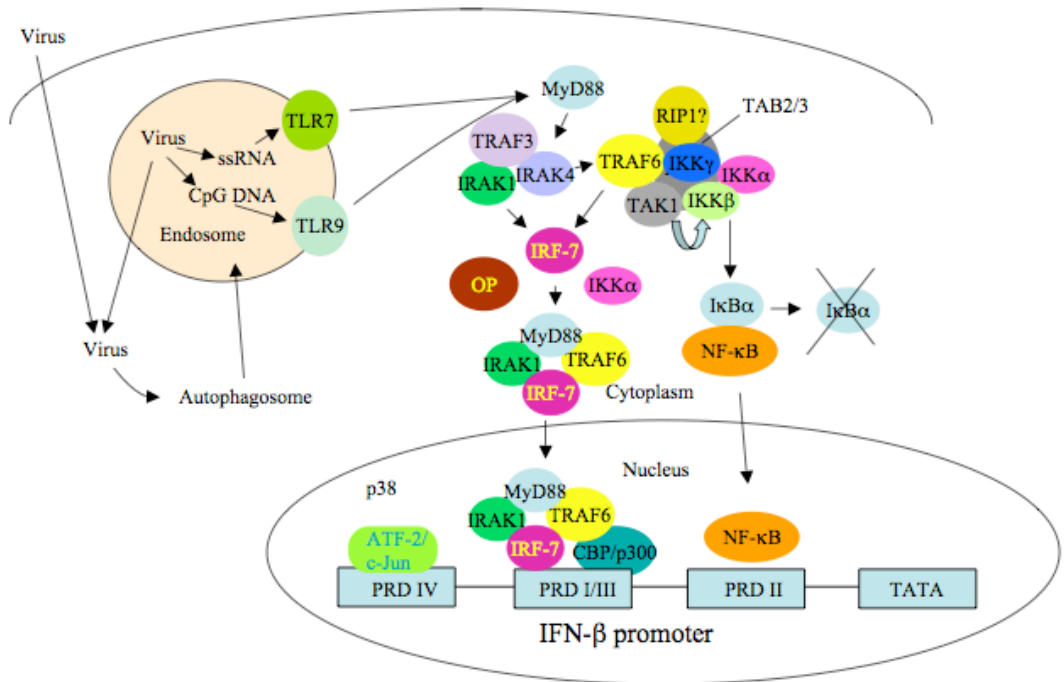
(Randall and Goodbourn, 2008)

induced is unclear and there are questions as to whether or not NF-κB is involved (Ishii et al., 2006; Takaoka et al., 2007).

1.1.2.5. Viral Proteins

Several viral envelopes or particles have been reported as inducers of IFN. The most studied systems regarding viral proteins that induce IFN are herpes viruses. In response to either Cytomegalovirus (CMV) or Herpes Simplex virus-1 (HSV-1), human fibroblasts have been shown capable of activating the IRF3 complex formation without the need for protein synthesis. It is still not clear if viral entry is required to initiate IRF3 activation (Navarro et al., 1998; Preston et al., 2001; Mossman et al., 2001).

1.1.3. IFN Signaling Pathway

Once produced, IFN-β activates the Jak/STAT pathway by binding to the appropriate receptor on the surfaces of neighboring cells (Figure 1.2). The receptor complex is composed of at least two different subunits (IFNAR1 and IFNAR2). In the absence of stimulatory signals, IFNAR1 constitutively associates with the kinase Tyk2 while IFNAR2 associates with the kinases Jak1 and STAT2. However, when stimulation occurs, the receptor subunits rearrange themselves and heterodimerize. Then, Jak1 and Tyk2 transphosphorylate. Tyk2 phosphorylates a tyrosine residue Tyr466 on IFNAR1 allowing the SH2 domain of STAT2 to bind to IFNAR1. Tyk2 also phosphorylates STAT2 at its Tyr690 enabling STAT1 to bind to the complex. The novel heterodimer then dissociates from the receptor and forms a heterotrimeric complex with IRF-9, a DNA binding protein of the IRF family. The new complex, called ISGF3, translocates to the nucleus and binds to the IFN-stimulated response elements (ISREs) present in the promoters of certain

IFN-stimulated genes (ISGs) initiating their transcription (reviewed in Haller et al., 2006) (Figure 1.2).

An alternate and contrasting process of signal transduction is called attenuation. Though little is known about this process, a number of proteins including IRF2 and IRF-8 have been reported to bind to ISREs and negatively regulate their expression. This helps to prevent IFN-response (Harada et al., 1989; Nelson et al., 1993).

1.1.4. Antiviral Proteins

There are several types of ISGs that code for antiviral proteins. Three of these proteins are the protein kinase R (PKR), 2'-5' oligoadenylate synthetase (2-5OAS) proteins, and Mx proteins.

PKR

The IFN-inducible PKR is a serine threonine kinase. PKR has two domains with different functions: the C-terminal domain has a catalytic function and contains all the conserved motifs for protein kinase activity whereas the N-terminal domain contains the dsRNA-binding site. PKR is synthesized in an inactive form and activates in response to dsRNA (Katze et al., 1991; Meurs et al., 1990). A stress-activated protein called PACT can also activate PKR (Peters et al., 2001).

PKR also plays a decisive role in the induction of the transcription factor NF-κB (see chapter 1.1.1). PKR is important in cell-regulatory processes such as cell growth, antiviral states, and apoptosis. In order to regulate apoptosis, PKR is responsible for the phosphorylation of the α subunit of the eukaryotic translation initiation factor eIF2 resulting in the inhibition of translation required for protein synthesis (Balachandran et al., 1998; Gil et al., 1999). Another process by which PKR can regulate apoptosis involves Fas, a widely known cell surface receptor. The gene that codes for Fas is

upregulated upon PKR induction leading to the recruitment of the Fas associated protein named FADD. Subsequently, caspase 8 is also recruited and this protein is responsible for executing apoptosis in the cells (Gil et al., 2000; Nanduri et al., 1998).

Although the protein PKR is extremely important for antiviral response, studies with mice have shown that it is not sufficient to entirely mediate the antiviral response (Yang et al., 1995).

2'-5'Oligoadenylate Synthetase System

The 2'-5'Oligoadenylate Synthetase System (OAS) consists of a group of enzymes that use dsRNA as a cofactor. These proteins oligomerise ATP through a 2'-5' phosphodiester linkage producing 2'-5'oligonucleates that bind to the endoribonuclease RNase L thereby activating it. The RNase L protein degrades viral and cellular RNAs. This system appears to be important for the defense against the vaccinia virus, the reovirus, and the encephalomyocarditis virus (Castelli et al., 1998; Silverman, 1996).

Mx Proteins

An alternative pathway for an antiviral response involves the Mx proteins. The IFN-inducible Mx proteins are highly conserved large GTPases with homology to dynamin (reviewed in Staeheli et al., 1993). Some examples of Mx proteins include the Mx1 and the MxA. The Mx proteins interfere with virus replication by inhibiting virus polymerase activity at certain points in a virus lifecycle. This results in much slower virus growth (Stranden et al., 1993). Mx 1 proteins have been shown to inhibit growth of *Orthomyxoviridae* viruses whereas MxA proteins suppress the growth of *Orthomyxoviridae*, *Paramyxoviridae*, *Rhabdviridae*, *Bunyaviridae* and *Thogaviridae* viruses (reviewed in Goodbourn et al., 2000). Studies show that human MxA protein recognizes the viral nucleoprotein of the Thogoto virus and prevents incoming viral

nucleaocapsids from being transported into the nucleus and impairing a viral response (Weber at al., 2000).

While PKR, RNase L, and Mx are the most well understood antiviral proteins, several others exist. This has been demonstrated experimentally with cells from triple knockout mice still able to exhibit a limited IFN-induced antiviral state (Zhou et al., 1999). These other proteins include ISG15 (Pitha-Rowe et al., 2007), ISG54, ISG56 (Terenzi et al., 2005), PML bodies (Everett and Chelbi-Alix, 2007), APOBECs, TRIMs (Towers et al., 2007), and Adenosines Diamenases (reviewed in Toth et al., 2006).

1.1.5. Virus Countermeasures to IFN Responses

There are three primary methods by which a virus counteracts cell response to infection: inhibiting the production of IFN, inhibiting the IFN signaling pathway, or inhibiting the IFN-induced anti-viral proteins. Viruses are also capable of circumventing the IFN response if they have a replication system that is insensitive to it. However, it is not always advantageous for a virus to inhibit cellular gene expression and cause the destruction of the host cell. By doing so the virus allows itself little time to replicate.

Inhibition of IFN Induction

Viruses are able to inhibit IFN production. They can operate by sequestering dsRNA essential for the expression of the IFN-β gene. One example is the NS1 protein of the Influenza virus (Garcia-Sastre et al., 1998). The sequestration of dsRNA minimizes its dependent activation of antiviral gene products and also blocks possible dsRNA-induced apoptosis. Viruses can also interfere with important proteins of the IFN induction pathway. For instance, Paramyxovirus*es* use their V protein in order to block MDA-5 activity (Andrejeva et al., 2004). The transcription factor IRF3 is also a target for some viruses such as bovine viral diarrhea virus (BVDV), which uses its

Npro protein to block IRF3 targeting it for degradation (Hilton et al., 2006). The ML protein of the Thogoto virus can also inhibit IRF3 dimerisation and the recruitment of the transcriptional coactivator CBP (Hagmaier et al., 2004). Likewise, the human papillomavirus contains the protein E6 that binds to the IRF3 inhibiting its activation function (Ronco et al., 1998). However, because there are substitutes for the IRF3 function in the IFN pathway (such as IRF1) inhibition is not complete.

The other main transcription factor, NF-κB, can also be impaired by viruses. In fact, the African swine fever virus encodes a homologue of IκB that inhibits the activity of NF-κB (Powell et al., 1996). However, it has been established that NF-κB also induces anti-apoptotic genes and if the virus blocks NF-κB such genes will not be produced. This may lead to an enhancement of apoptosis in the infected cell that would be detrimental to the virus.

Inhibition of IFN Signaling

The inhibition of the IFN signaling pathway may occur at various stages by numerous kinds of viruses. Most Paramyxovirus*es* inhibit STAT proteins by a variety of methods. Parainfluenza Type 5 virus (PIV5) employs the V protein to target STAT1 for degradation (Didcock et al., 1999), whereas Human Parainfluenza virus Type 2 (HPIV2) targets STAT2 (Young et al., 2000). The Mumps virus uses the V protein to target both STAT1 and STAT3 (Ulane et al., 2003). The V and the P proteins of the Nipah and Hendra viruses sequester STAT1 and STAT2 (Rodriguez et al., 2003). The Sendai virus also sequesters STATs thus increasing their turnover and altering the pattern of STAT1 phosphorylation (revewed in Stock et al., 2005). Despite interfering with STAT proteins, some other Paramyxovirus*es* inhibit the Jak kinase signaling pathway by interacting with the protein RACK1 (Kubota et al., 2002; Yokota et al., 2003).

Other viruses responsible for blocking the IFN signaling pathway include the Rabies virus that has the P protein (which interacts with STAT1

and STAT2 establishing an inactive complex) (Brzozka et al., 2006), the Hepatitis C virus (HCV) that has the core protein (which interacts with STAT1 inhibiting its phosphorylation and its interaction with STAT2) (Lin et al., 2006), and the Ebola virus that has the VP24 protein (which interacts with karyopherin alpha 1 inhibiting the nuclear transport of STATs) (Reid et al., 2006). In addition, the HSV virus rapidly induces the expression of SOCS3, a cellular inhibitor of the Jak/STAT pathways (Yokota et al., 2003). Finally, Dengue (Ho et al., 2005), Kunjin, and West Nile (Liu et al., 2005) viruses partially block the IFN signaling pathway.

Inhibition of IFN Induced Antiviral Enzymes

Several viruses (such as Pox, Herpes, Influenza, and Reoviruses) encode proteins that are responsible for sequestering dsRNA and therefore preventing the activation of IFN-induced antiviral proteins (such as PKR and OAS).

Viruses can inhibit PKR by producing small and highly structured RNA molecules that prevent PKR dimerisation and consequently its activation. One example is the VA-RNA of Adenoviruses (Clarke et al, 1995). Viruses can also produce proteins that directly bind to PKR inhibiting it; examples include NS5A of HCV or NS1 of Influenza (Lu et al., 1995). Finally viruses can also produce proteins that induce the expression of PKR inhibitors such as the p58 protein of Influenza virus (Melville et al., 1999).

The OAS group suffers inhibition by viruses as well. Some viral proteins (such as the NS1 protein of the influenza virus) bind to dsRNA that, in turn, binds to OAS (Min & Krug, 2006). Other viruses induce RNase L inhibitors causing the down-regulation of this protein. The HIV-1 virus is an example of this process (Martinand et al., 1999).

HBVcore/precore proteins are responsible for blocking Mx proteins (in particular the MxA protein) by interacting directly with their promoters (Fernandez et al., 2003).

1.1.6. Consequences of Infection for the Host

Cells infected by viruses show damage to their IFN response. However, if this damage is not severe the cell may survive. Some cellular functions seem to be controlled by IFN even in the absence of a viral infection. One example is the activity of osteoclasts that are known to be involved in bone reabsorption and that are negatively regulated by the release of small amounts of IFN (Takayanagi et al., 2002; Takayanagi et al., 2005). An increase of osteoclast activity may cause a disease called Paget's Bone Disease. Although it is not completely accepted, recent studies reveal that persistent infection by Paramyxovirus*es* causes an increase in osteoclast activity and therefore an increase of this disease (Bender, 2003). Oddly enough, there are some reported advantages for hosts due to chronic/latent viral infections. Mice latently infected with herpes viruses are more resistant to a bacterial infection than non-infected mice. Also, since IFNs induce the production of Il-15, which is responsible for stimulating memory CTLs, the continuous production of IFN may assist the maintenance of immune memory (Boyman et al., 2007).

1.1.7. IFN Response and Viral Host Range

Several viruses differ in their ability to circumvent the immune response according to their current host. This can be due to the fact that in some non-original species the viral replication cycle is slower allowing the cell to mount more significant antiviral responses that would impair the virus from blocking the IFN pathways. For instance, Myxoma virus, a rabbit-specific pox virus, does not replicate in murine or human cells because it is incapable of counteracting the IFN response in these animals.

PIV5 is non-pathogenic in Severe Combined Immunodeficiency (SCID) mice because it fails to block the IFN signaling pathway (its V protein does not target STAT1 for degradation). A single amino acid change may, however,

allow the V protein to target STAT1 for degradation and therefore block the IFN signaling pathway (Young et al., 2001).

Some viruses are, however, perfectly capable of counteracting IFN in species which are not their natural host. Some examples are the Npro of BVDV, which blocks IFN production in human, monkey, and dog species (Hilton et al., 2006). Also, the V protein of the Mapuera virus is capable of blocking IFN signaling in horse, dog, pig, monkey, and human cells (Hagmaeir et al., 2007).

It was reported (Ida-Hosonuma et al., 2005) that poliovirus can replicate in Human-Polivirus receptor (PVR) transgenic mice. The expression of PVR can be observed in both target and non-target tissue. It was revealed that in mice where IFN was knockout, the infection had spread to tissues in areas other than the nervous system such as the liver or the spleen. Therefore, it was implicit that in this case IFN had an important role in determining the tissue tropism. It protected tissues that were potentially susceptible to infection.

1.2. Viral Proteins

Several viral proteins have been reported to block the IFN action whether during its initial production or on the following signaling cascades. Some examples of those viral proteins are the core and precore proteins of the Hepatitis B virus (HBV), the core protein of the Hepatitis C virus (HCV), the V protein of Paramyxovirus*es*, and the P protein of the Rabies virus.

1.2.1. Hepatitis B Virus

The Hepatitis B virus infects a great number of people all over the world. The high-risk groups are well defined: homosexuals, drug addicts, health workers, and children of immigrants from countries where the

Hepatitis B virus is endemic (Alexander, 1986). The majority of individuals chronically infected with HBV experience severe liver disease and risk developing hepatocellular carcinoma (Waris et al., 2003). Patients treated with IFN-α show a 40% recovery rate (Fernandez et al., 2003).

HBV is a small DNA virus with a circular genome of 3.2Kb. Its genes C, S, P, and X code for different viral proteins. The C gene codes for the core protein and the serum e antigen; the S gene codes for three related viral envelope proteins known as surface antigen; the P gene codes for the viral polymerase P protein; and the X gene codes for a 16.5 kDa X protein (Waris et al., 2003).

The X protein is essential for the productive infection of mammalian cells with HBV. It does not directly bind to DNA but instead functions via protein-protein interaction. The X protein has been shown to function as a transcriptional transactivator via different molecules such as NF-κB, ATF/CREB, NF-AT, AP-1, C/EBP, p53, Egr-1, and STAT3. It can also activate signaling pathways including the mitogen-activated protein kinase (MAPK), the c-jun N-terminal kinase (JNK), and the Src tyrosine kinases (Waris et al., 2003) pathways.

The HBV virus produces a secreted form of the structural nucleocapsid core protein called precore protein. The precore protein has all the amino acids of the core protein plus 29 N-terminal residues (Guidotti et al., 1996). This precore protein was suggested to be important in the progression of chronic Hepatitis B when mutant strains with the same protein were reported to be more susceptible to treatment with IFN-α (Wang et al., 2005).

It has been shown that HBV defective particles are implicated in a deficient response to IFN-α in huh7 cells. The authors have proposed that the core protein performs the transcriptional inhibition of IFN-α in these cells. Recent studies suggest a direct interaction between the precore and the core proteins of the HBV virus and two regions of the MxA promoter. In this way, these proteins contribute to the inhibition of the action of IFN-α (Fernandez et al., 2003; Rosmorduc et al., 1999). There are also studies that show that HBV induces a significant up-regulation of PP2Ac (the catalytic subunit of PP2A).

PP2A is a key serine/threonine phosphatase that physically interacts with PRMT1 (an enzyme expressed in cells). This PRMT1 enzyme is responsible for the arginine methylation of many proteins including histones, RNA binding proteins, and STAT1. Therefore, by up-regulating PP2Ac there is an inhibition of PRMT1 and a subsequently reduced methylation of STAT1. Unmethylated STAT1 has a high affinity to PIAS1 (inhibitor of DNA binding to STAT dimmers), which confers STAT1 a reduced ability to stimulate IFN-α target genes. By up-regulating PP2Ac, the virus limits the ability of STAT1 to stimulate the expression of IFN-α target genes (Christen et al., 2006).

1.2.2. Hepatitis C Virus

The Hepatitis C virus (HCV) is classified under its own genus (*Hepacivirus*) in the *Flaviridae* family. Like other members of this family (such as the *Flavivirus* and the *Pestivirus*) the *Hepacivirus* has a positive-sense single stranded RNA genome (Yanagi et al., 1998). This genome has 9,500 nucleotides containing a single open reading frame (ORF) that encodes a large poly-protein of 3,000 amino acids. This polyprotein is processed by both host encoded and virus encoded proteases in order to create various mature polypeptides. This process yields three proteins with important structural functions: the core protein (C), the envelope 1 protein (E1), and the envelope 2 protein (E2). It also yields six non-structural proteins called NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The ORF is flanked by a 5'non-coding region (NCR) that acts as an internal ribosome site (IRES) and a 3'NCR that consists of anywhere from 27 to 66 nucleotides followed by a poly(U) tract. The ORF is followed by 98 highly conserved base elements thought to be required for replication and/or packaging (Tanaka et al., 1995).

The majority of HCV isolates can be classified into six groups designated genotypes 1 through 6. More genotypes (7, 8, 9, and most recently 10a and 11a) have also been proposed (Tokita et al., 1995). The genotypes 1 and 2 are distributed throughout Far East Europe, the USA, and also parts of Africa; genotype 3 is distributed throughout Europe, the USA, Thailand, and

India, and also rarely in Japan. Unlike the others, genotype 4 is specific to the Middle East and Central and North Africa where it is particularly prevalent in Egypt (Chamberlain et al., 1997). Over 80% of HCV infections lead to chronic hepatitis and approximately 20-30% of patients in this group develop cirrhosis.

Though HCV is a major cause of morbidity and mortality worldwide and there is a significant need for prevention and effective treatment, neither a vaccine nor an absolutely efficient treatment exists. There is, however, a therapy that utilizes IFN-α alone or in combination with Ribavirin (Chamberlain et al., 1997; de Lucas et al., 2005). Ribavirin is a pro-drug (a chemical precursor to the drug itself) that when activated by cellular kinases interferes with the viral RNA metabolism. The treatment using Interferon and Ribavirin appears to work in only 40-60% of infected cases. This indicates that HCV has a mechanism for counteracting the effect of IFN-α (de Lucas et al., 2005). If treatment utilizes only IFN-α, the response rate drops to 20%.

It has been reported that the HCV proteins in cell lines and in transgenic animals interfere with the IFN signaling pathway (Heim et al., 1999). This interference may be connected to the core protein. The HCV core protein induces a reduction in the formation of the ISGF3 complex thereby inhibiting the Jak/STAT pathway (de Lucas et al., 2005). Studies reveal that an interaction exists between the HCV core protein and the STAT1 protein. The N-terminal 23 amino acids of the HCV core protein interact with the SH2 region of STAT1. STAT1 is then unable to bind to STAT2, thus impairing the IFN signaling pathway. Several experiments indicate that point mutations in other key domains of STAT1 show no signs of disrupting HCV core and STAT1 interaction which means that the SH2 domain is indeed the essential domain for HCV core and STAT1 interaction (Lin et al., 2006).

It has also been shown that the same protein (HCV core) interferes with the MxA protein expression. It has been proven that the expression of the HCV core protein leads to a decrease in MxA levels in a dose-dependent manner. This suggests that the core protein inhibits IFN-α induced transcription of the gene that codes for the MxA protein. Several studies (Li et

al, 2005; Meyland et al., 2005) have demonstrated that the HCV proteins NS3-4a may also efficiently block the IFN production pathway. These viral proteins colocalize with the adaptor IPS-1/Cardif/VISA/MAVS at the mitochondrial membrane and cleave the adaptor at its cys508, dislocating it from the mitochondria and impairing it from activating the IRF3 and NF-κB pathways.

1.2.3. Paramyxoviruses

Some viruses, members of the *Paramyxoviridae* family, have evolved the ability to antagonize IFN functions by blocking the IFN signaling pathway. These viruses have a linear non-segmented negative-sense RNA genome of 15-18kb. The genome consists of six genes (N, P/V(C), M, F, G, and L) that encode the nucleocapsid protein, the phosphoprotein (or the V protein), the matrix protein, the fusion glycoprotein, the attachment glycoprotein, and the large protein. The phosphoprotein and the large protein form the RNA-dependent RNA polymerase. Concerning the V protein, its C-terminus has seven cysteine residues and is highly conserved among Paramyxovirus*es*. The V protein has been reported to be responsible for the inhibition of the IFN signaling pathway in some Paramyxovirus*es*. This process is carried out by inhibiting the Jak/STAT pathway.

1.2.3.1. Mumps Virus

Mumps virus (MuV) uses its V protein to degrade the STAT1 protein. Several studies show that the V protein also inhibits STAT1 and STAT2 phosphorylation (Kubota et al., 2005; Yokosawa et al., 2002). The Cys-region in the C-terminal region of the MuV V protein decreases STAT1 production and thereby contributes to the blockage of the IFN signaling pathway (Kubota et al., 2005). The mechanism involved in the suppression of STAT2 phosphorylation is still unknown. However, since STAT2 associates with

STAT1, it must be considered that such suppression could be due to the decrease in STAT1 production (Yokosawa et al., 2002).

1.2.3.2. Parainfluenza Type 5 Virus

The Parainfluenza Type 5 virus (PIV5) expresses the V protein. This protein is the 222 amino-acid product of an mRNA copy of the second open reading frame (V/P gene) of the viral genome. Similar to other Paramyxovirus*es*, the C-terminal domain of the V protein of PIV5 does not induce the degradation of STAT1 in the absence of STAT2. It appears that a particular interdependence exists between these two proteins for degradation (Yokosawa et al., 2002; Precious et al., 2005). Studies have established that the degradation of the STAT1 protein is mediated by ubiquitination and that the 127 kDa protein DDB1 seems to play an important role in this process. In fact, the V protein does not interact directly with the target molecule STAT1 but instead with STAT2. STAT2 interacts with DDB1 and the created complex targets STAT1 for ubiquitination (Precious et al., 2005). Studies to determine the host range of PIV5 have shown that it does not degrade STAT1 in murine cells and thereby does not block the signaling pathway in these cells. The differences between mouse and human STAT2 seem to be the reason for this. The expression of the human STAT2 in mice can enable the virus to overcome the antiviral state in a murine host (Parisien et al., 2002).

1.2.4. Rabies Virus

Members of the *Rhabdoviridae* family such as the Rabies virus (Rv) are also capable of counteracting the IFN response. Rv encodes only five viral proteins (though all are essential for virus replication): the nucleoprotein N, the phosphoprotein P, the matrix protein M, the glycoprotein G, and a large L RNA-dependent RNA polymerase. It has been shown that Rabies has the

ability to interrupt the IFN-stimulated Jak/STAT signaling pathways using the P protein. The P protein binds both STAT1 and STAT2 and impairs the translocation of the STAT1/STAT2 dimer to the nucleus. Subsequently, there is an accumulation of the complex in the cytoplasm. Research has shown that the C-terminal 10 amino-acid residues of P are the ones responsible for binding this protein to the STAT proteins (Brzozka et al., 2006).

1.3. IFN Sensitive Viruses and Antiviral Drugs

There are two viable approaches toward the control of viral diseases: prevent the infection by producing a vaccine or treat the infection using an antiviral drug.

Concerning the vaccine, there are safety issues that must be analyzed (including the reduction of side effects to a minimum). Vaccines should also provide a long-term protection to the host.

Live attenuated virus vaccines have been developed. Such vaccines are produced using a weakened virus strain that allows the activation of all components of the vaccinee immune system. Although they are considered easy to administer and not very expensive, there are some disadvantages to this kind of vaccine. For example, they can reverse to their virulence and cause illness to the vaccinee. Also, attenuated viruses can be difficult to grow in culture because most cells will produce IFN in order to respond to an infection. Vero cells are a good alternative in which to grow these viruses because they do not produce IFN. However, only certain viruses will grow in them. Therefore, cells that will constitutively express viral antagonists without producing or responding to IFN must be engineered.

Another alternative to using control viruses that encode IFN antagonists is to develop antiviral drugs. These can impair viruses from blocking the IFN production and signaling pathways and result in either a complete host recovery or at least an improvement in the therapy. Antiviral drugs for acute infection must be administered early in the infection process or prophylactically to populations at risk. Some viruses, however, replicate

too quickly with moderately high mutation frequencies that may be resistant to antiviral drugs. Antiviral drugs also play an extremely important role in chronic virus diseases such as those caused by the Hepatitis B and C viruses (Flint et al., 2000).

The development of antiviral drugs has been slow. A significant reason for this is that compounds used to build antiviral drugs often have adverse effects on the host. Also, the compounds must be completely efficient in blocking the virus. The search for compounds that can be used in antiviral drugs can be done by a screening cell-based assay. Compounds are generally arrayed in multi-well plates and robots apply the compounds to other plastic dishes containing the cells expressing viral proteins. After incubation an output is recorded. Many companies, pharmaceutical and chemical, use extensive libraries of chemical compounds. Some antiviral drugs have already been developed: Ribavirin, Acyclovir, Ganciclovir, and Amantadine. These antiviral drugs must be safe and are tested for toxicity in cells and animals.

Another use for viruses with advantageous results for the host concerns cancer therapy. Some viruses called oncolytic viruses can be useful in treating cancers in which the cells are deficient to the IFN response. These viruses can replicate and destroy only cancer cells by lysing them and thereby eliminate the cells from the human body. Some examples of these viruses include the Newcastle disease virus (NDV) (Elankumaran et al., 2006), the vesicular stomatitis virus (VSV) (Wollmann et al., 2007), and mutants of the herpes simplex virus (HSV) (Hunter et al., 1999).

Aim of the Project

Antiviral drugs can be used toward the purpose of eliminating viral infections. One starting point for the development of an antiviral drug is the use of a screening assay with engineered cell lines to search for chemical compounds that can block viral proteins and therefore qualify as potential candidates for an antiviral drug.

A novel method for finding viral proteins that block the IFN signaling pathway and could therefore be targets for certain antiviral drugs was suggested by Clarke et al. in 2004. The method aimed to access the ability of a certain viral protein (E7 of Human Papiloma virus (HPV)) in blocking the IFN signaling pathway. The approach involved both a quantitative and a qualitative assay based on cell survival. They developed cell lines expressing the viral protein E7 and tested those cells in the presence of IFN. The results showed that the cells not expressing E7 died in the presence of a certain compound. In fact, in these cells the IFN signaling pathway was activated and through this pathway the cells upregulated a gene the product of which metabolized the toxic compound resulting in cell death. The cells expressing the viral protein however, survived, indicating impairment of the IFN signaling pathway (Clarke et al., 2004).

In light of this new screening method, the aim of our project is to develop reporter cell lines expressing viral proteins and access those proteins for their ability to block the IFN signaling pathway using phenotypic cell assays and also quantitative assays. The viral proteins in this work are chosen based on their proficiency for blocking the IFN signaling pathway according to the literature. The proteins to be used are the Hepatitis B core and precore, the Hepatitis C core protein of two geotypes (1a and 4a), and the P protein of two strains of Rabies (SADL 16 and BH). The Hepatitis B core and precore and the Hepatitis C core protein are going to be expressed in the Hep2 Mx1TIPSE cell line and the Rabies P proteins in the A549Luc cell line.

2. Materials and Methods

2.1. DNA processing and analysis

2.1.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was used to amplify any DNA segments required for cloning these segments into vectors. The DNA sequences were obtained using the enzyme Taq polymerase (Promega Ltd., UK) or PfuI (produced in-house) according to the manufacturer's recommendations on a GeneAmp PCR System 2400 (Applied Biosystems). The oligonucleotide primers used in PCR reactions (obtained from Eurogentec Ltd., UK) were designed considering the restriction enzymes sites (Figure 3.2).

The PCR condition parameters were generally as follows: melting at 95°C for 30 seconds (sec), annealing at 55°C for 60 sec, and strand extension at 72°C or 68°C (for Taq or Pful, respectively) for 90 sec. The PCR set had a duration of 30 cycles.

2.1.2 Agarose Gel Electrophoresis

The DNA was analyzed by gel electrophoresis in horizontal mini-gels of 0.7-1% w/v agarose (sigma-Aldrich Co Ltd., UK) in TBE buffer (0.045M Tris-borate, 0.001M EDTA). Before the electrophoresis, the DNA samples were mixed with DNA loading buffer (0.03% bromophenol blue, 0.03% xylene cyanol FF, 10mM Tris-HCl (pH 7.5), and 50mM EDTA (pH 8.0)) (Promega Ltd., UK) in the proportion of 1/6 (v_{buffer}/v_{DNA}) sample. The samples were run along with DNA markers (1kb) ladder (Promega Ltd., UK) at 90-100 V in TBE buffer supplemented with 1mg/ml ethidium bromide. The electrophoresed DNA was visualized through UV irradiation (Gel Doc 2000 UV transilmuminator/photography system; Bio-Rad, UK). The exposure of

the gel was kept to a minimum to prevent the formation of thymidine dimmers.

2.1.3. Plasmid DNA purification

Procedures for plasmid DNA purification follow the recommended protocol of the QIAprep Miniprep Kit. E.coli, harboring the desired plasmid, was cultured in LB (Luria-Bertani containing 10g NaCl, 10g tryptone, and 5g yeast extract per litre) medium at 37°C, 200rpm overnight. The next day, 1ml of the culture was centrifuged at 1300rpm for 1 min. The pellet bacterial cells were resuspended in 250µl of Buffer P1, containing RNase A. Under the alkaline condition, the cell resuspension was mixed by gently inverting the tube 6 times with 250µl of buffer P2. This buffer is composed of NaOH/SDS. Leaving these lyses for more than 5 min is not recommended because long exposure to alkaline conditions can cause an irreversibly denatured form of plasmid, which is resistant to the restriction enzyme digestion; during the lysis reaction, vigorous treatment will shear the bacterial chromosome leading to the contamination of the plasmid with chromosomal DNA. Therefore, gently inverting the tube is sufficient. Following this reaction the lysate was immediately, though gently, mixed with 350µl of buffer N3 containing sodium acetate. The high salt and low pH value properties of this buffer lead to the coprecipitation of chromosomal DNA, cellular debris, and denatured proteins. The mixture was then centrifuged at 1300 rpm for 10 minutes and supernatants were applied to the QIAprep column by pipetting. The columns were placed in the centrifuge for 1 min at 1300 rpm. After the first centrifuging, columns were washed by applying 0.75ml of buffer PE and centrifuged and then centrifuged for another min to remove the residual wash buffer. The columns were then transferred to clean centrifuge tubes, rinsed with 30µl of buffer EB, and centrifuged for a min.

2.1.4 Treatment of DNA with Enzymes

2.1.4.1 Restriction Enzyme Digestion of DNA

Analytical Restriction Enzyme Digestion

In this kind of digestion, 1-3μg of DNA were incubated at 37ºC with 2 units of the appropriate restriction enzymes (Promega Ltd., UK, or New England Biolabs Ltd., US) with the corresponding reaction buffer in a total of 10μl. They were incubated for 3hrs.

Preparative Restriction Enzyme Digestion

The protocol for this type of digestion is identical to the analytical enzyme digestion, though the total volume of DNA was of 1-10μg for a total reaction volume of 20μl.

2.1.4.2. T4 DNA Ligase Reaction

For generating proper plasmid constructions, the desired DNA fragments and plasmid vectors were ligated by T4 DNA ligase (200 units/reaction) in the presence of 1x ligation buffer (50mM Tris-HCl, pH 7.5; 1mM ATP, 25mg/ml bovine serum) in the reaction volume of 20μl at 4ºC overnight.

2.1.4.3. Screening for Positive Colonies

Following the transformation of ligation reactions (see 2.2.2) into competent cells and the amplification of the obtained colonies, DNA was purified using miniprep kits (Qiagen Ltd., UK) (see 2.1.3). The miniprep samples were then screened to find positive clones. 5μl of the miniprep DNA was incubated with 2U-appropriate restriction enzymes and the respective

buffer in a total of 10-20μl at the temperature of 37ºC for 3h. DNA samples were then analyzed by agarose gel electrophoresis.

2.1.5. Preparation of Plasmid DNA

For small-scale preparations, bacterial cell cultures of 2-3ml were grown overnight at 37ºC in a shaking incubator. DNA was extracted from cells using the Qiagen DNA miniprep kit (see 2.1.3) (Qiagen Ltd., UK) according to the manufacturer's instructions. This extraction of DNA was based on the alkaline lysis of bacterial cells followed by the adsorption of DNA onto silica in the presence of high salt. For large-scale preparations, bacterial cultures of 200-250 ml were grown overnight at 37ºC in a shaking incubator. Similarly to small-scale preparations, DNA was extracted from cells and purified on silica gel membrane columns using the Qiagen DNA maxi-prep kit (Qiagen Ltd., UK) according to the manufacturer's instructions.

2.1.6. Plasmid DNA

The vectors below were used as backbone vectors or as provided DNA sequences for the construction of new plasmids.

—pdl HBV (provided by Dr. Alvin Patel, University of Glasgow); this vector was used to PCR amplify the sequences corresponding to the proteins core and precore of HBV.

—pdl HCV 1a and pdl HCV 4a (provided by Prof. Richard Elliot, University of St Andrews); these vectors were used to PCR the sequence corresponding to the core protein of the HCV of both genotypes 1a and 4a.

—pdl RabiesPSADL16 and pdl RabiesPBH (provided by Dr. Nadin Hagendorf, Pettenkofer Institut Genzentrum Institut F. Virologie); these vectors were used to PCR the sequences corresponding to the P protein of the Rabies virus on both strains SADL16 and BH.

—pdl MumpsV Enders pkIB (provided by Dr. Monica Galiano, University of St Andrews); this plasmid was used for a transient transfection in a luciferase reporter assay.

—pdl PIV5 V pkIP (provided by Dr. Yun-Hsiang Chen, University of St Andrews); this vector was used for a transient transfection in a luciferase reporter assay.

—pdl Surv pkIB (Figure 2.1)(provided by Dr. Yun-Hsiang Chen, University of St Andrews); these vectors were used as backbone vectors for the construction of the vectors pdl HBVcorepkIB, pdlHBVprecorepkIB, pdl HCVcore1apkIB, pdl HCVcore4apkIB, pdl RabiesPSADL16pkIB, and pdl RabiesPBHpkIB.

Several lentivirus vectors were constructed for transient transfections and the generation of stable cell lines.

—pdl HBVcorepkIB (Figure 3.3); this lentivector was generated to express the HBV core protein in a stable cell line using the Hep2Mx1TIPSE cell line.

—pdl HBVprecore pkIB (Figure 3.4); this lentivector was generated to express the HBV precore protein in a stable cell line using the Hep2Mx1TIPSE cell line.

—pdl HCV1apkIB and pdl HCV4apkIB (Figure 3.5); these lentivectors were generated to express the HCV core protein of both genotypes of HCV(1a and 4a) in stable cell lines using the Hep2Mx1TIPSE cell line.

—pdl RabiesPSADL16pkIB and pdl RabiesPBHpkIB (Figure 3.6 and 3.7); these lentivectors were generated to express the Rabies P protein of both strains SADL16 and BH in stable cell lines using the A549Luc cell lines.

2.2. Bacterial Transformations

2.2.1. Preparation of Competent *E.coli* Cells
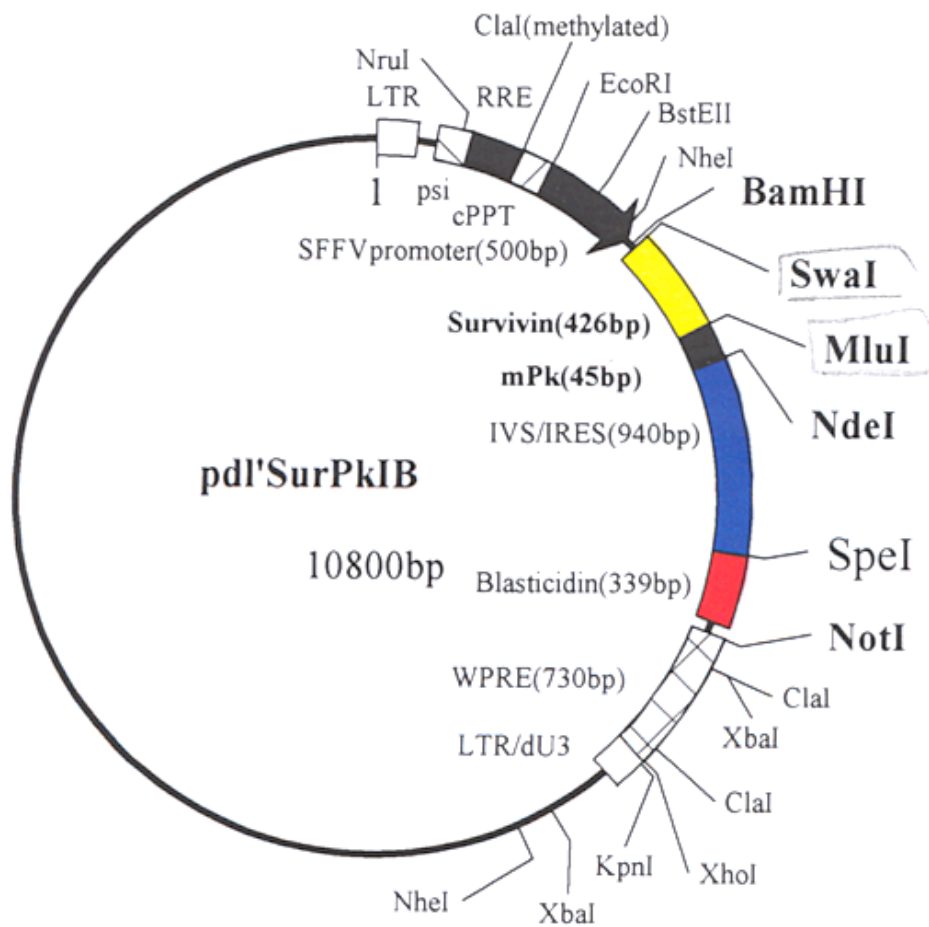
Figure 2.1. Schematic representation of the pdl'SurPkIB expression vector (Dr. Yun-Hsiang Chen, University of St Andrews).

Representation of the pdl'SurPkIB plasmid to be used as a backbone vector for the cloning of the viral genes. This plasmid has the epitote Pk and the reporter gene that confers blasticidin resistance under the control of the SFFV promoter. The viral genes were cloned into the survivin site using the restriction sites for the enzymes BamHI and MluI.

This procedure usually produces competent bacteria with transformation efficiency of about 10^6-10^7 transformed colonies per 1mg of supercoiled plasmid DNA. The efficiency is high enough for routine plasmid cloning in *E.coli* even though it will be affected by bacterium strains. In preparing competent cells, LB was inoculated with DH5α cells of overnight culture in a 1/100 amount. The incubation took 2 hrs at 37°C at 120 rpm. The A_{600nm} was measured after this time and when this value was between 0.4 and 0.6 the culture was transferred into a 50ml pre-chilled sterilized tube and kept on ice for 30 min in the cold room (4°C). The cells were then centrifuged at 2800rpm for 5 min at 4°C and the supernatant was disposed. 2.5ml of a $CaCl_2$ 100mmol solution and 2.5ml of a 40 mmol $MgSO_4$ solution were added and the pellet was resuspended gently in that mixture and kept on ice in the cold room. Pre-chilled glycerol was added until a concentration of 10% and the mixture was aliquoted in 1.5ml tubes and quickly frozen in liquid N_2. Later, it was stored at -70°C.

2.2.2. Transformation of Competent Bacterial Cells *(E. coli)*

For the transformation of a cloning plasmid, 5µl of DNA ligation reaction solution were transferred into 100µl of freshly prepared or stored competent cells and mixed well by swirling gently. Then this mixture was treated with heat-shock for 1min at 42°C and placed on ice for 2min. 900µl of LB broth were added to the 100µl and the mixture was incubated at 37°C in a shaker (220rpm). 1ml of the mixture was transferred to an eppendorf and 50µl of the recovered culture were spread into a LB plate with ampicillin. The rest of the mixture in the eppendorf was centrifuged at 1300rpm for 1 min and the pellet was resuspended in the remains of the supernatant. The mixture was then spread into another LB ampicillin plate. The plates were placed in an incubator for 24h at 37°C.

2.3. Cells and Cell Lines

2.3.1. Cell Lines

Cell lines of human origin were used during this project.

Basic cell lines:

—HEp2; human larynx carcinoma epithelial cells provided by ECACC (Chen, 1988).

—293T; human embryo kidney cells; the 293T cell line was provided by Prof. Richard Iggo, University of St Andrews.

—A549; human alveolar basal epithelial cells.

Permanent cell lines:

—HEp2Mx1TIPSE cells that express tk and puromycin resistance genes under the control of the murine Mx1 promoter (Figure 2.2).

In addition to the cell lines mentioned above, human stable cell lines were generated and used:

—HEp2Mx1TIPSEHBVcore; cells that express the HBV core protein and the resistance genes tk and puromycin under the control of the murine promoter Mx1.

—HEp2Mx1TIPSEHBVprecore; cells that express the HBV precore protein and the resistance genes tk and puromycin under the control of the murine promoter Mx1.

—HEp2Mx1TIPSEHCVcore1a and HEp2Mx1TIPSEHCVcore4a; cells that express the HCV core protein of two different genotypes, 1a and 4a, and the resistance genes tk and puromycin under the control of the murine promoter Mx1.
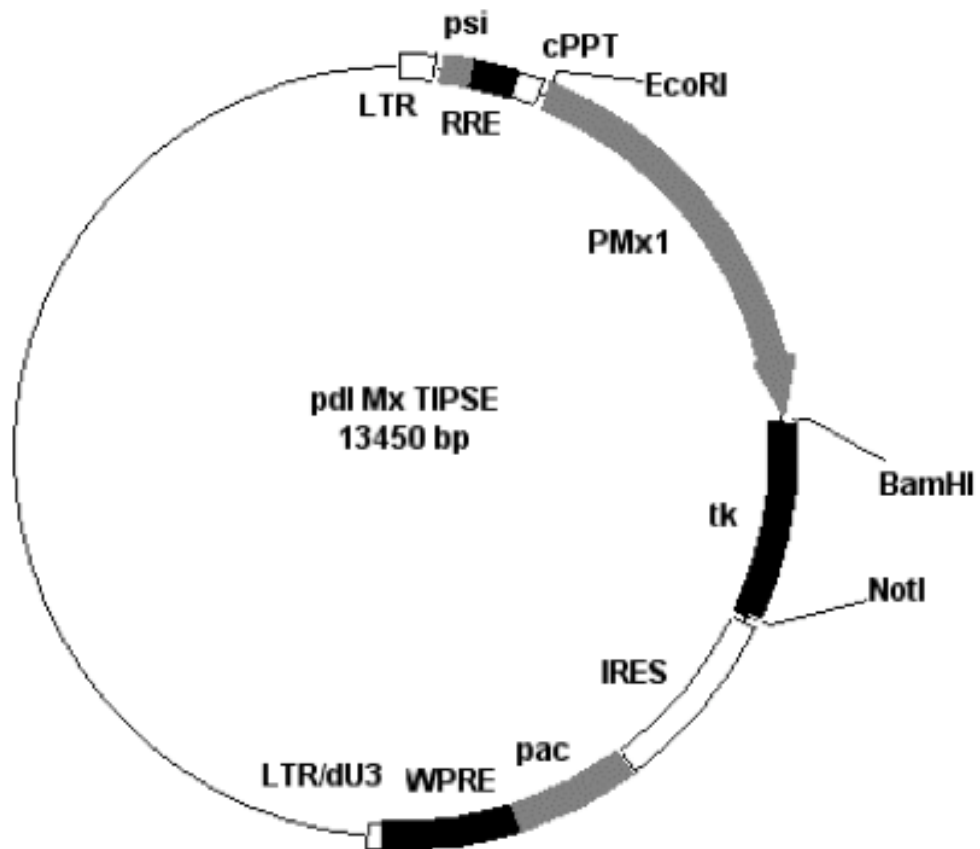
Figure 2.2. **Schematic representation of the pdl' Mx1TIPSE vector.**
(Rodrigues, 2008)

Representation of the pdl' Mx1TIPSE vector used to produce the Hep2Mx1TIPSE cell line. This plasmid contains the promoter gene that upon activation controls the reporter genes TK and pac. TK is the gene that expresses Tymidine Kinase, which interacts with the drug Ganciclovir, and pac the gene that confers resistance to puromycin.

— A549LucRabiesPSADL16 and A549LucRabiesPBH; cells that express the Rabies P protein of two strains (SADL16 and BH)

2.3.2. Transient Transfection in 293T Cells

Adherent 293T cells were transfected with DNA 24hrs after trypsinisation using the Fugene™ 6 reagent. The proportion of Fugene used was 3/2 according to the amount of DNA transfected. Eppendorfs containing a mixture of DMEM, Fugene, and DNA to transfect were left to rest for 30-45min and then applied to 6-well plates. Each well contained 2ml of DMEM with 10% Fetal Calf Serum. The plates were incubated at 37°C.

2.3.3. Production of Lentivirus

The 293 cells were maintained in Fetal Calf Serum 10% on DMEM in 75cm³ flasks. The plasmids were transfected into these cells according to the following steps: the cells were set up one day before this experiment to be 50-80% confluent and the volume of the serum was 5ml. On the day of the transfection, an eppendorf for each cell line to transfect was prepared with 100μl of DMEM, 30μl of Fugene (Fugene™ 6 Transfection Reagent 2000), 4μl of the DNA to be transfected, and 3μl of each of the two vectors pCMV and pVSV-G. These eppendorfs were left to rest on the hood for 30-45min. The medium of each flask was replaced with 5ml of Fetal Bovine Calf Serum 10% on DMEM. After 30-45min, the content of the eppendorfs was emptied to the flasks, which were then incubated at 37°C. The next day, the medium was replaced by a fresh 5ml of the medium. On the third day, the medium was collected and 5 new milliliters of medium were added to each flask, all of which were then collected the following day. The medium collected was centrifuged for 3min at 1500rpm and filtered through a Millipore Millex GP 0.45μm membrane. It was then aliquoted in 1.5ml tubes and frozen at -70°C.

2.3.4. Preparation of Stable Cell Lines by Lentivirus Infection of Mammalian Cells

The cells were adherent in a 6-well plate with 10% Newborn Calf Serum solution on DMEM and were 30-50% confluent on the experiment day. On the day of the experiment, the medium was aspirated from the wells and 1ml of the lentivirus and 1ml of DMEM were added to each well for both types of lentivirus. Then polybrene, a cationic polymer used to enhance the efficiency of the infection, was added in the dilution of 1ml polybrene to 500ml of medium. The plates were then centrifuged for 30min at 1500rpm and incubated at 37°C for 48 hours. Some wells, where cells were not infected with the lentivirus, the cells were in 1ml of DMEM and 1ml of 10% Newborn Calf Serum in DMEM solution.

2.3.5. Sub-cloning

Cells in suspension in 10ml of DMEM 10% Calf Serum were added to a 50ml sterile tube with the dilution of 5000 cells/ml. 200μl of medium of this tube were then added to 9.8ml of DMEM 10% Calf Serum in a second 50ml tube. 6ml of the previous tube were added to 24ml of DMEM 20% Calf Serum in a third 50ml tube. In a fourth tube with 22.5ml of DMEM 20% Calf Serum, 7.5ml of the third tube were added. From this tube, 6ml were taken and added to a fifth 50ml tube with 24ml of DMEM 20% Calf Serum. Finally, 6ml of the fifth tube were added to a sixth 50ml tube with 24ml of DMEM 20% Calf Serum.

200μl of the third 50ml tube were added to each well in a 96-well plate and the wells were topped up with DMEM 10% Calf Serum. This procedure was repeated for tubes 4, 5, and 6. After 7-10 days, the medium of the plates was replaced with fresh medium (DMEM 10% Calf Serum). After approximately one week, single colonies were checked for in each of the plates.

2.3.6. 96-Well Plate Drug Assay

Cells were set up in a 96-well plate (some infected with the virus, others not) in a total medium (DMEM 10% Newborn Calf Serum) volume of 100µl. On the next day, 10µg/µl of IFN were added to alternate rows of the plates in a dilution of 1/1000 in DMEM containing 10% Newborn Calf Serum. 6 hours later, puromycin and Ganciclovir were added to wells (some with and some without IFN) of both infected and uninfected cells in dilutions of 1/1000 puromycin and 1/250 Ganciclovir in DMEM with 10% Newborn Calf Serum. The concentration of both drugs was 10µg/µl.

2.4. Protein Analysis

2.4.1. Antibodies

The antibodies used in this project are listed below:

— Anti-mouse Ig Texas Red conjugated Ab (Oxford Biotechnology Ltd., UK).

— Anti-mouse Ig HRP linked Ab (Amersham Bioscience, UK).

— PIV5-Pk (mAb) (Randall et al., 1987).

— STAT1 p91 mouse monoclonal IgG (Santa Cruz Biotechnology, US).

2.4.2. Immunofluorescence

Cells adherent to coverslips on 24-well plates were cultured in DMEM medium containing 10% Fetal Calf Serum at 37ºC with 5% CO_2. The coverslips were washed with washing buffer (PBS containing 1% (v/v) Fetal Calf Serum) twice and then fixed with the fixing solution containing 5% (v/v) formaldehyde and 2% (w/v) sucrose in PBS for 10min at room temperature.

After fixation, the cells were washed twice again and permeabilized with a solution that contained 0.5% (v/v) IGEPAL, 10% (w/v) sucrose, and 1% (v/v) azide in PBS for 5min. Because the tested proteins have the PIV5-tag on the C-terminal end, the cells were stained with a mAb PIV5-Pk for 30min with a dilution of 1/200 in PBS containing 1% (v/v) Fetal Calf Serum plus azide and washed twice. Then the second mAb was added (sheep anti-mouse IgG conjugated TXRD) with DAPI in a dilution of 1/200, the same dilution as the first mAb.

The coverslips were then re-fixed to avoid dissociation of the antibodies and washed first in PBS and then in water. They were then mounted on the slides with a mounting gel called citifluor. The slides were examined at the microscope.

2.4.3. Immunoblotting

Cells were adhered to a 6-well plate and incubated at 37°C and 5% CO_2. The cells were washed with PBS and put on ice. The wells were aspirated and 200μl of the disruption buffer (0.15 M NaCl, 5mM EDTA pH 8, 1% Triton X100, 10mM Tris HCl, pH7.4, and 5M DTT, 100mM PMSF in isopropanol, 5M e-aminocaproci acid all in (1/1000) dilution) were added to each well in order to remove the cells and collect them in eppendorfs. Then the cells were sonicated and the tubes were boiled at 100°C for a few minutes. A SDS page gel was run for about 1 hour with 1x running buffer (5x SDS Running Buffer (1L) Tris 15g, Glycine 72g, SDS 5 g) at 200V. At the same time, the blotting pads and a nitrocellulose membrane (previously activated in Methanol) were soaked with transfer buffer (1x Running Buffer in 205 ml methanol). The proteins were then transferred to the membrane by electro blotting with a charge of 28V for 1h30m. The nitrocellulose membrane was soaked in a 5% milk solution for 1h. The primary antibody was added to the membrane in a 5% milk solution for 1 hour with a dilution of 1/2000 (v/v). Then the membrane was washed for 1 hour, changing medium (PBS tween) every 15 minutes. After washing with PBS Tween, the secondary antibody was added

to the membrane in a dilution of 1/2000 in a 5% milk solution for another hour. The membrane was washed again for 1 hour. Antibody binding was detected by the enhanced chemiluminescence's assay (ECL). A mixture of ECL 1 and ECL 2 in the ratio of 1:1 was added to the membrane and the latter was incubated for 5min. The membrane was then exposed to an auto-radiographic film.

2.4.4. Luciferase Reporter Assay

Cell monolayers were incubated in 6-well plates at 37°C, 5% CO_2, and 50% confluent. They were transiently transfected with both the IRES-luciferase plasmid and the vector coding for each viral protein. 48 hours after transfection, IFN was added to some of the cells and then 6 hours later the cells were harvested and lysed. For that purpose, 200µl of Luciferase buffer A (5ml of Luciferase Master buffer (50mM Tris-phosphate pH7.8, 2mM DTT, 2mM EDTA, 2% TritonX100, and16mM $MgCl_2$) and 5ml water) were added for 2 min. Then 200µl of Luciferase buffer B (5ml of Luciferase Master buffer, 3ml of Glycerol, 80µl of 100mM ATP, and 2ml of 10% BSA) were added to the cells. 300µl of this mixture were then transferred to a luminometer cuvette and 50µl of Luciferin were added. The samples were then read for the amount of luciferase.

2.4.5. β-Galactosidase Assays

900µl of buffer A–β-Mercaptoethanol mixture (100mM NaH_2PO_4; 10mM KCl; 1mM $MgSO_4$; 50mM β-Mercaptoethanol) were added to the cell lysates (from the lucifearse assay) in microcentrifuge tubes and the tubes were incubated in a 37°C water bath for 5 minutes. 200µl of o-Nitrophenyl-β-D-Galactopyranoside (ONPG) substrate in the concentration of 4mg/ml were added to 100 mM NaH_2PO_4 buffer (pH 7.5) resulting in a 1xONPG solution. This mixture was added to the tubes and the latter were vortexed for 5 seconds. The time of ONPG addition was recorded and the reaction was

incubated for 30 minutes or more in a 37°C water bath until the sample turned bright yellow.

The reaction was stopped by adding 500μl of stop solution (1M Na_2CO_3).

The OD_{420nm} was measured and the specific activity was determined. (OD_{420}/0.0045 = nmoles formed per milliliter; nmoles/ml × total assay volume (lysate, buffer A, ONPG, and stop solution) = nmoles)

3. Results

Part I - Generation of the Reporter Cell Lines Expressing a Viral Protein Responsible for Blocking the IFN Signaling Pathway in Hep2Mx1TIPSE Cells

Hep2Mx1TIPSE cell line

The Hep2 (human epithelial cells established from a laryngeal carcinoma) cell line available in our lab had been engineered by infection using a lentiviral based system transferring the genes in the vector pdl Mx1TIPSE (Figure 2.2). This vector contains the selection marker genes thymidine kinase (TK) and puromycin resistance (pac) under the control of an IFN-inducible promoter called the Mx1 promoter. Whenever these cells are treated with IFN(α/β) the expression of hundreds of genes is up-regulated as a result of the antiviral state the cells enter. One of these genes codes for the protein Mx1. Therefore, if IFN is present, the Mx1 promoter is activated and the cells will express the genes TK and pac. The TK gene works as a selective marker due to its interaction with the antiviral drug ganciclovir (GCV) resulting in life or death for the cells. The reason for this is that ganciclovir can work like a substrate for the enzyme TK and although GCV by itself is not toxic for the cells, when phosphorylated by TK it becomes toxic and once incorporated in the DNA it can cause cell death. Therefore, in the presence of GCV, the expression of TK results in cell death. Puromycin is an antibiotic

commonly used for selection in mammalian cells and it interferes with protein synthesis leading to the death of the cells.

The engineered Hep2Mx1TIPSE cells were tested in the presence of IFN for their markers. When the cells were subjected to the drugs, they responded as expected (Figure 3.1). The cells to which IFN was added survived in the presence of puromycin (the pac gene was active) and died in the presence of ganciclovir (TK was active). The Hep2Mx1TIPSE cells are easy to grow and maintain in cell culture and are able to be passaged indefinitely. They can be considered good candidates for expressing IFN antagonists.

Engineering Hep2Mx1TIPSE reporter cell lines expressing viral proteins

The process to engineer the Hep2Mx1TIPSE reporter cells lines expressing certain viral proteins was initiated with the cloning of the viral genes into the reporter plasmid pdl'SurPkIB.

The viral DNA was obtained from different labs (see 2.1.6.) and primers were designed (Figure 3.2) with consideration for the genetic sequences of the viral genes and the restriction enzymes present in the vector pdl'SurPkIB where the genes were to be cloned. These enzymes are SwaI or BamHI (for forward primers) and MluI (for reverse primers) (Figure 3.2.).

A PCR was then performed in order to amplify the DNA coding for each viral protein to be cloned in the vector.

Following the PCR, the PCR products were digested with the appropriate restriction enzymes and cloned into the same sites present in the pdl'SurPkIB vector thus generating the different plasmids (Figures 3.3-3.5).

The vector pdl'SurPKIB was chosen for this cloning because it contains a blasticidin resistance gene under the control of the SFFV (Spleen Focus-forming virus) promoter. This allowed us to select the cells that contained the plasmid of interest by adding blasticidin. The viral genes were cloned upstream of a gene which codes for a 14 amino acid sequence derived from the P and V proteins of PIV5 designated by Pk epitote. The Pk protein worked as a tag to the viral protein and since we had an antibody targeting Pk we

Figure 3.1. Response of Hep2Mx1TIPSE cells to puromycin and Ganciclovir after IFN treatment

Hep2Mx1TIPSE cells were set up in 96-well microtitre plates. IFN was added to every other row twenty-four hours after the cells were set up. Puromycin and ganciclovir were then added to the cells at a concentration of $10\mu g/\mu l$ eight hours after IFN had been added. Four days later the cells were fixed using a solution with PBS 5% formaldehyde and 2% sucrose and stained with crystal violet.

The live cells were stained in violet whereas the dead ones were washed off. From Figure 3.1 we observe that, as expected, cells to which IFN was added survived in the presence of puromycin and died in the presence of ganciclovir.

	No-drug addition	puromycin addition	ganciclovir addition
+IFN			
-IFN			
+IFN			
-IFN			
+IFN			
-IFN			
+IFN			
-IFN			
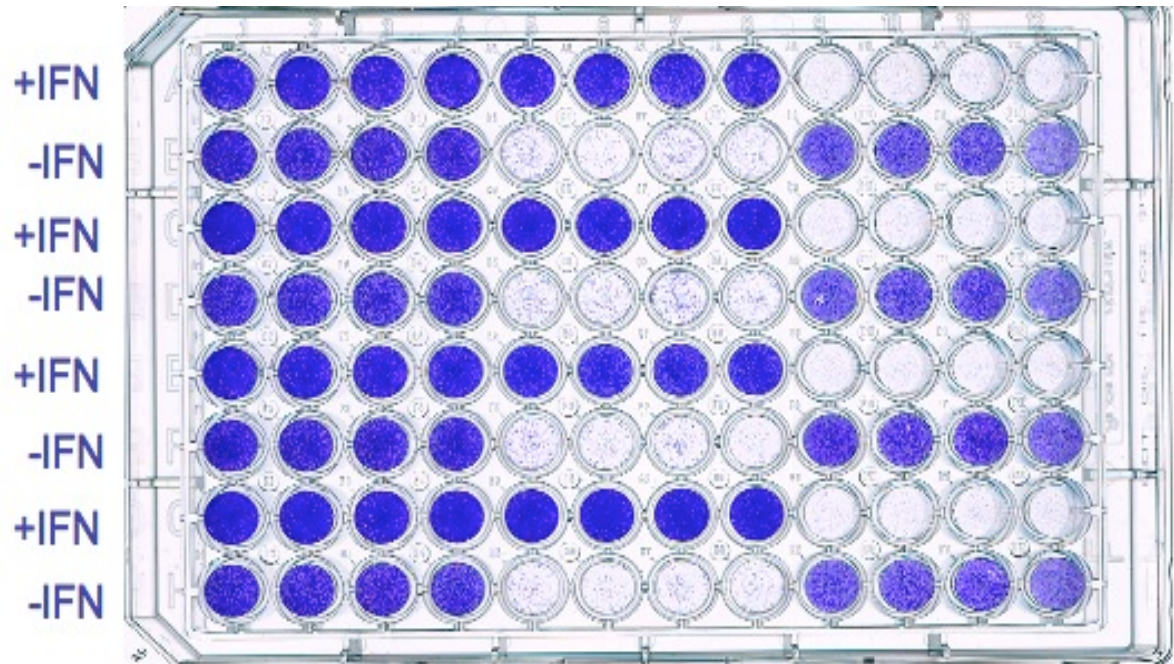
Figure 3.2: **Primers designed to clone PCR fragments into plasmid pdl′ SurPkIB**.

The primers were designed using the program DNA Strider while considering the restriction enzymes necessary to cut the PCR fragments in order to insert them into the plasmid pdl′ SurPkIB. The restriction enzyme used for designing the reverse primers was MluI and the enzymes used for designing the forward primers were BamHI or SwaI.

| Primers	Restriction enzymes	Primer sequences
HBV precore fwd	BamHI	5' agc tag gga tcc atg caa ctt ttt cac ctc tgc 3'
HBV core fwd	BamHI	5' cat ccg gga tcc atg gac att gac cct tat aaa 3'
HBV rev (core and precore)	MluI	5' act gca acg cgt aca ttg aga ttc cg aga ttg 3'
HCV 1a rev	MluI	5' tag ccg acg cgt ggc tga agc ggg cac agt cag 3'
HCV 4arev	MluI	5' cga tcg acg cgt ggc cga agc ggg gac agt cag 3'
HCVfwd (1a and 4a)	BamHI	5' ccg cgc gga tcc atg agc acg aat cct aaa cct 3'
Rabies P fwd (SADL16 and BH)	SwaI	5' cgc att taa ata tga gca aga tct ttgtc 3'
Rabies P SADL16 rev	MluI	3' cgc acg cgt gca aga tgt ata gcgattt 5'
Rabies P BH rev	MluI	3' cgc acg cgt ata cgc aag ata tctgtt 5'
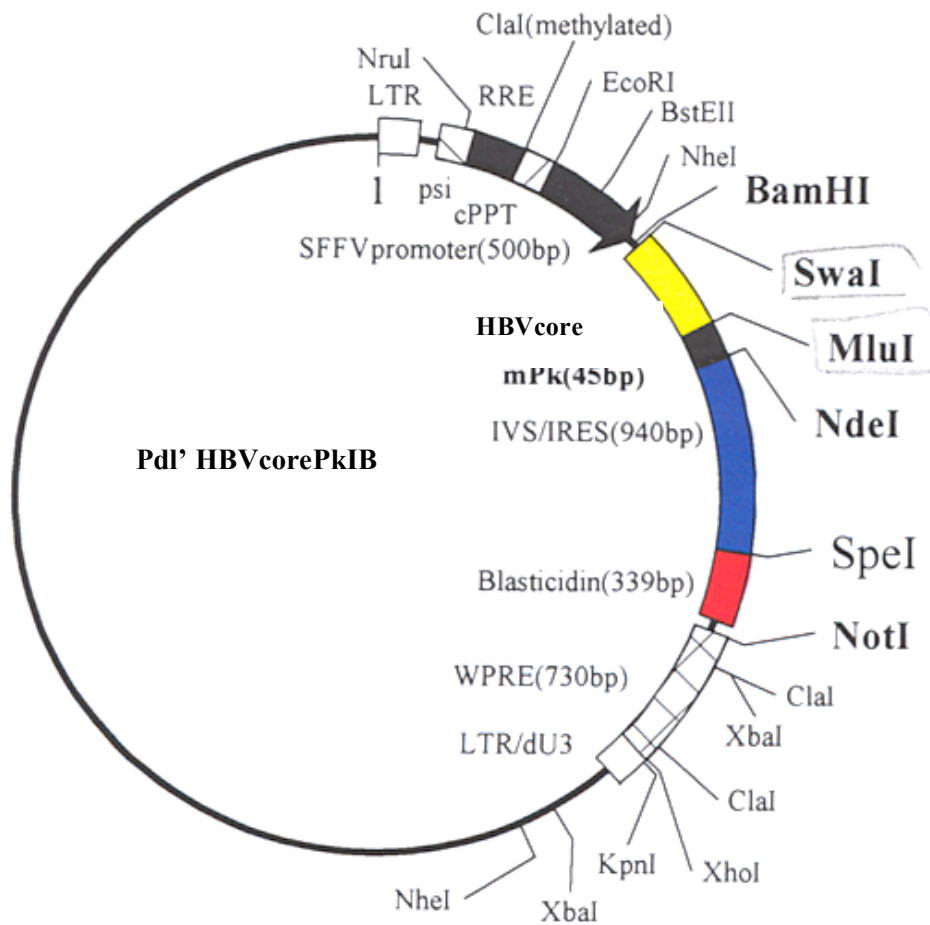
Figure 3.3. Schematic representation of the pdl′ HBVcorePkIB vector.

Representation of the pdl′ HBVcorePkIB plasmid to be tranfected into 293T cells along with pCMV and pVSV-G in order to produce lentiviral particles to infect the Hep2Mx1TIPSE cells. In this representation, the locations are shown for the HBVcore promoter gene cloned right before the epítote Pk and the blasticidin gene which are under its control. The cloning site for HBVcore is between the enzymes BamHI and MluI.
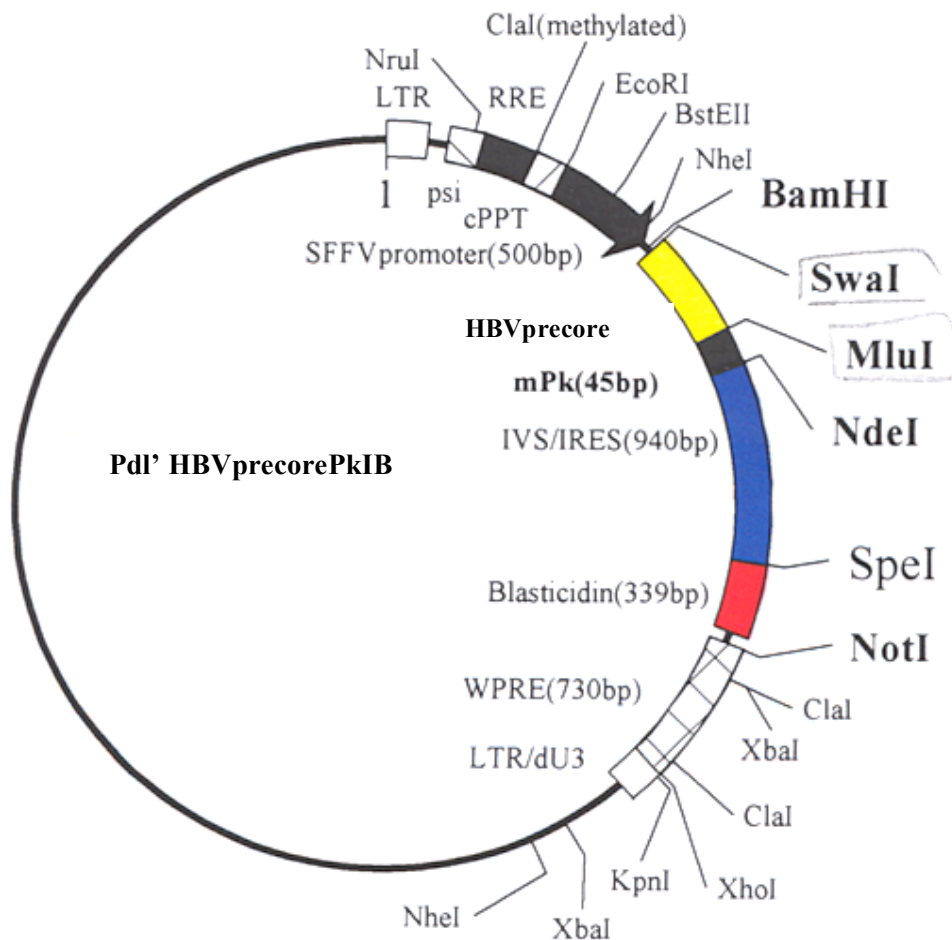
Figure 3.4. **Schematic representation of the pdl' HBVprecorePkIB vector.**

Representation of the pdl' HBVprecorePkIB plasmid to be tranfected into 293T cells along with pCMV and pVSV-G in order to produce lentiviral particles to infect the Hep2Mx1TIPSE cells. In this representation, the locations are shown for the HBVprecore promoter gene cloned right before the epítote Pk and the blasticidin gene, which are under its control. The cloning site for HBVprecore is between the enzymes BamHI and MluI.
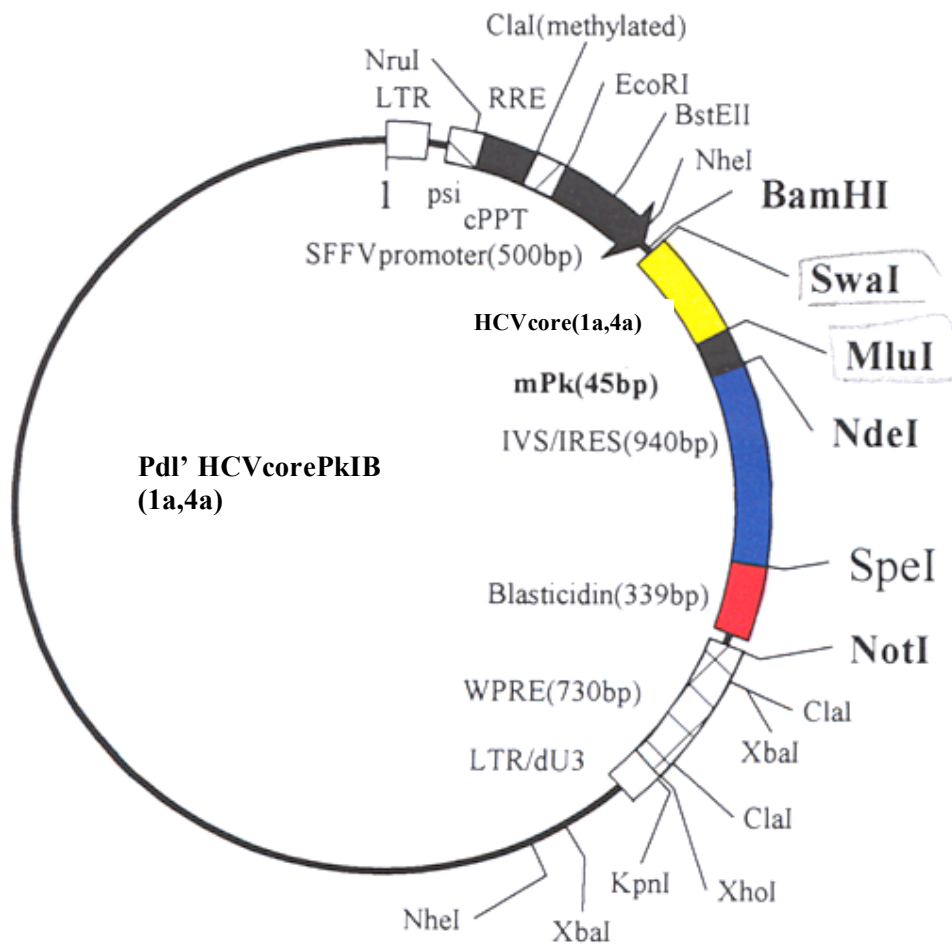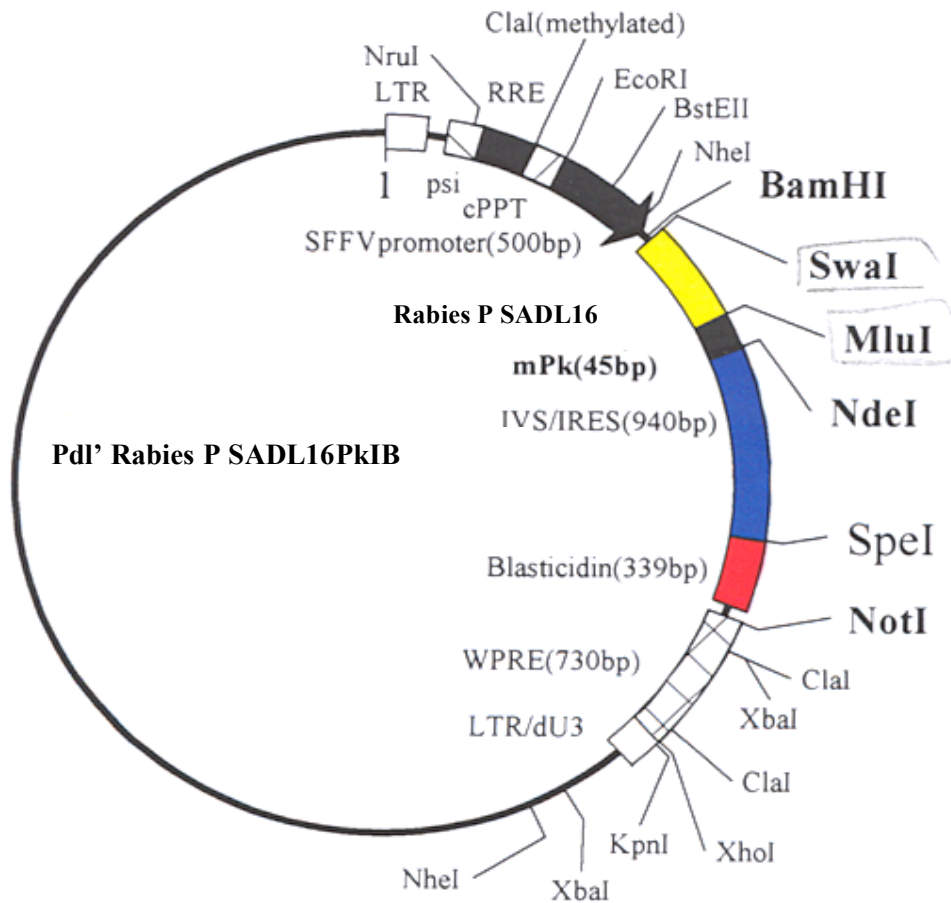
Figure 3.5. **Schematic representation of the pdl' HCVcorePkIB vector (1a and 4a).**

Representation of the pdl' HCVcorePkIB plasmid to be tranfected into 293T cells along with pCMV and pVSV-G in order to produce lentiviral particles to infect the Hep2Mx1TIPSE cells. In this representation, the locations are shown for the HCVcore promoter gene cloned right before the epítote Pk and the blasticidin gene, which are under its control. The cloning site for HCVcore is between the enzymes BamHI and MluI.

Figure 3.6. **Schematic representation of the pdl' RabiesPSADL16PkIB vector.**
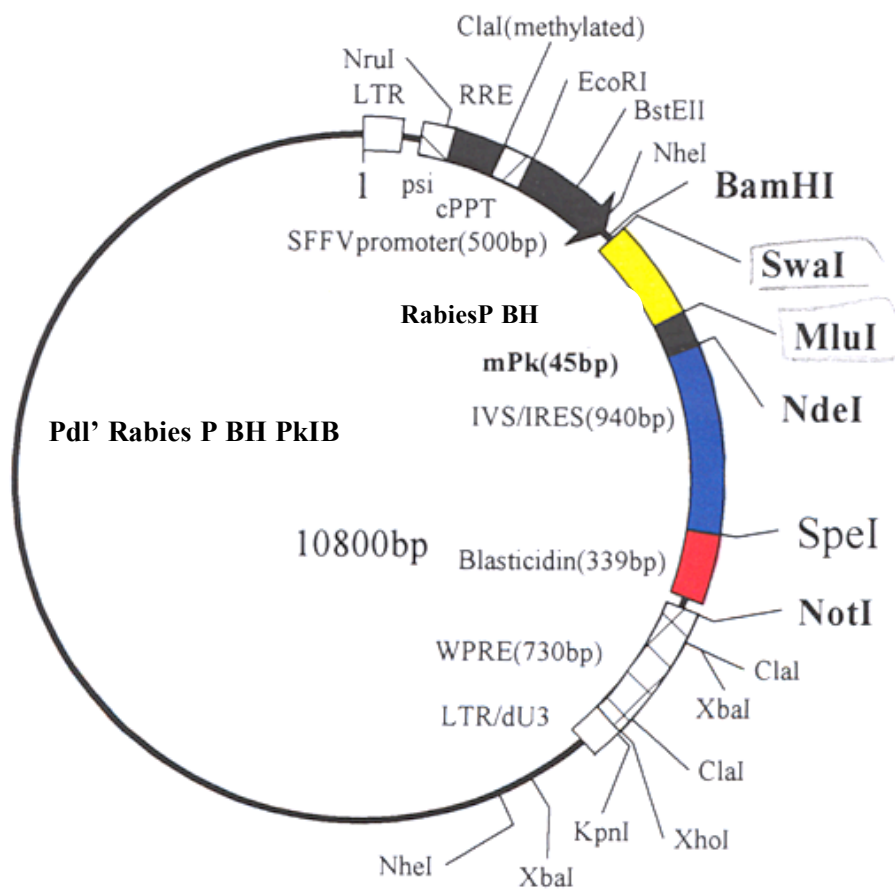
Representation of the pdl' RabiesPSADL16PkIB plasmid to be tranfected into 293T cells along with pCMV and pVSV-G in order to produce lentiviral particles to infect A549Luc cells. In this representation, the locations are shown for the RabiesPSADL16 promoter gene cloned right before the epítote Pk and the blasticidin gene, which are under the control of this promoter. The cloning site for RabiesPSADL16 is between the enzymes SwaI and MluI.

Figure 3.7 **Schematic representation of the pdl' RabiesPBHPkIB vector.**

Representation of the pdl' RabiesPBHPkIB plasmid to be transfected into 293T cells along with pCMV and pVSV-G in order to produce lentiviral particles to infect A549Luc cells. In this representation, the locations are shown for the RabiesPBH promoter gene cloned right before the epítote Pk and the blasticidin gene, which are under the control of this promoter. The cloning site for RabiesPBH is between the enzymes SwaI and MluI.

conducted several immunoblotting and immunofluorescence assays for the viral proteins of interest.

The cloned vectors expressing the viral proteins pdl'HBVcorePKIB, pdl'HBVprecorePKIB, and pdl'HCVcorePKIB (1a and 4a) (Figures 3.3-3.5) were submitted for DNA sequencing to confirm a successful cloning (data not shown).

Lentiviral Infection of Hep2Mx1TIPSE Cells and Viral Expression

A lentivirus-based vector system was used to produce the reporter cell lines in the Hep2Mx1TIPSE cells. Lentiviruses, such as HIV, are retroviruses capable of infecting both dividing and non-dividing cells (Amado & Chen, 1999).

In this experiment, lentiviral particles were produced in the 293T (Human embryonic kidney) producer cell line and released in the medium where they were later harvested and used to infect the Hep2Mx1TIPSE cell line.

The production of the lentiviral particles started with the transfection of the 293T cells by three different plasmids. The first plasmid, our construct (Figures 3.3-3.5), was engineered to contain the sequences that code for the viral proteins of interest to our project and that we aimed to transfer into the Hep2Mx1TIPSE cells. The second plasmid, pCMV (also referred to as packaging plasmid), contained the structural proteins (except envelope protein (env)) and enzymes that were crucial for the generation of the lentiviral particles. The third plasmid, pVSV-G, contained a sequence for the Glycoprotein of vesicular stomatitis virus (VSV-G). This protein acts as the env protein being responsible for mediating the entry of the lentiviral particles in the Hep2Mx1TIPSE cells (Kahl et al., 2004; Yang et al., 2006). Once inside the 293T cells, the DNA was transcribed into RNA and the particles were formed and released in the medium. The medium of the 293T cells was after collected and used for the infection of the Hep2Mx1TIPSE cells. This process was initiated when the lentiviral particles entered the Hep2Mx1TIPSE

cells (due to the env protein) and their RNA was reversely transcribed into DNA by a reverse transcriptase enzyme. This DNA, along with other components of the viral particle (protein Vpr and matrix and enzyme integrase), formed a pre-integration complex. The complex then entered the nucleus of the Hep2Mx1TIPSE cells and the DNA was inserted into the cell's genome by the enzyme integrase (Amado and Chen, 1999).

Using the lentivirus vector based system involved some precautionary measures. The major concern was that the lentiviruses could self-replicate by recombination; the lentiviral particles could infect humans, self-replicate and behave like HIV (Amado and Chen, 1999). To avoid this problem the packaging plasmid did not contain some HIV genes that would be essential for self-replication.

Two days after infection blasticidin was added to the cells and three days after (the period of time necessary for blasticidin action) the new cell lines were assayed for viral expression. The viral expression was analyzed in HBVcore/precore Hep2Mx1TIPSE cells by immunofluorescence using the anti-pk-tag antibody. The cells were set up as monolayers and stained with the primary antibody anti-pk-tag and the secondary anti-mouse FITC-conjugated antibody TX red. The use of these antibodies allowed us to see the cells that are expressing the viral protein colored red. The cells were also stained with DAPI to facilitate the observation of cell nucleus. The cell lines expressing HBV precore and core proteins exhibited a good expression of the viral proteins in question (Figure 3.9).

An immunoblot to determine the expression of the HCV core in both HCV genotypes (1a and 4a) was successfully conducted. The molecular weight of this protein is approximately 21kDa (Figure 3.8).

Blocking of the IFN Signaling Pathway

To determine exactly if the viral proteins were blocking the IFN signaling pathway, two separate assays were performed. The first consisted of an immunoblot targeting the protein STAT1. In this assay, we intended to

Figure 3.8: Immunoblotting analysis targeting for the HCVcore protein using anti Pk-tag antibody

In order to check if the Hep2Mx1TIPSE cell lines expressed the viral protein HCVcore the cells were set up in a 6-well plate and an immunoblot was conducted using the anti-pktag antibody.

In this figure the band of 21 kDa represents the HCVcore protein. The band was present for both genotypes of HCV (1a and 4a), although the expression for 4a is much stronger than 1a.

Hep2Mx1TIPSE
HCV core
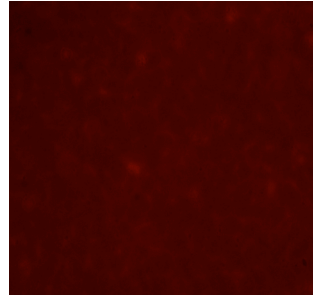**1a**   **4a**

HCVcore
(21kDa)
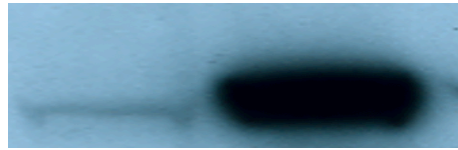
Figure 3.9: **Immunofluorescence analysis revealing the expression of the proteins precore and core of HBV in the Hep2Mx1TIPSE cells**

The Hep2Mx1TIPSE cells were infected with lentivirus containing the viral RNA in order to engineer cell lines expressing the HBV core and precore proteins. To ensure that the cell lines were in fact expressing the viral proteins we performed an immunofluorescence analysis using the anti Pk-tag antibody and human TXRD as a secondary antibody. From these figures we can observe that there is a good expression of these proteins in the Hep2Mx1TIPSE cells. DAPI staining was also performed in order to help locating the cell nucleus and aid on the interpretation of the results.

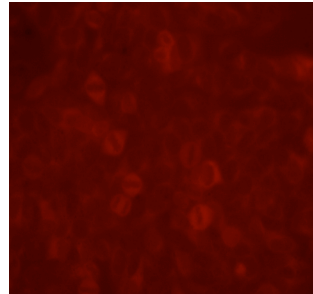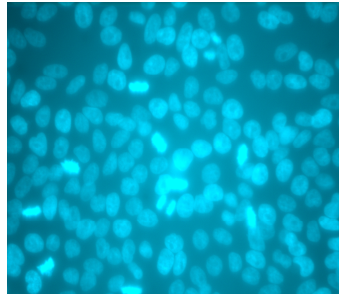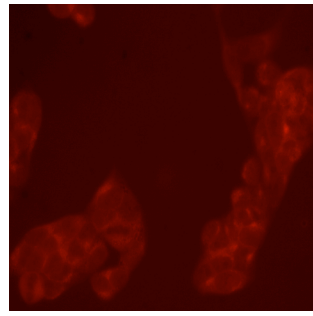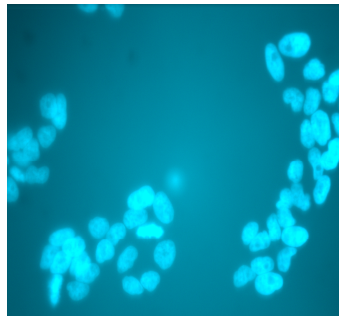|	DAPI	TXRD
Hep2Mx1TIPSE		
Hep2Mx1TIPSE HBVprecore		
Hep2Mx1TIPSE HBVcore		
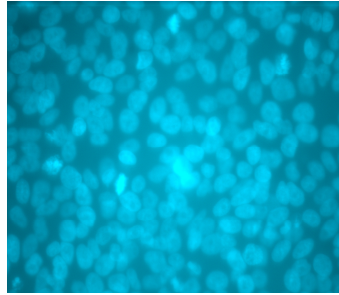
observe any alteration in the amount of expression of this protein by comparing the intensity of the band representing STAT1 in the presence of IFN in the Hep2Mx1TIPSE cells with the band in the cells expressing a viral protein.

It is known that when IFN is produced in cells (such as Hep2Mx1TIPSE) as the result of a viral infection or due to any other stimuli the IFN signaling pathway is activated and STAT1 is phosphorylated forming a heterodimer with STAT2. This heterodimer then translocates to the nucleus of the cell up-regulating different ISGs (including the gene that codes for the same protein STAT1) that will act as an antiviral response. Therefore, in the presence of IFN the amount of this protein in the cell is greater than in the absence of it and so we should expect the band corresponding to STAT 1 to be more intense in the presence of IFN. In fact, after setting up the Hep2MX1TIPSE cells in monolayers in six well plates and performing an immunoblot analysis targeting STAT1, we noticed a band around 90kDa corresponding to the molecular weight of the STAT1 protein. This band was more intense when IFN was present (Figure 3.11). In the same immunoblot analysis we observed that the cells expressing HCV core protein (both HCV genotypes 1a and 4a) (Figure 3.11) show the same result as the normal Hep2Mx1TIPSE cells. This result was not expected since in the cells expressing the HCV core the latter should reduce the quantity of phosphorylated STAT1. By binding to STAT1, the viral protein would avoid the formation of the STAT1/STAT2 heterodimer. In this manner, the ISGs would not become activated and the protein STAT1 would not be up-regulated. Thus, we would not see an increase in the expression of the band corresponding to the molecular weight of STAT1 in the Hep2Mx1TIPSEHCVcore cells upon IFN addition. The above result seems to suggest that the HCV core protein is not blocking the up-regulation of STAT1 contradicting what was expected (Basu et al., 2001).

However, the protein HCV core might be able to block the interferon signaling pathway by affecting other key components of this pathway. For instance, it was reported that the HCV core protein was able to induce a

Figure 3.10: Immunoblotting analysis targeting the STAT1 protein indicating that the HBVcore and precore proteins were not blocking STAT1 activity.

The Hep2Mx1TIPSEHBVcore cells and the Hep2Mx1TIPSEHBVprecore cells were analyzed in an immunoblot targeting the protein STAT1. As observed when IFN is added to the Hep2Mx1TIPSE cells, there is an increase in the intensity of the band corresponding to the protein STAT1 (90kDa). This result is the same in the cells expressing the viral proteins HBV core and precore and therefore we can suggest that the viral proteins are not blocking the expression of STAT1.
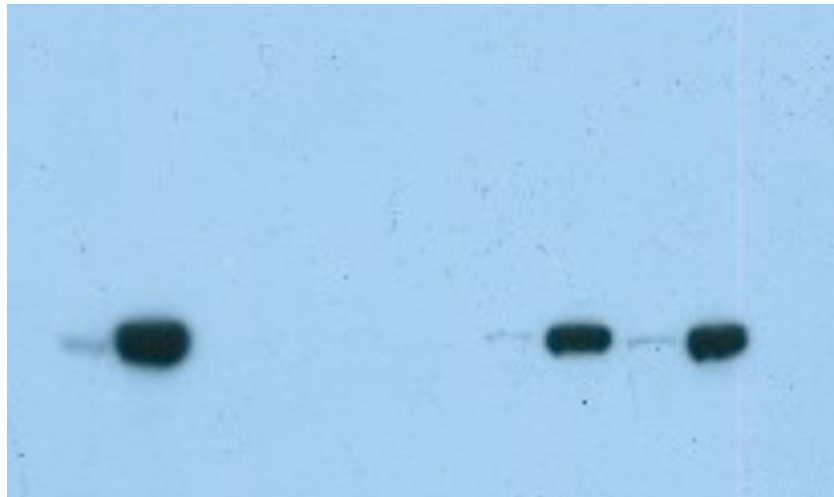
Figure 3.11: **Immunoblotting analysis targeting STAT1 protein indicating that the HCVcore protein was not blocking STAT1.**

The Hep2Mx1TIPSEHCVcore cells were analyzed in an immunoblot targeting the protein STAT1. As observed when IFN is added to the Hep2Mx1TIPSE cells, there is an increase of the band corresponding to STAT1 (90kDa). The result is the same in the cells expressing the viral protein HCVcore and therefore we can suggest that the core protein is not blocking the protein STAT1. This result was obtained for both the HCV genotypes 1a and 4a.
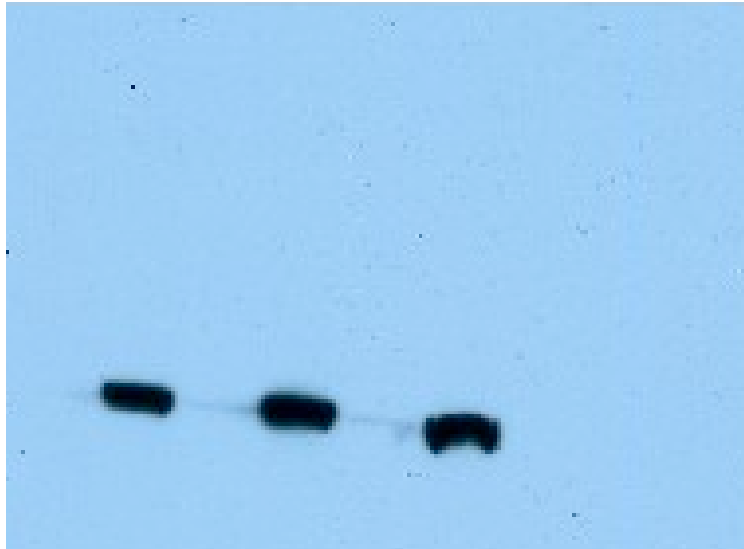
decrease in the binding of the ISGF3 complex to the ISRE domain by means other than inhibiting the transcription of STAT1 (de Lucas, 2005).

Also, the same protein, HCV core, has been shown to be capable of inhibiting the expression of the antiviral IFN-sensitive MxA gene and down-regulating the expression of PKR and 2′-5′ OAS proteins (de Lucas, 2005).

Contrastingly, previous studies (Dansako et al., 2003) have shown that the HCV core protein activates the 2′-5′ OAS gene. However, the activation of this gene appeared to involve a separate mechanism from the activation of the signaling pathway. There was no enhancing in the expression of the transcription factors STAT1 and STAT2 or the kinases JAK1 and Tyk2 by the core protein.

The results for the Hep2Mx1TIPSEHBVprecore cells and the Hep2Mx1TIPSEHBVcore cells (Figure 3.10) were the same as for the Hep2Mx1TIPSEHCVcore cells: in none had the viral proteins appeared to be blocking the IFN signaling pathway by interfering with STAT1 production or/and phosphorylation.

The Hepatitis B core and precore proteins may also be able to block the IFN signaling pathway by affecting other components of this pathway. This is strongly supported by the results in Figure 3.12. This figure shows the results of an assay performed to the cells aimed to determine if the core protein of Hepatitis C and B and also the precore protein of Hepatitis B were involved in inhibiting the expression of the protein Mx1.

This experiment involved a vital test in the presence of the antibiotic puromycin (Figure 3.12). The cells were set-up in 96-well plates and after IFN treatment puromycin was added to them. From Figure 3.1 we can observe that the Hep2Mx1TIPSE cells survived the puromycin addition when IFN was present. This happened because in the presence of IFN the IFN signaling pathway is activated and, as mentioned above, different Interferon stimulated response elements (ISREs) are also activated starting the production and up-regulation of thousands of antiviral genes such as the one coding for the Mx1 protein. Therefore, the Mx1 promoter, present in the Hep2Mx1TIPSE cells, is activated and the gene pac, which is under its control, is also activated

Figure 3.12: Response of Hep2Mx1TIPSE (HBVprecore, HBVcore and HCVcore) cells to puromycin addition while IFN treatment.

The Hep2Mx1TIPSEHBVprecore (a) cells, the Hep2Mx1TIPSEHBVcore (b) cells, and the Hep2Mx1TIPSEHCVcore 1a (results are the same for 4a; data not shown) (c) cells were set up in 96-well microtitre plates. Puromycin in the concentration of 10μg/μl was added to the wells and the plates were fixed after two days. In the figure 3.12 we can observe that the cells expressing HBVprecore and HCVcore 1a to which we added puromycin and IFN survived. The cells to which IFN was not added died after two days in the presence of puromycin. These results are the same for the Hep2Mx1TIPSE cells (Figure 3.1). In all these cells the Mx1 protein is being activated as a result of the IFN signaling pathway. The above result therefore suggests that the viral proteins HBVprecore and HCVcore are not interfering with the IFN signaling pathway. The HBVcore infected cells (b), however, did not survive puromycin addition in the presence of IFN, indicating impairment on the IFN signaling pathway.

a) **Hep2Mx1TIPSE HBVprecore**

No-drug addition **Puromycin addition**

-IFN
+IFN
-IFN
+IFN
-IFN
+IFN
-IFN
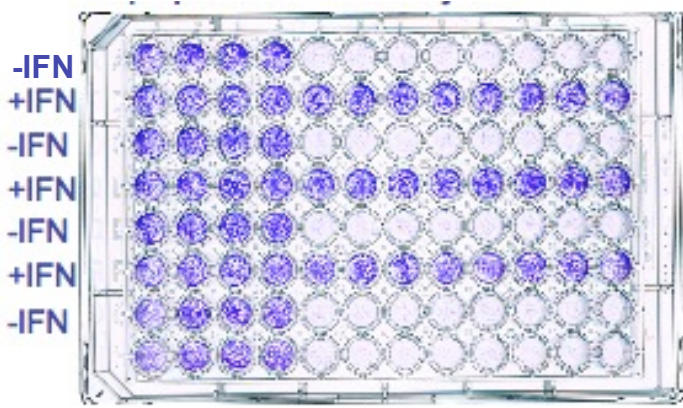
b) **Hep2Mx1TIPSE HBVcore**

No-drug addition **Puromycin addition**

-IFN
+IFN
-IFN
+IFN
-IFN
+IFN
-IFN
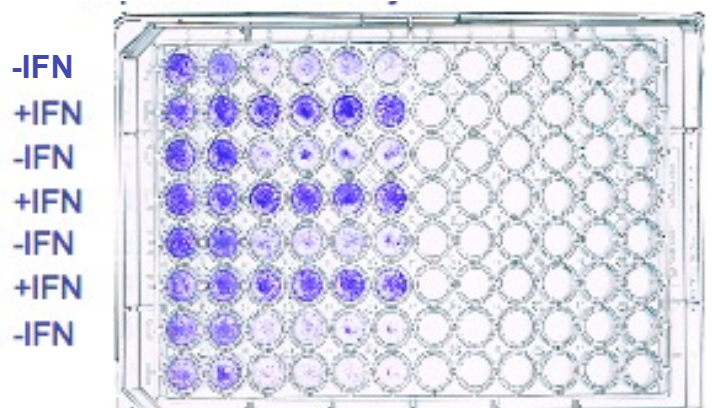
c) **Hep2Mx1TIPSE HCVcore1a**

No-drug addition **Puromycin addition**

-IFN
+IFN
-IFN
+IFN
-IFN
+IFN
-IFN
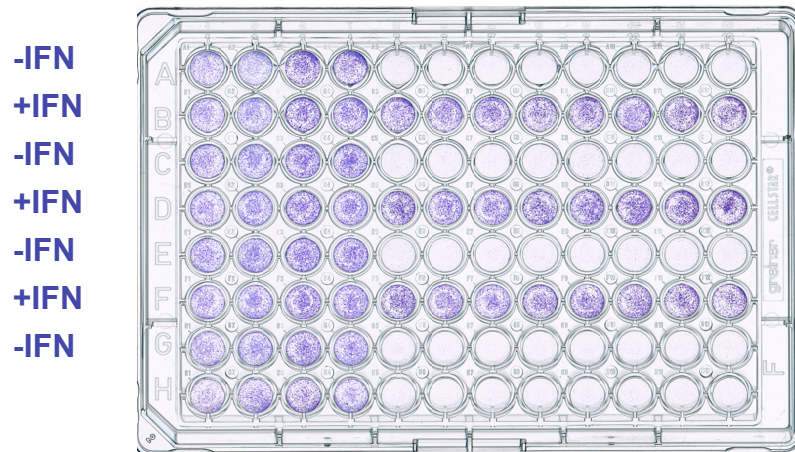
enabling the cells to resist to puromycin. In Summary, in the presence of IFN, the Mx1 promoter is activated leading to the activation of the puromycin resistance gene and the cells live (Figure 3.1). By observation of Figure 3.12c, we notice that the cells expressing the viral protein HCV core present the same result as the Hep2Mx1TIPSE cells, which leads us to conclude that in these cells the IFN signaling pathway is not being blocked by the viral proteins. However, Figure 3.12b shows us that the cells expressing the HBV core protein are unable to survive puromycin addition. In these cells the viral protein is therefore clearly affecting the transcription of Mx1 leading us to believe that it interferes with the IFN signaling pathway. For the cells expressing the HBV precore protein however, the results were the same as for normal Hep2Mx1TIPSE cells.

Part II- Generating A549lucRabies P Cell Lines

The above results and the recent publications led us to speculate whether the viral proteins in question really did not block STAT1 induction or interfere in other ways with the IFN signaling pathway, or instead the expression of those proteins in the cell lines engineered was not sufficient to allow us to have enough viral protein to observe a significant blockage of the IFN signaling pathway.

With that in mind, we thought about developing a different cell line that instead of giving us a subjective qualitative result would give us a quantitative analysis of how much the viral proteins interfere with the IFN signaling pathway. Therefore, we chose to engineer a reporter cell line using the pre-existing A549Luc (human alveolar basal epithelial), cell line available in our lab. This cell line contains a gene that expresses luciferase. The luciferase reporter gene in A549Luc cells is under the control of an interferon stimulated promoter gene and expresses the Luciferase protein. This protein is able to produce bioluminescence when exposed to the right substrate. It catalyses the oxidation of the supplemented luciferin pigment (substrate) in a two-step reaction to yield oxyluciferin. The energy supplied to the reaction in

the form of ATP is converted to light. The emitted protons can be detected with a light sensitive apparatus such as a luminometer.

However, before attempting to develop permanent cell lines expressing a viral protein in A549Luc cells, we performed a transient transfection in 293T cells followed by a luciferase reporter assay.

In this assay, 293T cells were transiently transfected with a luciferase reporter vector containing an interferon stimulated response element (ISRE) encoding sequence promoter. They were also co-transfected with a vector with the viral gene of interest. The chosen viral protein was the Rabies P protein, which had also been reported to block the IFN signaling pathway (Brzozka et al., 2006). The gene for the P protein, obtained from Conzelmann lab in Germany, had been cloned into the pdl'SurPkIB plasmid (Figure 3.6-3.7). We used two different strains of the Rabies virus: SADL16 and BH. The strains vary in their ability to block the IFN pathways. While SADL16 P is a mutant only able to inhibit the STAT1 protein, the P protein from the BH strain is able to block the TBK protein as well.

For this transient transfection we also used a negative control (293T cells not transfected with any vector containing a viral gene) and two positive controls (293T cells co-transfected with plasmids containing the gene PIV5 V and the gene Mumps Enders V). It has been proven that both the viral proteins PIV5 V and Mumps Enders V are responsible for the blocking IFN signaling pathway (Precious et al., 2005; Kubota et al., 2005; Yokosawa et al., 2002).

We measured the luciferase expression therefore measuring the activity of the ISRE promoter in the presence and absence of IFN in 293T cells transfected with viral genes and 293T cells not transfected with the viral genes. We then observed the difference between the values of luciferase when IFN was present and when it was absent and compared these values in the cells lines transfected with the viral genes and the ones that weren't. In short, we wanted to see if the activity of the ISRE promoter was being affected by the expression of the viral proteins. If that were the case, then it would mean

that the viral proteins would be blocking the IFN signaling pathway somehow, causing the down-regulation of the ISRE.

The results for luciferase activity are expressed in Figure 3.13. From this figure, we observe that when IFN is added to the 293T cells there is a considerable increase in luciferase expression compared to the cells that were not treated with IFN. This indicates that the ISRE is being up-regulated in the presence of IFN by activation of the IFN signaling pathway. However, in the cells transiently transfected with vectors containing the genes that code for the viral proteins Mumps Enders V, PIV5 V, and Rabies P BH or Rabies P SADL16 the increase of luciferase expression is not as significant. This indicates that the activity of the ISRE promoter is not as high in cells transfected with viral genes, which suggests a partial blockage of the IFN signaling pathway.

The values for standard error were calculated using n=3 (n=number of assays performed). The error bars were then included in the chart according to the standard error calculated. From the chart (Figure 3.13) we can observe that the error bars between 293T cells with and without IFN addition do not overlap, however, the distance between the bars is less than the length of each bar, which means that these measurements are statistically significant. Also, for all the cells transfected with viral genes, the error bars for cells with IFN added and the error bars for cells with no IFN addition overlap confirming that these results are also statistically relevant.

The transfection efficiency was measured using the β-galactosidase assay system. In order to do this, the cells were also transfected with the plasmid containing the gene that codes for the protein β-gal and the absorbance at 420nm was measured. These measurements (data not shown) allow us to correct for any differences in transfection efficiency.

We then attempted to generate a permanent cell line expressing this protein in the A549Luc cells. The A549Luc cells were infected using the lentiviral system previously described. From there, the new cell lines were subjected to an immunoblot to reveal the presence of the desired viral protein in the cells. In the Figure 3.14, we can see a band of 36kDa that corresponds to

Figure 3.13: **Transient co-transfections in 293T cells with a vector containing the viral protein (Rabies P, PIV5V or MumpsEndersV) and the pISRE-luc reporter plasmid.**

293T cells set to grow in 75cm^3 flasks, were co-transfected with the pISRE-luc reporter plasmid and with the plasmid reporter pdl' PkIB where the viral gene was cloned. The cells were then stimulated by the addition of IFN. Figure 3.13 shows the average activity measurements for 3 assays on which the cells were incubated with and without IFN and measured for luciferase expression using a luminometer.

We can observe that when IFN is added to the cells there is an increase in the amount of luciferase expressed. This increase is due to the up-regulation of the ISG. However, the increase is less accentuated in the cells transfected with a viral gene, which suggests that in these cells the IFN signaling pathway is being affected by the viral protein. The represented error bars indicate that the difference between the amount of luciferase expression in cells with no IFN addition and cells with IFN is statistically significant.
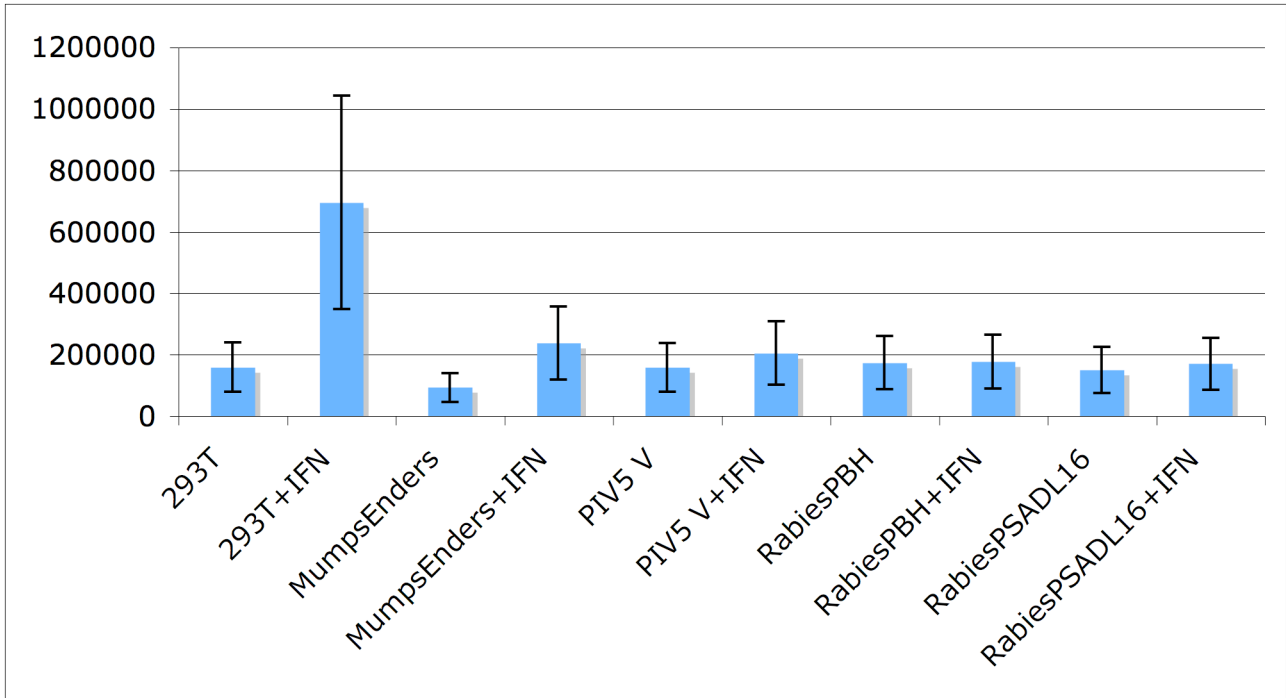
Figure 3.14: **Immunoblot analysis targeting the Rabies P protein using anti-Pk-tag antibody**

In order to check if the A549lucRabiesP cell lines expressed the viral protein the cells were set up in a 6-well plate and an immunoblotting assay was conducted using the anti-pk-tag antibody. Bands representing the protein for both Rabies strains are visible at 36kDa in this figure. Two samples for each strain were analyzed and the same results were obtained.

A549luc RabiesP SADL16   **A549lucRabiesP BH**

Rabies P (36kDa)
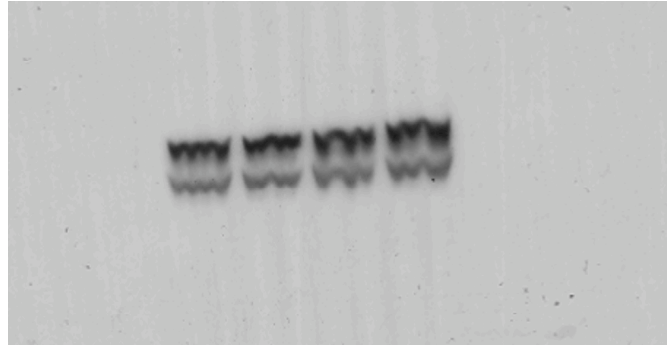
the molecular weight of the P protein suggesting that these cells are expressing Rabies P. After we obtained a stable cell line, we assayed the cells by measuring luciferase expression and checked the affects of the viral protein on its expression. If the viral protein down-regulates the expression luciferase, it means that the same viral protein is responsible for partially blocking the IFN signaling pathway, as explained. From the resulting chart (Figure 3.16) we can observe that (in the presence of IFN) there is, indeed, a reduction in the amount of luciferase expressed by the cells expressing the viral protein P when compared with cells not expressing the same protein. The expression of luciferase is, however, not the same for both strains of Rabies virus. In fact, we can observe that the amount of luciferase expression in the cells expressing the Rabies P SADL16 protein is, in the presence of IFN, higher than the amount of luciferase in cells expressing the Rabies P BH protein. A reasonable explanation for this result is that in cells expressing Rabies P SADL16 protein only the STAT1 nuclear import is being blocked, whereas in the cells expressing Rabies P BH the viral protein is most likely able to circumvent the IFN signaling pathway by interfering as well with other members of this pathway. Since only one measurement was taken for each sample, n=1 no standard error could be calculated. Therefore, these results can only be considered preliminary since the repetition of this assay is essential to draw any conclusions.

An immunoblot targeting the protein STAT1 (Figure 3.15) shows that the band corresponding to STAT1 is slightly smaller and less intense for A549luc cells expressing the Rabies P protein when compared with those cells not expressing the Rabies P protein. Also, we can observe that the cells expressing the Rabies P SADL16 exhibit less STAT1 expression than the other strain of Rabies P protein. Once again, this result suggests that the target for the P protein in the SADL16 strain is only the protein STAT1 while, in the BH strain, the P protein may block IFN signaling by indirectly interfering with STAT1 expression.

Figure 3.15: Immunoblot analysis targeting STAT1 protein in the A549LucRabiesPSADL 16 cells and A549LucRabiesP BH cells

The A549LucRabiesPSADL 16 cells and the A549LucRabiesP BH cells were set up in 6-well plates and IFN was added to every other well 24 hours later. An immunoblot was then conducted in these cells. From Figure 3.15 we can observe that in the A549Luc cells expressing the Rabies P protein, and to which IFN was added, there is a band at 90kDa corresponding to the molecular weight of STAT1. This does not happen in the absence of IFN. However, in the cells expressing the P protein the band is not as intense as when compared with the same band in A549Luc cells. This tells us that in cells expressing the Rabies P protein there might be a partial blockage of the STAT1 protein and therefore of the IFN signaling pathway. This result is especially noticeable in the cell line A549luc RabiesPSADL16.
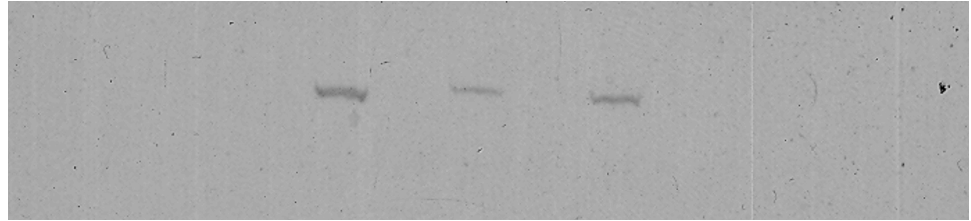
Figure 3.16: Luciferase Reporter Assay conducted in A549LucRabiesP (SADL16 and BH) cell lines.

A549 cell lines containing the luciferase reporter plasmid were infected with lentiviral particles containing the RNA coding for the Rabies P protein and were grown in 75cm^3 flasks. These cells were then stimulated by the addition of IFN 48 hours post-infection. After 8 hours a luciferase reporter assay was performed with these cells. As negative controls in the same assay, A549Luc cells were used. The obtained values were used to construct the following chart. In this chart we can observe that for A549Luc cells the expression of luciferase increases immensely when IFN is added to the cells. In the cells expressing the viral proteins there is also an increase in the amount of luciferase expressed. However, this increase is significantly lower than the one in the A549Luc cells. This result is especially noticeable in the A549luc RabiesPBH cells. This result indicates that in the A549Luc cells expressing the Rabies P protein there is a decrease in the ISG promoter expression. In turn, this suggests that the IFN signaling pathway is being blocked by the P protein, as expected.
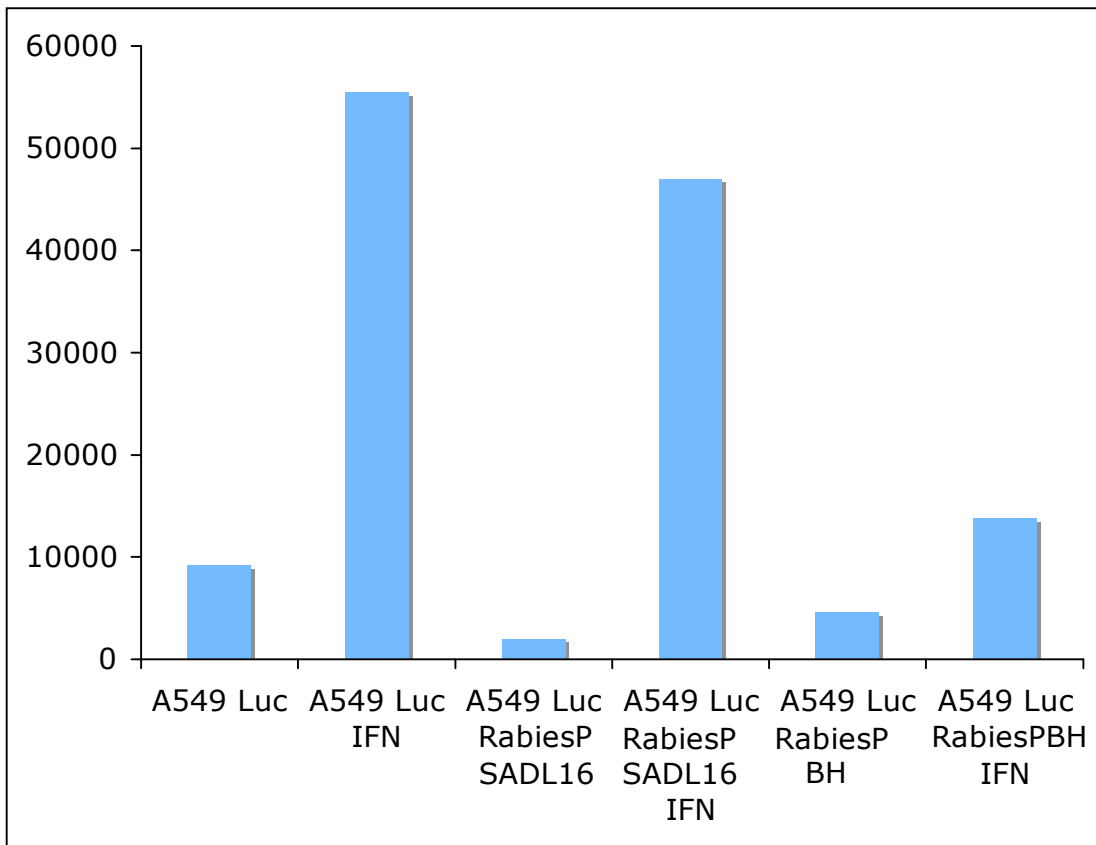
4. Discussion

4.1. Engineering Cell Lines for Compound Screening Assays in Hep2Mx1TIPSE Cells

In order to manufacture novel antiviral drugs, chemical compounds in high throughput screening assays are selected based upon their capability to block viral protein action in host cells. In order to be used in this kind of assay, reporter cell lines expressing certain viral proteins were engineered. The viral proteins (IFN antagonists) HCV core, HBV core and precore were reported to block the IFN signaling pathway by interfering with the Jak/STAT signaling cascade. As they block the pathway, these viral proteins interfere with the up-regulation of a significant number of genes that limit viral replication and proliferation.

The cell lines were generated using the previously characterized reporter cell line Hep2Mx1TIPSE, which contains the resistant genes TK and pac under the control of the inducible promoter Mx1. Mx1 is one of the antiviral proteins induced upon IFN production; its transcription is up-regulated by the Jak/STAT pathway.

In order to generate the cell lines, the sequences coding for the viral proteins were cloned into a reporter plasmid, which had the selective marker blasticidin. Through a lentivirus based vector system, the Hep2Mx1TIPSE cells were infected with the lentivirus containing the RNA that codes for the viral proteins. Hep2Mx1TIPSEHBVcore and Hep2Mx1TIPSEHBVprecore cells were tested for viral protein expression using immunofluorescence whereas Hep2Mx1TIPSEHCVcore cells were tested using immunoblotting techniques. Several assays were also performed to the viral Hep2Mx1TIPSE cells to determine if these viral proteins were able to block the IFN signaling pathway. The first of such assays was an immunoblot targeting the protein STAT1. When the IFN signaling pathway is activated, this protein is phosphorylated and forms a heterodimer with STAT2. This heterodimer then translocates to the nucleus of the cell up-regulating different ISGs (including

the gene that codes for the same protein STAT1). The purpose of this assay was to show that in the presence of IFN, the amount of produced STAT1 protein is diminished in the cells expressing viral proteins when compared with cells not expressing these proteins. However, the results stated the opposite: none of the viral proteins appeared to be blocking the IFN signaling pathway by interfering with STAT1 production and/or phosphorylation since the amount of this protein was the same for Hep2Mx1TIPSE cells and for the cells expressing the viral proteins (Figures 3.10 and 3.11).

Another assay performed on the cells tested them for survival in the presence of the antibiotic puromycin (Figure 3.12). If the viral proteins were, in fact, blocking the IFN signaling pathway, then the ISG that codes for the protein Mx1 would not be up-regulated and, since the pac gene is under its control, the cells would become sensitive to puromycin. Therefore, in the presence of IFN the cells expressing viral protein should die while the Hep2MX1TIPSE cells survive the puromycin addition. By observing the plates in Figure 3.12c we notice that the cells expressing the viral protein HCV core survived the antibiotic addition, suggesting that in these cells the viral proteins are incapable of preventing the induction of the puromycin resistance gene. The cells expressing the HBV core protein (Figure 3.12b), however, died, suggesting that in these cells, the IFN signaling pathway is being affected by the viral protein and that the Mx1 protein production therefore is being impaired. For the HBV precore protein, the results were similar to those of the HCV protein suggesting that the extra 29 nucleotides that this protein contains in comparison with the HBV core protein might make a difference in its ability to interfere with the expression of Mx1 protein.

There are, however, a number of considerations to be taken into account regarding the obtained results. The infections of Hep2Mx1TIPSE cells with the lentiviruses may not have been as successful as required. To have a maximum possibility of selecting a homogenous cell line where all the cells express the viral protein, the maximum number of cells should be infected with only one lentiviral particle. To ensure this happens, a sub-cloning

protocol may be employed on the cell lines. However, this procedure requires additional time not available for the completion of this report.

Finally, when the Hep2Mx1TIPSE cells were set up in the 96-well plates in the absence and presence of IFN subjected to GCV addition, we received clear and expected results (Figure 3.1). However, when several attempts were made to use GCV in an identical assay in the cell lines expressing the viral proteins HBV core, HBV precore, and HCV core, the result was unexpected cell death for all cells with or without IFN. This may have occurred because we were unable to optimize the concentration of GCV or for other unknown reasons.

4.2. Rabies P pdl'SurPkIB Transient Transfection

In this study, we attempted to engineer a permanent cell line expressing the viral protein Rabies P in A459Luc reporter cells.

But, initially, we transiently co-transfected 293T cells with the RabiesPSADL16PkIB or the RabiesPBHPkIB plasmid and a vector containing the luciferase gene under the control of an ISRE promoter and compared the activity of the promoter in these cells in the absence and presence of IFN by measuring the luciferase expression. As a negative control, we used 293T cells non-transfected with the vector expressing the viral protein. The two positive controls used were the viral proteins PIV5 and MumpsV. The genes coding these proteins were cloned into the same reporter vector as Rabies P. Both of these viral proteins are known to block the IFN pathways.

The aim of this assay was to discover if the ISRE promoter was being affected by the expression of the viral protein, indicating that this protein would be responsible for a blockage of the IFN signaling pathway. From the results, we conclude that there is a considerable down-regulation of the ISRE produced for both strains of Rabies when compared with the control cells (Fig 3.13). This result supports the published data (Brzozka et al., 2006) indicating

that Rabies P protein is, in fact, responsible for a partial blockage of the IFN signaling pathway by interfering with the protein STAT1.

By analysis of the error bars, we can confirm that the increase of luciferase expression when IFN is added to the cells is significant. However, we only performed 3 measurements for this experiment and an accurate statistical analysis requires a higher number of measurements.

Because 293T are extremely sensitive, using them for luciferase reporter assays is a very delicate process. They attach weakly to surfaces and monolayers and can be disturbed easily by any compound introduction. However, alternative cell lines, which can be used for transient transfections like Hep2 cells or Vero cells, have very low transfection rates.

4.3. A549Luc Rabies P reporter cell line

With the intention of constructing a reporter cell line expressing the viral protein Rabies P, the gene P was cloned into the pdl' SurPkIB reporter plasmid and, through the lentiviral-based system described above, the A549Luc cells were infected. The resulting reporter cells were tested according to their ability to block the IFN signaling pathway. This was done by measuring luciferase in the absence and presence of IFN and by comparing those values with the corresponding differences in A549Luc cells not expressing Rabies P.

The results (Figure 3.16) show that although there is an increase in the amount of luciferase expressed in the presence of IFN compared to when it is absent, this increase is not as intense as it is for A549Luc cells. However, since there is an increase on the luciferase expression in the presence of the IFN, it means that the IFN signaling pathway is not being completely blocked. This may be because the level of P protein necessary for a complete blockage would be much higher than what is probably being expressed. The level of P protein expression is important since it appears that this protein does not interfere with STAT1 by degrading it, as other proteins do (PIV5V), but instead, it (Brzozka et al., 2006) impairs its nuclear uptake and therefore

higher concentrations of P protein might be needed. Furthermore, it might be necessary to impair other IFN signaling proteins to ensure a complete blockage of the IFN signaling pathway. In fact, we observed that the amount of luciferase expression in the cells expressing the Rabies P SADL16 protein was higher in the presence of IFN, than the amount of luciferase in cells expressing the Rabies P BH protein. We therefore assume that the P protein in the RabiesBH strain is able to interfere with other proteins of the IFN signaling pathway. It is known that the P protein in this strain of Rabies is able to impair IFN production by blocking TBK-1, essential for IRF3 induction (Brzozka et al., 2006). In this particular project we were only testing the ability of the viral protein P in blocking the IFN signaling pathway. It would be, however, interesting to study the ability of this protein in impairing the IFN production pathway and compare it with the Rabies P SADL16 strain.

As mentioned above, a number of repetitions of the luciferase assays need to be performed and the statistical significance of these results has to be determined in order to apply these cell lines on further experiments.

Future Work

Obviously, a major difficulty in this study was the generation of stable cell lines that had a good efficiency of viral protein expression. The construction of cell lines had two purposes: first, as a short-term goal, to detect whether or not the viral proteins in question were responsible for blocking STAT1 and therefore the IFN signaling pathway; second, in the long-term, to obtain a stable cell line which could be used in compound screening assays (an essential step in the manufacturing of antiviral drugs).

Regarding the first objective, we can only conclude that the viral proteins (HBV core and precore and HCV core) at their current level of expression in the cell lines are not able to block the STAT1. In order to

increase the expression of the proteins, an alternative may be used that involves adenoviruses.

Adenoviruses are double-stranded DNA viruses. Recombinant adenoviruses are known to be extremely capable elements of gene delivery into mammalian cells. They can deliver genes into a huge selection of cell types including dividing and non-dividing cells, primary cells, or cell lines. In this alternative, a recombinant adenovirus vector is generated in order to express the protein in question. The recombinant adenovirus is then used to infect the Hep2Mx1TIPSE cells at a desired Moi (Multiplicity of infection). Next, an immunoblot and 96-well plate puromycin assays may be conducted in order to determine whether or not the viral proteins are blocking the IFN signaling pathway as expected. The adenoviruses are an extremely attractive vehicle of gene delivery because they can produce large amounts if highly purified recombinant virus (Wilson et al., 1996). Therefore, using recombinant adenoviruses could increase the expression of viral proteins to a level that would allow us to see the proteins blocking the IFN signaling pathway. Note that this procedure does not result in the generation of a stable cell line since the adenovirus infection is a transitory process.

In the long-term, however, by using procedures such as sub-cloning (see 2.3.5), we may be able to produce successful, stable reporter cell lines that will be paramount in the process of developing antiviral drugs.

5. Conclusion

With the emergence and reemergence of viral infections, the processes with which to discover drugs capable of preventing or curing viral infections have enjoyed enormous developments in recent years. Screening assays to find chemical compounds that qualify as good antiviral drug candidates have been conducted. The aim of this project has been to develop cell lines expressing viral proteins capable of efficiently blocking the IFN signaling pathway that can be used as targets for those screening assays. In this project, A549LucRabiesP reporter cell lines were developed and can now be used to

screen compounds with antiviral potential. They have however, to be improved regarding their ability to generate high viral protein expression.

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