

Interferon, Virus Vaccines and Antiviral Drugs

By

Ana Mara Lopes Rodrigues

School of Biology

University of St Andrews

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Abstract

The emergence of viruses with zoonotic potential, i.e. with the potential ability to cross species barriers to infect unnatural hosts, poses a huge threat to humans. It is therefore essential to develop new methodologies to rapidly and efficiently generate attenuated virus vaccine candidates to attempt to control the threat. Viruses need to be able to at least partially inhibit the host's innate defence mechanism, known as the interferon (IFN) system, to replicate efficiently *in vivo* and establish a productive infection. It has been previously reported that viruses that have lost their ability to circumvent the host's IFN response, or IFN-sensitive viruses, are promising candidates for live attenuated virus vaccines.

Here we report on the development of a cell-based method to attempt to rapidly select IFN-sensitive viruses that can not block IFN signalling, from wild-type virus populations. Lentivirus vectors containing selection markers (HSV-tk – Herpes Simplex virus thymidine kinase gene and pac – puromycin resistance gene) under the control of a tight IFN-inducible promoter (the murine Mx1 promoter) were generated and used to specifically engineer HEp2 cell lines, termed Mx GIPSE and Mx TIPSE, for this purpose. The developed methodology relies on the engineered cell lines and a selection procedure using exogenous IFN- α and puromycin: if a cell is infected with IFN-resistant virus, it will die in the presence of IFN- α and puromycin because IFN signalling will be blocked, thereby blocking the activation of the Mx1 promoter and consequent expression of pac; if a cell is infected with an IFN-sensitive virus, it will survive in the presence of IFN- α and puromycin because the Mx1 promoter will become activated through the IFN signalling pathway, leading to the expression of pac. IFN-sensitive viruses can then be rescued from the surviving cells, and amplified using IFN-permissive cell lines expressing viral IFN antagonist proteins (proteins that block the host's IFN response). When tested on PIV5 strains CPI- (an IFN-sensitive virus) and CPI+ (an IFN-resistant virus), the developed method allowed the survival and amplification of cells infected with CPI-, whilst cell death was observed for cells infected with CPI+. Whilst the developed methodology

seems promising, further developments of the system are required. The possibilities of using the developed methodology in combination with other techniques, such as FACS sorting and immune selection, to rapidly select IFN-sensitive mutant viruses from wild-type and mutagenised virus populations are discussed. The potential to use Mx TIPSE cells to select IFN-resistant revertant viruses from IFN-sensitive virus populations is also discussed.

In addition, a high throughput screening assay has been developed using the engineered Mx GIPSE and Mx TIPSE cell lines to search for compounds that block IFN signalling or that block the action of viral IFN antagonist proteins. Compounds that block IFN signalling would potentially be useful as anti-inflammatory drugs whilst compounds that block the action of viral IFN antagonist proteins would be valuable as antiviral drugs.

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ii) I was admitted as a research student in January 2004 as a candidate for the degree of Doctor of Philosophy in Molecular Virology; the higher study for which this is a record was carried out in the University of St Andrews between 2004 and 2007.

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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

nm – Nanometer

pfu – Plaque forming unit

sec - Seconds

U - Unit

V - Volt

Viruses

BUNV – Bunyamwera virus

BVDV – Bovine Viral Diarrhea virus

CoV – Coronavirus

CPI – Canine Parainfluenza virus

EMCV – Encephalomyocarditis virus

HBV – Hepatitis B virus

HCV – Hepatitis C virus

HeV – Hendra virus

HIV – Human Immunodeficiency virus

hPIV – Human Parainfluenza virus
HSV – Herpes Simplex virus
MeV – Measles virus
MuV – Mumps virus
NDV – Newcastle Disease virus
NiV – Nipah virus
PIV – Parainfluenza virus
PIV5 – Parainfluenza virus 5
PV - Poliovirus
RPV – Rinderpest virus
RSV – Respiratory Syncytial virus
SeV – Sendai virus
SFFV – Spleen Focus-Forming Virus
SV5 – Simian virus type 5
VV – Vaccinia 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
T - Thymidine

Proteins and complexes

ADAR – RNA-specific adenosine deaminase

BCR – B cell receptor

CBP – CREB-binding protein

CIAP – Calf intestinal alkaline phosphatase

DDB – Damage-specific DNA binding protein

F – Fusion glycoprotein

eGFP – Enhanced green fluorescent protein

GAF – Gamma-activated factor

GBP1 – Guanylate binding protein 1

GFP – Green fluorescent protein

GPT – Guanine-phosphoribosyl transferase

HBsAg – Hepatitis B virus surface antigen

HN – Haemagglutinin neuraminidase protein

HSV-tk – Herpes Simplex virus thymidine kinase

IFN – Interferon

IFNAR – Interferon-alpha receptor

IFNGR – Interferon-gamma receptor

IKK - IκB kinase

IL – Interleukin

IPS-1 – Interferon promoter stimulator 1

IRF – Interferon regulatory factor

ISGF3 – Interferon-stimulated gene factor 3

JAK – Janus tyrosine kinase

L – Large protein

M – Matrix protein

MAPK – Mitogen-activated protein kinase

MDA-5 – Melanoma differentiation-associated gene 5

MHC – Major histocompatibility complex

Mx – Myxovirus-resistance

MyD88 – Myeloid differentiation primary response protein 88

NP – Nucleocapsid protein
Nf- κ B – Nuclear factor- κ B
OAS – 2'-5' oligoadenylate synthetase
P - Phosphoprotein
Pac – Puromycin N-acetyltransferase
PACT – PKR-activating protein
PIAS – Protein inhibitor of activated STAT
PKR – dsRNA-dependent protein kinase R
rHuIFN- α - Recombinant human interferon alpha
RIG-I – Retinoic acid-inducible gene I
RLR – RIG-I-like receptor
RNase L – Endoribonuclease L
SH – Small hydrophobic protein
SOCS – Suppressor of cytokine signalling
STAT – Signal transducer and activator of transcription
TBK1 – TANK-binding kinase 1
TCR – T cell receptor
TIR – Toll/interleukin 1 receptor
Tk – Thymidine kinase
TLR – Toll-like receptor
TRAF6 – TNF associated-receptor factor 6
TRIF – Toll/interleukin 1 receptor domain-containing adaptor protein inducing IFN- β
TYK – Tyrosine kinase
V – V protein

Chemicals and reagents

5-FU – 5-Fluorouracil
6-TG – 6-Thioguanine
BUdR - Bromodeoxyuridine
DAPI – 4',6'-diamino-2-phenylindole
DMEM – Dulbecco's modified eagle's medium

DMSO – Dimethyl sulfoxide
FCS – Fetal calf serum
HAT – Hypoxanthine, aminopterin, thymidine medium supplement
LB – Luria-Bertani
PBS – Phosphate buffer saline

Miscellaneous

2'-5' A – 2'-5' linked oligomers of adenosine
AIDS – Acquired immune deficiency syndrome
APC – Antigen-presenting cell
CARD – Caspase recruiting domain
cDC – Conventional dendritic cell
CPE – Cytopathic effect
CTL – Cytotoxic T cells
FACS – Fluorescence activated cell sorter
GAS – Gamma-activated site
HTS – High throughput screening
ICTV – International committee on taxonomy of viruses
IRES – Internal ribosomal entry site
ISG – Interferon-stimulated gene
ISRE – Interferon-stimulated response element
M.o.i. – Multiplicity of infection
NK – Natural killer
ORF – Open reading frame
pa – Protease activation
PCR – Polymerase chain reaction
pDC – Plasmacytoid dendritic cell
p.i. – Post-infection
SARS – Severe acute respiratory syndrome
StaphA – *Staphylococcus aureus* strain A

TH – T helper cell

Ts – Temperature sensitive

UV – Ultraviolet

WHO – World Health Organization

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1. INTRODUCTION

1.1 Viruses

The word virus is derived from the Latin word *virus* meaning “poison”, “slime” or “venom”. One main motivation for the study of viruses is the fact that they cause many important infectious diseases, among them the common cold, influenza, rabies, measles, many forms of diarrhoea, hepatitis, yellow fever, smallpox and acquired immune deficiency syndrome (AIDS). Table 1.1 lists a few viral infections associated with human disease. According to the World Health Organization (WHO), viral infections of the respiratory tract account for almost half of all acute illnesses. Viruses are also common causes of gastrointestinal illness, and in less developed countries, this type of infection is a significant cause of mortality in infants and young children. Other viruses such as Poliovirus and Rabies virus cause infections of the central nervous system that can lead to paralysis or death in humans. Hepatitis B virus (HBV) and Hepatitis C virus (HCV) can cause acute and chronic liver disease and are one of the leading causes of cancer in humans. There are currently estimated to be 300 million infectious carriers of HBV. Human Immunodeficiency virus (HIV) probably infected humans in the 1930s but emerged in the 1980s to become a major pathogen. In December 2005, the WHO reported that AIDS had killed more than 25 million people since it was first recognised in 1981, making it one of the most destructive epidemics in recorded history. Furthermore, close to 5 million people were newly infected with the virus in 2005.

Some of the new viral strains, with high virulence for humans and animals of economic importance, are lethal to the host they infect. Why do lethal viruses evolve when it is obviously of no benefit to a virus to kill its host? Today it is believed that most viruses are relatively benign in their natural host; the lethal viral diseases are explained as resulting from an "accidental" jump of the virus from its natural host to a new one that is not accustomed to it (zoonosis). It is believed that the major factor contributing to the appearance of new zoonotic pathogens in human populations is increased contact

between humans and wildlife (Daszak *et al.*, 2001). This can be caused either by an increase of human activity in wilderness areas or by movement of wild animals into areas of human activity due to anthropologic or environmental disturbances. An example of this is the outbreak of Nipah virus (NiV) in peninsular Malaysia in 1999, when intensive pig farming intruded into the natural habitat of fruit bats carrying the virus. Unidentified spillover events caused infection of the pig population which acted as an amplifier host, eventually transmitting the virus to farmers and resulting in 105 human deaths (Field *et al.*, 2001).

Table 1.1 Common diseases caused by viruses in humans (adapted from Barker, 2004).

Virus Family	Virus	Diseases
<i>Filoviridae</i>	Ebola virus	Ebola haemorrhagic fever
<i>Herpesviridae</i>	Herpes Simplex virus type I	Cold sores
	Epstein-Barr virus	Infectious mononucleosis, Burkitt's lymphoma
	Varicella-Zoster virus	Chicken pox, Shingles
<i>Poxviridae</i>	Variola virus	Smallpox
<i>Hepadnaviridae</i>	Hepatitis B virus	Hepatitis
<i>Papovaviridae</i>	Papillomavirus	Warts
<i>Orthomyxoviridae</i>	Influenza virus	Respiratory diseases
<i>Togaviridae</i>	Rubivirus	Rubella
<i>Paramyxoviridae</i>	Mumps virus	Mumps
	Measles virus	Measles
	Respiratory Syncytial virus	Respiratory diseases
<i>Rhabdoviridae</i>	Rabies virus	Rabies
<i>Retroviridae</i>	Human Immune Deficiency virus	AIDS
<i>Coronaviridae</i>	Severe Acute Respiratory Syndrome Coronavirus	Severe acute respiratory syndrome

Ebola virus is a zoonotic virus and also emerged as a serious human pathogen. According to the WHO, since the Ebola virus was discovered, approximately 1850 cases with over 1200 deaths have been documented. Despite considerable effort by the WHO no animal reservoir capable of sustaining the virus between outbreaks has been identified; however, it has been hypothesized that the most likely candidate is the fruit bat.

Another example is the virus that causes severe acute respiratory syndrome (SARS) called SARS Coronavirus (SARS CoV). SARS was first recognized as a global threat in 2003. It first emerged in the People's Republic of China and is believed to be an animal virus that crossed the species barrier to humans when ecological changes or changes in human behaviour increased opportunities for human exposure to the virus and virus adaptation, enabling human-to-human transmission (Antia *et al.*, 2003). Within four to five months, the international spread of SARS-CoV resulted in 8098 cases in 26 countries, with 774 deaths. The outbreak of SARS was contained through the isolation of suspect patients and quarantine of potentially exposed individuals. This was possible because patients are most infectious during the second week of illness and transmission prior to illness onset is rare (Peiris *et al.*, 2003).

Avian influenza is a contagious disease of animals caused by viruses that normally infect only birds, and less commonly pigs. Avian Influenza viruses have, on rare occasions, crossed the species barrier to infect humans. The outbreaks of highly pathogenic avian influenza, which began in South-East Asia in 2003, have affected many countries simultaneously, resulting in the loss of many birds. The causative agent is the H5N1 virus. When the virus passes from poultry to humans, the result is very severe disease, with high fatality. According to the WHO, in the outbreak that began in 2003, more than half of those infected by the virus died.

These are just a few examples of recent outbreaks of virus infections that caused disease and death in humans and animals. The truth is that viruses have the ability to infect virtually any type of cell, from bacterial to human; they have rapid generation times and they often have high mutation rates. These characteristics make viruses the key pathogens

capable of generating new variants, and thus new infectious diseases, in very short periods. Consequently, it is essential to be vigilant and to have ways of quickly responding to diseases caused by emergent viruses, such as the availability of rapid methodologies for generating vaccine candidates (see section 1.5).

1.1.1 The *Paramyxoviridae* family

The *Paramyxoviridae* family includes important pathogens of vertebrates, causing a number of significant diseases in animals and humans. One of the most infectious viruses known, that has been targeted by the WHO for eradication, Measles virus (MeV), is included in this family. Measles kills nearly one million children each year, half a million of those in Africa alone, despite the availability of a safe and effective vaccine for the past 40 years. The *Paramyxoviridae* family also includes some of the most prevalent viruses known, such as Respiratory Syncytial virus (RSV), Mumps virus (MuV) and Parainfluenza viruses (PIV). RSV is the most common cause of lower respiratory tract disease in infants and children worldwide and human PIVs are second to RSV as common causes of lower respiratory tract disease in young children. In animals, Newcastle disease and rinderpest affect poultry and animal farming. Parainfluenza virus 5 (PIV5), also referred to as canine Parainfluenza (CPI) virus and Simian virus type 5 (SV5), causes respiratory illness in dogs, and is also suspected to infect humans. PIV5 has been isolated from humans, dogs, monkeys and pigs (Chatziandreou *et al.*, 2004). Newly emergent viruses, including Hendra virus (HeV) and NiV, have been discovered over the past decade in a wide range of animals (reviewed in Wang *et al.*, 2001). These viruses, whose natural reservoir appears to be fruit bats, have caused infectious outbreaks in farm animals, domestic animals and humans.

1.1.1.1 Classification of paramyxoviruses

The *Paramyxoviridae* family is part of the order *Mononegavirales* together with three other families, *Rhabdoviridae* (Rabies and Vesicular Stomatitis viruses), *Filoviridae* (Marburg and Ebola viruses) and *Bornaviridae* (Bornavirus). This order includes all viruses with single-stranded, non-segmented, negative strand RNA genomes. The

genomic RNA (-) of these viruses serves as a template for synthesis of mRNAs and also synthesis of the antigenome (+; positive sense) strand and therefore these viruses are required to encode and package their own RNA polymerase. mRNAs are synthesized after the virus has been uncoated in the infected cell, after which viral replication occurs. The newly synthesized antigenome (+) strand then serves as a template for further copies of genomic (-) RNA.

Table 1.2 Current International Committee on Taxonomy of Viruses (ICTV) classification of the *Paramyxoviridae* family with examples of viruses of each genus (The Eighth Report of the International Committee on Taxonomy of Viruses, 2005).

Family <i>Paramyxoviridae</i>		
Subfamily	Genus	Species
<i>Paramyxovirinae</i>	<i>Respirovirus</i>	Sendai virus, human Parainfluenza virus 1, human Parainfluenza virus 3
	<i>Rubulavirus</i>	human Parainfluenza virus 2, human Parainfluenza virus 4, Mumps virus, Parainfluenza virus 5
	<i>Morbillivirus</i>	Canine Distemper virus, Measles virus, Rinderpest virus
	<i>Henipavirus</i>	Hendra virus, Nipah virus
	<i>Avulavirus</i>	Newcastle disease virus
<i>Pneumovirinae</i>	<i>Pneumovirus</i>	bovine Respiratory Syncytial virus, human Respiratory Syncytial virus
	<i>Metapneumovirus</i>	avian Metapneumovirus, human Metapneumovirus

The *Paramyxoviridae* family is divided into two-subfamilies, *Paramyxovirinae* and *Pneumovirinae*, which are further sub-divided into genera according to characteristics such as morphologic criteria, genome organization, virus morphology, protein

characteristics and relatedness of protein sequences (Lamb & Kolakofsky, 2001, see table 1.2).

1.1.1.2 Virion structure and viral proteins

The paramyxovirus virions are typically spherical enveloped particles ranging from 150 to 350 nm in diameter, but they can be pleiomorphic. Paramyxoviruses replicate in the cytoplasm and progeny viruses are formed and released from the plasma membrane of the host cell in which the virus is grown. In the course of this step, the viral particles acquire their lipid envelope, which contains the viral transmembrane glycoprotein spikes that project from the surface by about 8 to 12 nm. All paramyxoviruses have two major envelope glycoproteins for membrane fusion (F) and attachment (HN, H or G) which mediate virus entry into the host cell during infection, and exit from the host cell during maturation and release (budding). The F protein mediates the fusion of the viral envelope with the plasma membrane of the host cell, thereby playing a major role in virus penetration. Fusion proteins are fairly similar throughout the *Paramyxoviridae* family, whilst the characteristics of the attachment glycoprotein vary widely between genera. Viruses of the *Respirovirus* and *Rubulavirus* genera have an attachment glycoprotein with both haemagglutinin and neuraminidase activities (termed HN protein). Its haemagglutinin activity allows the protein to adsorb virus particles to the surface of the host cell by binding to sialic acid-containing molecules, while its neuraminidase activity is responsible for preventing the aggregation of newly formed virus particles at the cell membrane, by cleavage of sialic acid from the surface of virions and the surface of infected cells. Some members of the *Rubulavirus*, *Pneumovirus* and *Metapneumovirus* genera also encode an additional small hydrophobic integral membrane protein (SH). One role of the SH protein in PIV5 is to block apoptosis (programmed cell death) in virus-infected cells (He *et al.*, 2001).

One of the most abundant proteins in the virion is the matrix (M) protein. The M protein is considered to be the central organiser of viral morphogenesis, interacting with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer and the nucleocapsids. The remarkably stable helical nucleocapsid is present within the viral membrane and is composed of viral RNA that is encapsidated and thereby protected with

nucleoprotein (NP). The nucleocapsid is associated with a polymerase complex composed of phospho (P) and large (L) proteins. The P and L proteins are required for polymerase activity with NP:RNA templates, thereby playing an essential role in RNA synthesis and viral replication. A schematic representation of a paramyxovirus particle is shown in figure 1.1 (adapted from Carlos, 2005).

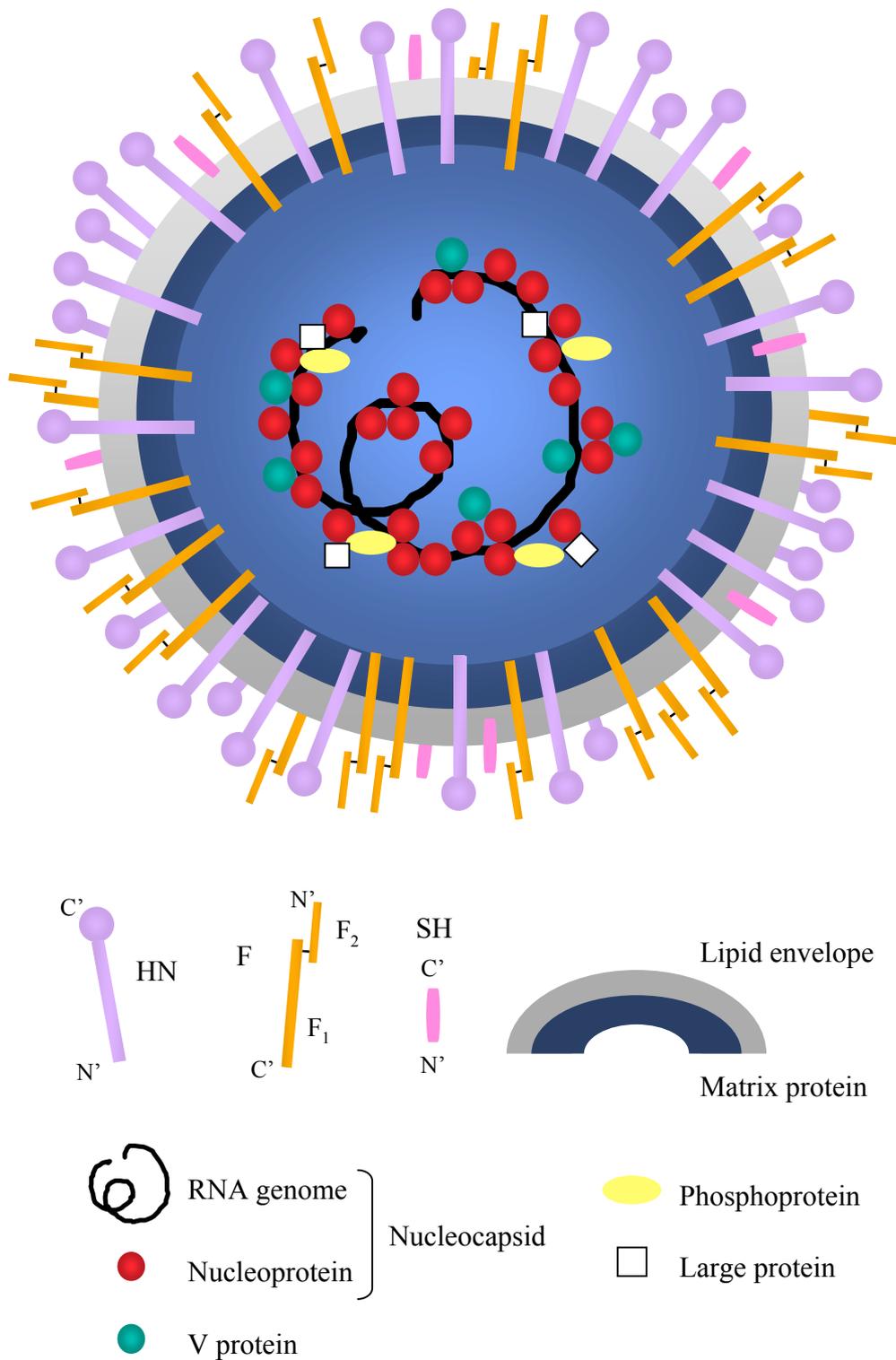
In addition to the essential genes for viral replication, paramyxoviruses also encode a subset of accessory proteins, largely unique to each genus. Some of these accessory proteins are present in the virion. In rubulaviruses, copies of the V protein (an accessory protein) are associated with the nucleocapsid (Paterson *et al.*, 1995, Randall & Bermingham, 1996).

The structure of the P gene is one of the relevant characteristics used to classify this virus family into subfamilies and genera. In members of the *Pneumovirinae* subfamily, the P gene encodes a single protein, the phosphoprotein P, but in all members of the *Paramyxovirinae* subfamily, the P gene encodes multiple gene products. Their P genes are subject to co-transcriptional RNA editing whereby the viral polymerase pauses at a conserved 'editing sequence' in the gene during transcription. When the viral polymerase resumes gene transcription, it stutters in a certain number of cases and slips backwards, adding one or two extra guanosine (G) residues. This results in a change in the reading frame downstream of the editing site, giving rise to different proteins with a common amino-terminal domain (upstream of the editing site) but different carboxy-terminal domains (downstream of the editing site). The paramyxovirus co-transcriptional editing site was first described for PIV5 in the *Rubulavirus* genus (Thomas *et al.*, 1988). In members of the *Morbillivirus*, *Respirovirus* and *Henipavirus* genera, in addition to the production of additional mRNAs by RNA editing, some P genes encode a C protein. The C protein open reading frame (ORF) has an alternative start site downstream to the P ORF and is accessed by ribosomal choice.

Figure 1.1 Schematic representation of the virion structure of Parainfluenza virus type 5 (PIV5), a typical paramyxovirus (adapted from Carlos, 2005).

The outer layer grey circle represents the lipid envelope which coats the virus, while the dark blue layer represents the matrix (M) protein, which is essential for the architecture of the virion. The M protein is thought to hold the virus particle together, bridging the surface proteins with the nucleocapsid. The haemagglutinin-neuraminidase (HN) and fusion (F) surface proteins are embedded into the lipid bilayer, and project from the surface of the virus particle. The small hydrophobic protein (SH) is also represented, although it only exists in some rubulaviruses including PIV5. The orientation of the membrane-anchored proteins is indicated in the figure. The helical nucleocapsid is found in the core of the particle and comprises the single-stranded, non-segmented, negative-sense genomic RNA of the virus encapsidated with the nucleocapsid (NP) protein. The phospho- (P) and large (L) proteins form the viral RNA polymerase and are associated with the nucleocapsid. The V protein is also associated with the nucleocapsid in all rubulaviruses, whereas for other genera of the *Paramyxoviridae* family, the V protein is only found in infected cells (reviewed in Lamb & Kolakofsky, 2001).

Figure 1.1



adapted from Carlos, 2005

1.1.1.3 Parainfluenza Virus 5

PIV5 is a typical paramyxovirus and as such, is frequently used in our laboratory as a model. PIV5 was originally isolated from rhesus monkey kidney cells, but as mentioned before, can cause respiratory illness in dogs and can also infect humans (Hsiung, 1972, Robbins *et al.*, 1981, Goswami *et al.*, 1984). PIV5 has been isolated on multiple occasions from different species, including humans, dogs, pigs and monkeys (Chatziandreou *et al.*, 2004).

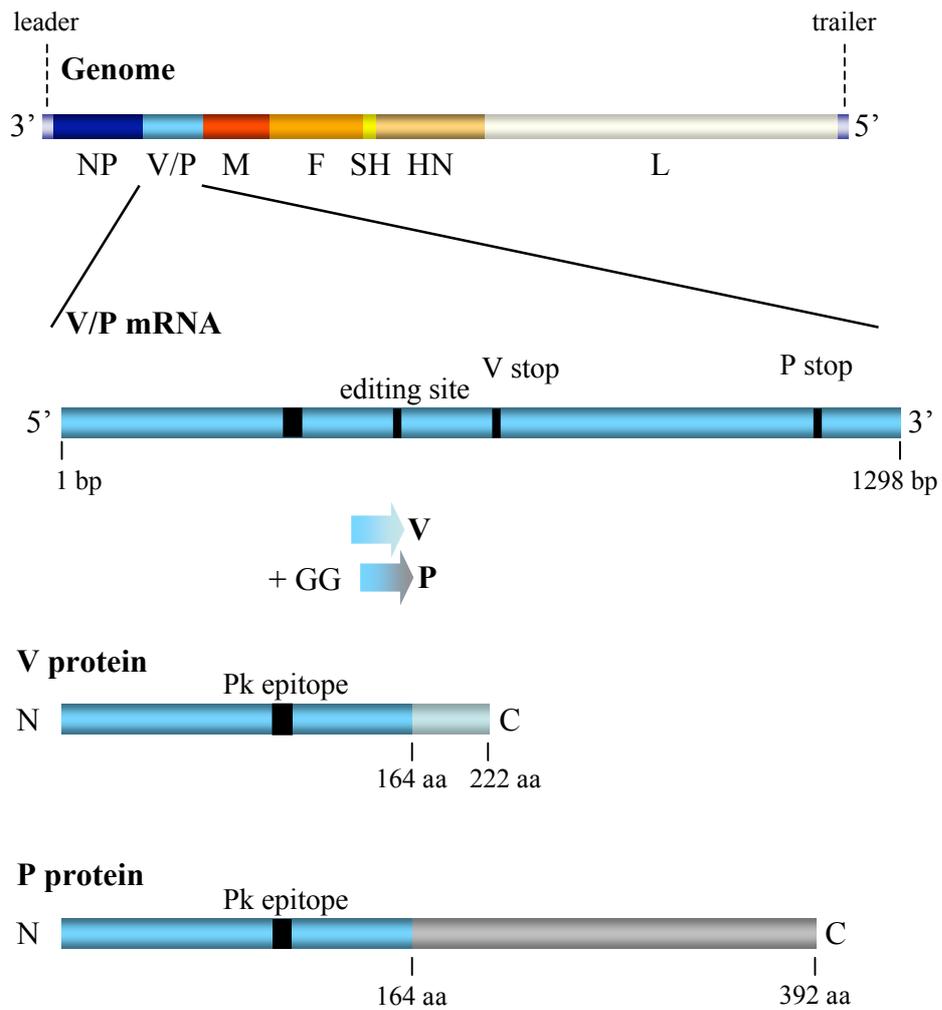
PIV5 contains one of the smallest genomes in the *Paramyxovirinae* subfamily (15246 bp; GenBank Accession number AF052755) and includes seven genes which encode eight proteins (HN, F, SH, M, L, NP, P and V). The V/P gene of PIV5 codes for two proteins. A faithful transcript of the V/P gene of PIV5 generates the V protein, while the P protein is created by RNA editing in which two guanosine residues are inserted. Thus, the V and P proteins of PIV5 are amino-coterminal, having the first 164 amino acids in common, but their carboxy-terminal domains are different. The structure of the PIV5 V/P gene is schematically represented in figure 1.2 (adapted from Lamb & Kolakofsky, 2001).

The V protein of PIV5 has 222 amino acids and a molecular mass of 23925 Da (Thomas *et al.*, 1988). The cysteine-rich carboxy-terminal domain of V (67 amino acids) is highly conserved in all paramyxoviruses, including seven perfectly conserved, identically positioned cysteine residues. The V protein of PIV5 (and other rubulaviruses) is a structural protein which is associated with the nucleocapsid and is incorporated into virions. It is a multifunctional protein and plays important roles in viral pathogenesis. V interacts with the nucleocapsid structure and can bind to free NP via its amino-terminal domain (Randall & Bermingham, 1996, Lin *et al.*, 1997). It has been suggested that V interacts with NP with the aim of keeping it soluble in the cell prior to encapsidation of viral RNA into nucleocapsids (Precious *et al.*, 1995). Thus, V plays an important role in viral encapsidation (and therefore in regulating viral transcription and replication) since encapsidation of the RNA genome is essential for the recognition of the RNA genome as a template by the viral polymerase. It has also been demonstrated that V downregulates the cell cycle, holding the intracellular environment in a virus replication-permissive

Figure 1.2 Representation of the PIV5 genome and the structure of the V/P gene, as well as the V and P proteins, encoded by the V/P gene (adapted from Lamb & Kolakofsky, 2001).

The PIV5 genome is single-stranded, non-segmented, negative sense RNA and contains seven genes that code for eight proteins, as well as two extragenic regions, the 3'-terminal leader sequence and the 5'-terminal trailer sequence. As a result of mRNA editing, the V/P gene codes for two distinct proteins, the V and P proteins. V mRNA is a faithful gene transcript, while the insertion of two non-templated G residues alters the open reading frame, giving rise to the P mRNA. Consequently, the amino-terminal parts of V and P are identical, whilst the carboxy-terminal domains are unique for each protein. Also shown in the diagram is the pk epitope, which maps to the amino-terminal domain (adapted from Lamb & Kolakofsky, 2001).

Figure 1.2



adapted from Lamb & Kolakofsky, 2001

state, by binding to DDB1 (the 127-kDa subunit of the damage-specific DNA binding protein, which is involved in damaged DNA repair) (Lin *et al.*, 1998). The V protein is also responsible for the ability of PIV5 to circumvent the host interferon (IFN) response (Didcock *et al.*, 1999b) (discussed in section 1.4).

PIV5 strain isolates

Two of the original monkey isolates of PIV5 are referred to as WR and W3A (or W3) (Choppin, 1964, Hull *et al.*, 1956) and are considered as “wild-type” viruses. Besides the “wild-type” virus, two canine strains are frequently used in our laboratory. One of the strains, termed CPI+, was isolated from the cerebrospinal fluid of a dog with temporary posterior paralysis (Evermann *et al.*, 1981, Evermann *et al.*, 1980), while another PIV5 strain, termed CPI-, was isolated from the brain tissue of a dog experimentally infected with CPI+, at 12 days post-infection (p.i.) (Baumgartner *et al.*, 1982). Characterization of CPI- showed that it was attenuated, causing only a mild cytopathic effect *in vitro*, when compared to giant syncytial cell formation (fusion of cellular membranes to form large cells with several nuclei) and cytolysis induced by CPI+ (Baumgartner *et al.*, 1991). It was also shown that, out of a panel of 50 antibodies, only one (the anti-pk antibody or PIV5-p-k mAb) could distinguish between these two closely related isolates (Southern *et al.*, 1991). Furthermore, sequence analysis of the CPI- V/P gene revealed that the V (and P) protein of CPI- has three amino acid changes compared to that of CPI+, one of which maps to the pk epitope. More recently, it was shown that CPI+ targets STAT1 for degradation, thereby blocking IFN signalling, whilst CPI- fails to target STAT1 for degradation due to the three amino acid differences in the V/P amino-terminal domain (Chatziandreou *et al.*, 2002). Thus, given that V enables PIV5 to circumvent the IFN response by targeting STAT1 for degradation (see section 1.4), changes in the ability of the pk epitope to bind to the monoclonal anti-pk antibody may serve as an indicator of potential changes in the ability of the virus to block IFN signalling.

1.2 The Immune System

The immune system is of relevance to vaccines and vaccine design and is therefore briefly overviewed in this section. For a more detailed description of the immune system refer to ‘Immunology, Infection, and Immunity’, edited by Pier, Lyczak and Wetzler (2004). For detailed information about the specific immune responses to viruses, refer to Whitton & Oldstone, 2001.

The mammalian immune system consists of specialised cells and organs that mediate protection against infectious agents. It has the ability to discriminate between the individual’s own body (self) and foreign (non-self) antigens (molecules that bind to specific immune receptors and elicit an immune response). It is often divided into two broad categories: innate or non-specific immunity and adaptive or specific immunity, although there is interplay between these systems (components of the innate immune system influence the adaptive immune system and vice-versa). The innate immune system is the first line of defence against invading organisms, while the adaptive immune system acts as the second line of defence and affords protection against re-exposure to the same pathogen.

The adaptive immune system is antigen specific and reacts only with the pathogen that induced the response. It often takes days to develop, so it is not effective in preventing an initial infection, but it generally prevents subsequent infections and helps in clearing long-lasting infections. It also demonstrates immunological memory; it ‘remembers’ it has encountered an invading pathogen and reacts more rapidly on subsequent exposure to the same pathogen.

The mechanisms of the innate immune system are fast-acting and non-specific, which implies that it recognises and responds to a wide spectrum of foreign stimuli, such as pathogenic proteins, nucleic acids and carbohydrates, within minutes to hours of infection. If it does not completely succeed in preventing pathogens from gaining access to the tissues, it does at least succeed in limiting the extent of the infection.

1.2.1 Adaptive immunity

The cells of the adaptive immune system are special types of leukocytes (white blood cells), called lymphocytes. There are two major types of lymphocytes which are derived from stem cells in the bone marrow: B lymphocytes are involved in humoral immune responses, while T lymphocytes are involved in cell-mediated immune responses.

The major components of cell-mediated responses, T lymphocytes (or T cells), are produced in the bone marrow, but complete their maturation in the thymus. T cells express T-cell receptors (TCRs) on their surface. TCRs recognize a ‘non-self’ target: a complex formed of a major histocompatibility class (MHC) molecule and an antigenic peptide exposed on the surface of antigen-presenting cells (APCs). As the name suggests, APCs present antigens on their surface in a context that T lymphocytes can recognize: APCs take up foreign antigens, process the antigens by cleaving them into many small peptides (antigenic peptides) and load these peptides onto MHC molecules. The MHC-peptide complex is then displayed on the cell surface, allowing T lymphocytes to recognise and bind to the complex, thereby becoming activated.

There are two major populations of T cells, the T helper (TH) cells and the cytotoxic T (CTL) cells. TH cells produce cytokines, signalling molecules that increase the activity of other immune cells. Functionally, they can be divided on the basis of the cytokines they produce, into those that preferentially stimulate antibody production, TH2 cells, and those that stimulate inflammatory responses, TH1 cells. They recognise MHC class II molecules, whilst CTL cells recognise MHC class I molecules. When activated by binding to the peptide-MHC class I complex on the surface of infected cells, CTL cells have the ability to destroy these infected cells by releasing perforin and granzymes, inducing their lysis. T cell killing of host cells is particularly important in preventing the replication of viruses.

The major components of the humoral immune response, B lymphocytes (or B cells), are produced and mature in the bone marrow. B cells are APCs that display B-cell receptors (BCRs) on their surface, which are transmembrane molecules of immunoglobulin (or

antibodies). Following engagement of the BCR with its cognate antigen, the antigen is endocytosed, degraded, and presented on MHC class II molecules (forming the MHC-antigenic peptide complex) on the surface of the B cells. When activated by binding to foreign antigen and after interacting with TH cells to receive additional signals, B cells begin to divide and differentiate into antibody-secreting plasma cells. These antibodies then circulate in blood plasma and lymph, bind to pathogens expressing the antigen and thereby mark them for destruction.

There are also specialised subsets of B and T cells called memory cells, which have a prolonged life span. Once in contact with a specific invader, memory cells will specifically and rapidly recognise it when challenged by a subsequent infection, leading to the immediate activation of the immune system and rapid clearance of the invader.

1.2.2 Innate immunity

Innate immunity comprises a wide array of defence mechanisms, including physical and physiological barriers (for example, skin and mucosal surfaces, temperature and pH), enzymatic and cellular effectors that kill or inactivate foreign agents, and non-specific defences tailored to prevent viral infections.

Inflammation is one of the first responses of the immune system to infection. Inflammation is a response to the release of cytokines and mediators of the complement system (see below) at the site of infection and serves to establish a physical barrier against the spread of infection and to promote healing of any damaged tissues. It helps in controlling infections, but is also essential in initiating the adaptive immune response. The main signs of inflammation - redness, heat, swelling and pain – are due to the infiltration of the tissues by plasma and leukocytes.

Platelets are small anucleate blood elements that play a role in blood clotting and inflammation. Once activated, platelets adhere to each other via adhesion receptors and to the endothelial cells in the wall of the blood vessel, leading to the formation of blood

clots. In addition, platelets are rapidly deployed to sites of infection and modulate inflammatory processes by interacting with leukocytes and by secreting cytokines.

The complement system is a biochemical cascade included in both innate and adaptive immunity: it comprises about 30 serum proteins that circulate in the blood stream in an inactive state until activated by the recognition of a pathogen. Their functions include triggering the recruitment of inflammatory cells, 'tagging' pathogens for destruction by other cells and killing of target cells by disruption of the plasma membrane.

The principal cellular effectors of innate immunity are phagocytes, cells capable of ingesting foreign antigens, and natural killer (NK) cells, cells capable of destroying tumours and infected cells (reviewed in Levy, 2004).

The phagocytic cells of the immune system include monocytes/macrophages, neutrophils and dendritic cells. Macrophages are large phagocytic leukocytes which are able to move outside of the vascular system and enter the areas between cells in the pursuit of invading pathogens. Neutrophils, along with two other cell types (eosinophils and basophils), are known as granulocytes due to the presence of granules in their cytoplasm. Neutrophils are phagocytic cells, destroying mainly bacteria and fungi, and contribute to early stages of inflammation. All granulocytes produce cytokines that are important in innate immunity and also in lymphocyte responses to infection. Dendritic cells are phagocytic cells present in tissues that are in contact with the external environment. In addition, dendritic cells are the most potent of APCs and also produce large amounts of cytokines, such as IFNs that have the ability to directly inhibit replication of virus at the onset of infection.

NK cells are another type of lymphocyte. Their function is to recognise damaged or altered cells (such as virally infected or tumour cells) and kill them. They are one of the first lines of defence against viral infections and also play a role in certain bacterial infections. When NK cells recognise infected cells, they release a mix of cytokines including IFN- γ , which has the ability to confer resistance to viral infections in the target cells. NK cells are activated in response to cytokines produced by other innate immune cells during early infection.

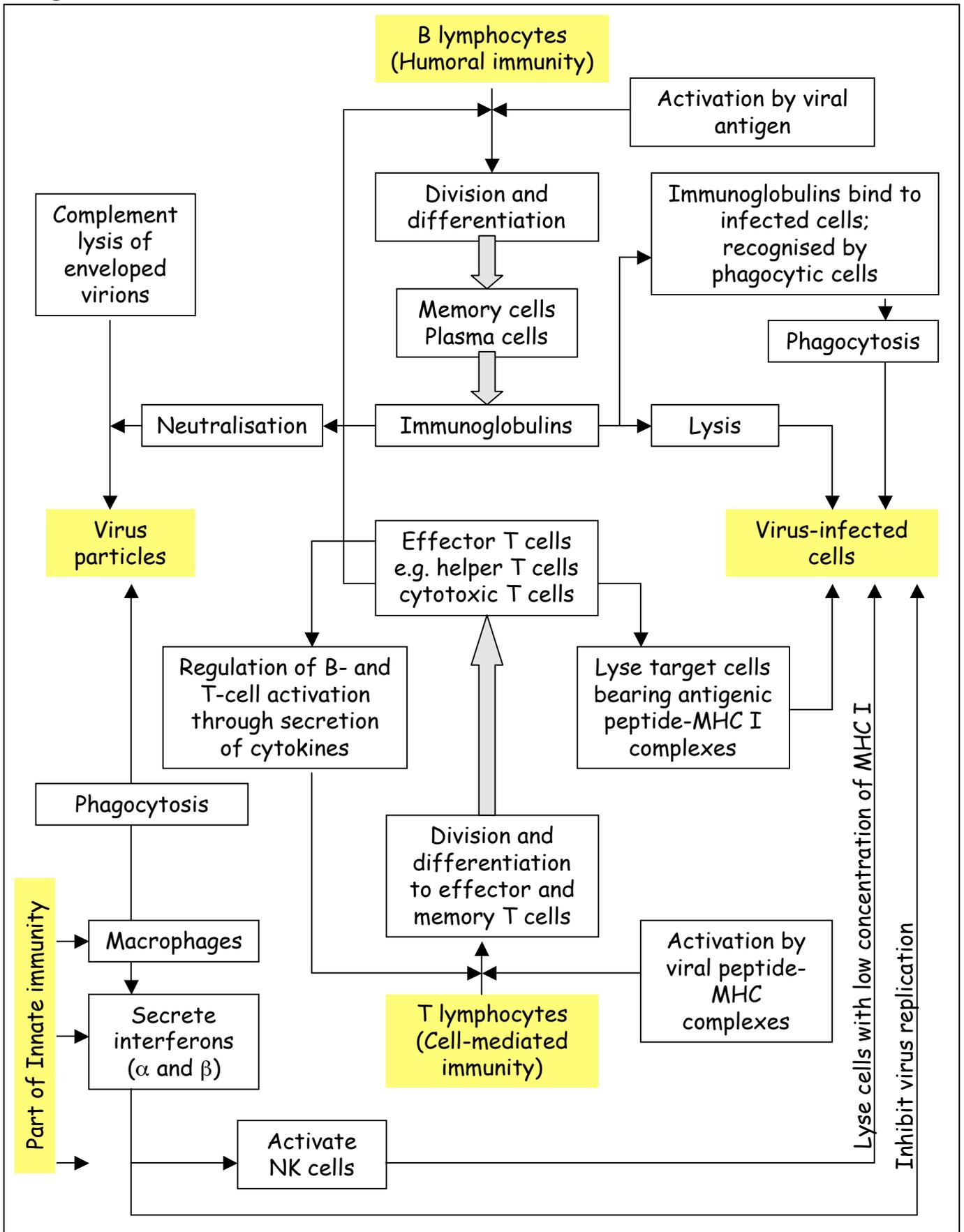
Cytokines are small proteins secreted by cells, particularly those of the immune system. They are the main regulators of immune function, ensuring that immune responses occur only under appropriate conditions, that they last for the appropriate length and have the appropriate magnitude. Cytokines influence the production, growth or differentiation of other cells, including white blood cells. Furthermore, they trigger several signalling pathways in infected cells or by engaging receptors on adjacent uninfected cells and on different effector cells of the immune system. Cytokines are not only important for innate but also for adaptive immunity. An example of a group of cytokines vital in innate immunity is IFN. IFNs mediate many innate defences against viruses, although IFN- γ also participates in adaptive immunity. The IFN system will be discussed in more detail in section 1.3.

Figure 1.3 (adapted from Dimmock *et al.*, 2001) summarises some of the responses of the immune system to viruses and virus-infected cells.

Figure 1.3 Representation of some of the responses of the immune system to viruses and virus-infected cells (adapted from Dimmock *et al.*, 2001).

The immune system is comprised of two main parts: the innate immune system and the adaptive immune system. B lymphocytes (humoral immunity) and T lymphocytes (cell-mediated immunity) are the main effector cells of the adaptive immune system. The main effector cells of the innate immune system are phagocytic cells (such as macrophages) and NK cells. Cytokines such as IFN also play a vital role in innate immunity. The mechanisms of the innate and adaptive immune systems act in an interlocking and orchestrated fashion. The essential parts of the complex action of the immune system against virus infection are summarised in this figure. There are two targets: virus particles and virus-infected cells. MHC, major histocompatibility complex; NK, natural killer.

Figure 1.3



adapted from Dimmock et al., 2001

1.3 Defence against virus: the Interferon System

Among the host innate immune mechanisms involved in antimicrobial defence, the interferon (IFN) system constitutes a powerful response dedicated to fighting viral infections. IFNs were discovered half a century ago and since then have been subject of intensive research. The history of IFN started in 1957 when Isaacs and Lindenmann described a cytokine secreted from chick cells that had been in contact with heat-treated influenza virus. They verified that this cytokine interfered with viral replication, hence the name interferon (Isaacs & Lindenmann, 1957). Fifty years after its discovery, enormous progress has been made in understanding the IFN system and the way it functions, but many questions remain unanswered.

1.3.1 Interferons are multifunctional

Viruses can not establish infections *in vivo* without having to replicate in the face of complex and powerful immune defence mechanisms. The IFN system is one of the first barriers viruses have to overcome (reviewed in Haller *et al.*, 2006, Weber *et al.*, 2004). IFNs are a large family of multifunctional cytokines and as mentioned earlier, one of the major components of innate immunity. The mechanisms involved in this response are triggered soon after viral infection and effectively limit viral spread, buying time for the specific adaptive immune responses to kick in. IFNs have the unique ability to establish an antiviral state in both infected and neighbouring uninfected cells through the activation of intracellular signalling cascades that lead to expression of many IFN-stimulated genes (ISGs). This induction of enzymes that interfere with cellular and viral processes either limits or completely abolishes viral replication, making it difficult for the virus to spread. The IFN system also plays major roles in cell growth regulation and activation of cells of the adaptive system (reviewed in Goodbourn *et al.*, 2000, Haller *et al.*, 2006). IFNs are capable of inhibiting cell growth by up- or down-regulating gene products directly involved in cell cycle regulation. They are also involved in the control of apoptosis (programmed cell death), inducing cells to become apoptotic whenever infected by a

virus. Finally, IFNs have many immunomodulatory roles, stimulating both innate and adaptive immune responses. They are involved in promoting and regulating T and B cell responses, and also enhancing the proliferation and cytotoxicity of NK cells. They serve as both positive and negative regulatory controls in the expression of innate and adaptive immune responses. One very important effect is the up-regulation of expression of MHC class I molecules by IFN- α and IFN- β , and of both MHC class I and II molecules by IFN- γ (Dimmock *et al.*, 2001).

1.3.2 Classes of interferons

IFNs can be divided into two main categories: type I and type II.

Type I IFNs are produced in direct response to viral infection and consist of many structurally-related members. Members of this family that are existent in humans are IFN- α , IFN- β , IFN- ϵ , IFN- κ and IFN- ω (Pestka *et al.*, 2004, Plataniias, 2005). There are at least 13 different IFN- α genes, whilst there is only one IFN- β gene product (Katze *et al.*, 2002, Plataniias, 2005). Type I IFNs all bind to the same cell surface receptor termed type I IFN receptor. The best-characterized type I IFNs are IFN- α and IFN- β , and most cell types can produce both. It is mostly leukocytes that synthesize the products of the IFN- α multigene family, while the product of the IFN- β gene is particularly synthesized by fibroblasts (Stark *et al.*, 1998, Goodbourn *et al.*, 2000). Several studies have demonstrated that the type I IFN system is essential for vertebrates to control viral infections. Knockout mice which are unresponsive to IFN- α/β due to targeted deletions in the type I IFN receptor, or which lack the IFN- β gene altogether are extremely sensitive to viral infections despite having a normal adaptive immune system (Deonarain *et al.*, 2000, Muller *et al.*, 1994).

Type II IFN consists of a single protein, the product of the IFN- γ gene. IFN- γ does not have a marked structural homology to type I IFNs, but it was still classified in the IFN family because of its ability to interfere with viruses. Additionally IFN- γ binds to a different cell-surface receptor than type I IFNs called the type II IFN receptor. As

opposed to type I IFNs that are directly induced by viral infection, type II IFN is produced in response to the recognition of infected cells by certain cells of the immune system: NK cells, TH1 cells and CTL cells (reviewed in Katze *et al.*, 2002). The main role of IFN- γ is immune regulation. IFN- γ has multiple direct antiviral effects: it stimulates the cytotoxicity of T cells and macrophages, influences naïve T cells to differentiate into TH1 cells and it induces increased class I and class II MHC expression on a wide variety of cell types.

Although the two types of IFN are structurally different, the types of genes that the two systems induce overlap. Moreover, when it comes to the signalling systems and the effectors used, there is an analogy between the two families. However, the two systems are non-redundant in many cases, allowing the IFN system to mount efficient responses to many different and specific virus infections.

Novel IFNs called IFN- λ 1, IFN- λ 2 and IFN- λ 3 (or IL-28A, IL-28B and IL-29, respectively) have recently been identified (Kotenko *et al.*, 2003, Sheppard *et al.*, 2003). This new family, sometimes termed type III IFNs, is structurally related to type I IFNs, but signals via its own receptor. The synthesis of type III IFNs is induced by virus infection or double-stranded RNA (dsRNA); they render cells resistant to virus infection and they activate the same signalling pathways as type I IFNs, so they are also functionally similar to type I IFNs. Similarly to the other classes of IFNs, they have been shown to induce antiviral enzymes and also enhance MHC class I antigen expression. More recent studies that compare the antiviral activity of IFN- λ to other IFNs in a range of human cell lines have shown that IFN- λ is weaker in inducing antiviral activity, and does so in fewer cell lines than other IFNs (Meager *et al.*, 2005).

1.3.3 Induction of interferon genes

As mentioned earlier, type I IFNs are induced generally as a part of innate immunity, while type II IFNs can be induced by mechanisms of both innate and adaptive immunity. Most cells of the body, including fibroblasts and conventional dendritic cells (cDCs),

produce type I IFNs, but they may differ with regard to the subtypes they predominantly synthesise. When infected by a virus, fibroblasts produce mainly IFN- β . However, during the subsequent amplification of the IFN response, they switch to the production of IFN- α . Plasmacytoid dendritic cells (pDCs), a specialised subset of dendritic cells also called natural IFN-producing cells, predominantly secrete high levels of IFN- α .

Type II IFN or IFN- γ is produced by NK cells when they recognize infected cells (innate immunity) and also by TH1 and CTLs after exposure to APCs (adaptive immunity).

1.3.3.1 Induction of type I interferons

Recently, exciting progress has been made toward elucidating the biochemical pathways by which the host recognises invading viral pathogens and triggers the production of type I IFNs.

Specialised receptors which recognise conserved molecular patterns characteristic of microorganisms trigger the induction of type I IFNs. These pattern recognition receptors are divided into two categories: Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) (reviewed in Samuel, 2007, Uematsu & Akira, 2007, Yoneyama & Fujita, 2007). RLRs are localised in the cytoplasm and recognise dsRNA produced upon viral infection and also uncapped single stranded RNA (ssRNA) bearing 5' triphosphate groups (Hornung *et al.*, 2006). TLRs are located on cell surfaces or in endosomes where they detect viral components or viral nucleic acid; some known ligands include dsRNA (recognised by TLR3, Alexopoulou *et al.*, 2001) and ssRNA (recognised by TLRs 7 and 8, Diebold *et al.*, 2004, Heil *et al.*, 2004) that are commonly found in virus infected cells, and unmethylated CpG dinucleotides (recognised by TLR9) found in bacterial and most viral DNA genomes (Bauer *et al.*, 2001, Hemmi *et al.*, 2000). CpG dinucleotides (CpG refers to a C nucleotide followed by a G nucleotide, with a phosphate group linking the two bases) are also found in cellular DNA, but in a methylated form; this allows the cell to distinguish between self (methylated CpG) and non-self (unmethylated CpG).

The RLR and TLR signalling pathways ultimately lead to the activation of transcription factors such as IFN regulatory factor (IRF) family members (IRF-3 and IRF-7) and nuclear factor κ B (NF- κ B) which are essential in the induction of type I IFNs. These transcription factors all bind to a relatively short regulatory element in the IFN- β

promoter, cooperating for maximal promoter activity and activate the induction of the IFN- β gene and some of the IFN- α genes. This early IFN triggers expression the up-regulation of IRF-7, which in turns controls transcription of many additional members of the IFN- α family. Figure 1.4 (adapted from Samuel, 2007) summarises aspects of the RLR and TLR signalling pathways leading to the induction of type I IFN expression in response to viral nucleic acids.

Signalling pathways activated by dsRNA

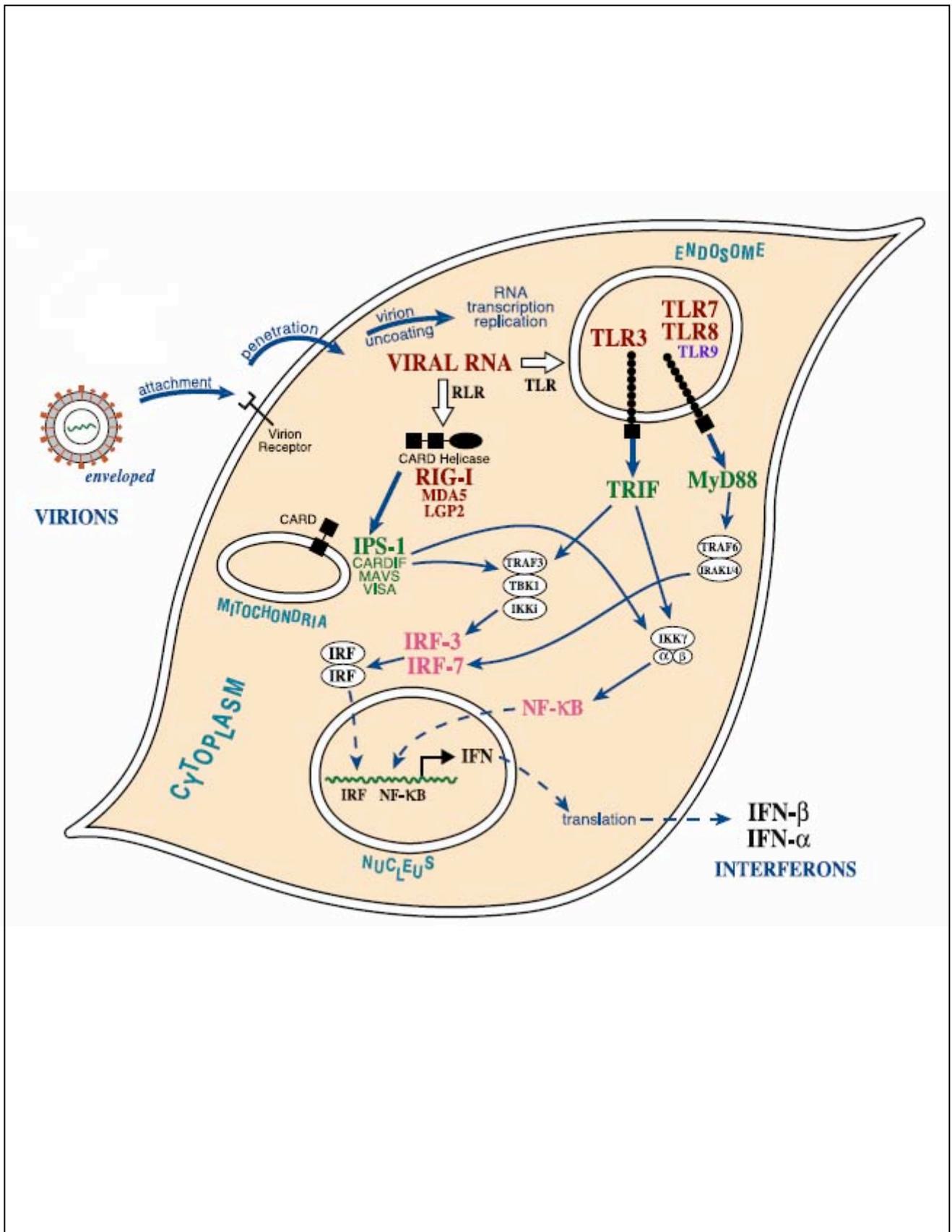
dsRNA is not naturally found in the intracellular environment; it may be provided by the viral genome or it may be formed as an intermediate of viral transcription during the infection process. Recognition of dsRNA by different cellular sensors leads to the activation of the different transcription factors that play central roles in the induction of type I IFNs (Levy, 2005).

The viral dsRNA can be detected by RIG-I-like receptors (RLRs). RLRs are intracellular RNA helicases that contain two repeats of the caspase recruitment domain (CARD)-like motif at their N terminus. Two proteins have been identified as RLRs: MDA5 (melanoma differentiation-associated gene 5; Andrejeva *et al.*, 2004) and RIG-I (retinoic acid-inducible gene I; Yoneyama *et al.*, 2004). Recent studies show that these two proteins, despite sharing overlapping roles, seem to function in parallel and are apparently non-redundant, having a degree of virus specificity (Yoneyama *et al.*, 2005). RIG-I exhibits strong dsRNA binding activity in vitro (Yoneyama & Fujita, 2007), which suggests that RIG-I is a specific sensor for dsRNA. In a recent study, it was also demonstrated that RIG-I is activated by viral genomic ssRNA bearing 5' phosphates in cells infected with Influenza A virus (Pichlmair *et al.*, 2006); more specifically, RIG-I is activated by uncapped 5'triphosphate ssRNA (Hornung *et al.*, 2006). Once activated by dsRNA (or ssRNA in the case of RIG-I), RLRs interact with a downstream molecule termed mitochondrial membrane-associated interferon promoter stimulator 1 (IPS-1, Kawai *et al.*, 2005, also known as MAVS, Seth *et al.*, 2005, VISA, Xu *et al.*, 2005 or Cardif, Meylan *et al.*, 2005) via their CARD domains to relay the signal. The signal is branched at IPS-1: IPS-1 coordinates the activation of TBK1/IKK ϵ kinases, leading to IRF-3/IRF-7

Figure 1.4 Representation of the type I IFN induction pathways (adapted from Samuel, 2007).

There are two main receptor types in cells that can recognise viral nucleic acids: RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs). RLRs are intracellular receptors containing CARD domains that are activated by binding to dsRNA (and ssRNA in the case of RIG-I) and that signal via an adaptor molecule (IPS-1, MAVS, VISA or Cardif) to induce the activation and nuclear translocation of IRF3 and NF- κ B. TLRs include TLR3 which is activated by binding to dsRNA, TLR7 and 8, which are activated by binding to ssRNA and TLR9 which is activated by binding to unmethylated CpG dinucleotides. TLR3 signals via an adaptor molecule termed TRIF which leads to the activation of IRF3 and NF- κ B. TLR7, 8 and 9 signal via another adaptor molecule called MyD88 that leads to the activation and nuclear translocation of IRF7. IRF3, IRF7 and NF- κ B are key transcription factors that bind to the promoters of type I IFN genes thereby leading to their expression.

Figure 1.4



adapted from Samuel, 2007

activation and consequently to IFN- β production. When kinases TBK1 and IKK ϵ are activated, they mediate phosphorylation of IRF-3 (Fitzgerald *et al.*, 2003, Sharma *et al.*, 2003). Activated or phosphorylated IRF-3 homodimerises and translocates to the nucleus, where it recruits the transcriptional coactivators p300 and CREB-binding protein (CBP), resulting in expression of IFN- β (Hiscott *et al.*, 1999, reviewed in Hiscott, 2007). IPS-1 also coordinates the activation of IKK α /IKK β /IKK γ kinases, leading to NF- κ B activation. In uninfected cells, NF- κ B is found in the cytoplasm associated with an inhibitor molecule termed I κ B. The activation of kinases IKK α , IKK β and IKK γ mediate the phosphorylation of I κ B; I κ B is therefore degraded, thereby releasing NF- κ B, which can then enter the nucleus and bind to the IFN- β promoter (reviewed in Israel, 2000). NF- κ B is not only important in activation of IFN- β ; it plays a key role in the transcriptional induction of many immunomodulatory genes, including other cytokines, MHC class I molecules and cell adhesion molecules (Baldwin, 1996).

Viral dsRNA can also be detected by TLRs, specifically TLR3. TLRs are type I transmembrane receptors that have an extracellular region and a cytoplasmic tail, which contains a toll/interleukin-1 receptor (TIR) domain. The TIR domain is the origin of the TLR-signalling pathways. TLR3 is located on the cell surface of fibroblasts, but it localises to endosomes in cDCs. TLR3 signalling activates the transcription factors IRF-3 and NF- κ B via the adaptor molecule TRIF (Toll/interleukin 1 receptor domain-containing adaptor protein inducing IFN- β). TRIF interacts with the two kinases TBK1 and IKK ϵ , which mediate activation of IRF-3 as previously explained. TRIF also mediates the activation and nuclear translocation of NF- κ B by activating the IKK α , IKK β and IKK γ kinases, as previously explained.

Signalling pathways activated by ssRNA or unmethylated CpG dinucleotides

IRF7 is necessary for the expression of IFN- α genes. In pDCs, also known as natural IFN-producing cells, IRF7 is constitutively expressed, allowing a rapid and comprehensive IFN- α response to danger signals (Kerkmann *et al.*, 2003, Prakash *et al.*, 2005). pDCs express TLRs in endosomes and thereby can sense the presence of RNA or DNA viruses in this compartment (Bowie & Haga, 2005). They mostly express TLRs

which can recognize viral ssRNA (TLRs 7 and 8) or unmethylated CpG dinucleotides present in viral DNA (TLR 9, Iwasaki & Medzhitov, 2004). After activation, TLR 7, 8 and 9 signal through their adaptor molecule MyD88 (myeloid differentiation primary response protein 88) which in turn forms a complex with TRAF6 and IRF7 (Kawai *et al.*, 2004). Upon phosphorylation of IRF7 by IRAK-1, an additional component of this receptor-associated multiprotein complex, multiple IFN- α genes are activated (Iwasaki & Medzhitov, 2004). Figure 1.5 (from Haller *et al.*, 2006) is a schematic representation of the TLR pathways in pDCs.

IRF7 is further upregulated in response to IFN which generates a positive feedback loop for high IFN- α and IFN- β production; in cells other than pDCs, IRF7 is only induced and expressed in response to IFN and consequently it takes longer for these cells to express IFN- α . Because of their enormous capacity to produce IFN- α , pDCs are regarded as the main sentinels for triggering a general response to viruses and represent a key interface between the innate and adaptive arms of the immune system (Colonna *et al.*, 2004).

1.3.3.2 Induction of type II interferons

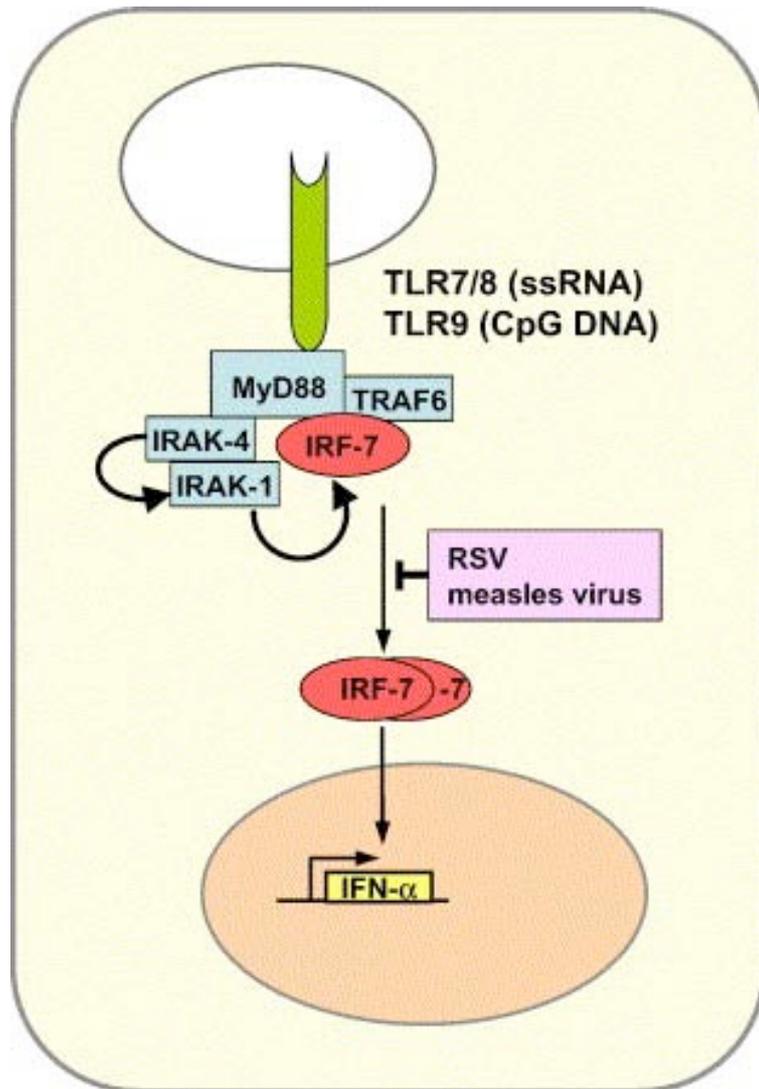
IFN- γ can be induced by effector cells of both innate and adaptive immunity. Type II IFN or IFN- γ is produced by NK cells when they recognize infected cells (innate immunity) and also by TH1 and CTLs after exposure to APCs (adaptive immunity). Two cytokines, IL-12 and IL-18 often play central roles in the induction of IFN- γ in both NK cells and T cells. Activated NK cells can produce IFN- γ when stimulated by IL-18 and APC-produced IL-12 (Singh *et al.*, 2000). Many of the NK cell-mediated antiviral effects depend on their being induced to make IFN- γ . IFN- γ is also produced by cells that take part in adaptive immunity, such as TH cells, when these are exposed to APCs (Young, 1996).

1.3.4 Interferon signal transduction

Figure 1.5 Representation of the TLR signalling pathway for IFN- α production in plasmacytoid dendritic cells (from Haller *et al.*, 2006).

TLR7 and TLR8 recognize ssRNA in endosomes, and thus respond to RNA viruses while TLR9 responds to DNA viruses by recognising unmethylated CpG dinucleotides. TLRs transmit their signals through an associated multiprotein complex containing the adaptor protein MyD88 and the transcription factor IRF7. IRF7 is constitutively expressed by pDCs (Kerkmann *et al.*, 2003, Prakash *et al.*, 2005). Some viruses, such as RSV and MeV, are capable of blocking IFN production in pDCs cells by an as yet unknown mechanism (Schlender *et al.*, 2005).

Figure 1.5



from Haller et al., 2006

IFNs interact with type-specific receptors to mediate their effects. These receptors are the starting point for complex signalling pathways. The result of these pathways is the induction and transcription of many IFN-stimulated genes (ISGs) that are normally expressed at low levels.

The classical pathway for IFN signalling is called the JAK-STAT signalling cascade and it involves molecules such as janus tyrosine kinases (JAKs) and signal transducers and activators of transcription (STATs) (shown in figure 1.6; from Plataniias, 2005).

Recent work in this area has demonstrated that alternative pathways also have important roles in IFN-mediated signalling (briefly discussed in section 1.3.4.2).

1.3.4.1 JAK-STAT pathways

The JAK-STAT pathways have been extensively studied (reviewed in Goodbourn *et al.*, 2000, Levy & Garcia-Sastre, 2001, Plataniias, 2005) and have been shown to be functionally relevant in the IFN system by many experimental approaches. Both IFN- α/β and IFN- γ signal via the JAK-STAT pathways.

Interferon α/β signalling

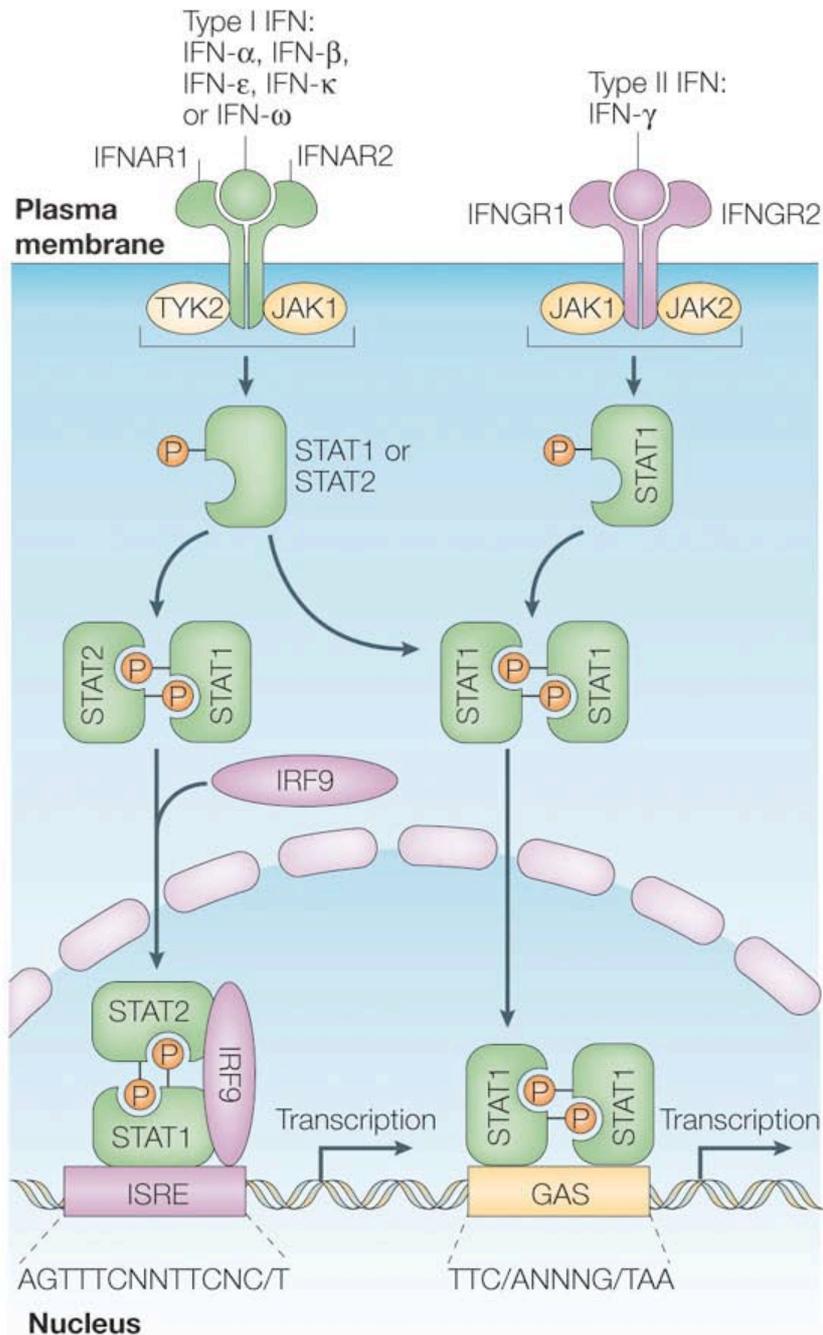
The type I IFN receptor has a multichain structure and is composed of at least two distinct subunits, IFNAR1 and IFNAR2 (reviewed in Mogensen *et al.*, 1999). Each of these receptors is constitutively associated by members of the JAK family: tyrosine kinase 2 (TYK2) interacts with IFNAR1 and JAK1 with IFNAR2. Binding of IFN to the receptors leads to autophosphorylation and activation of the associated JAKs, which in turn phosphorylate STAT1 and STAT2 on specific tyrosine residues. STAT1 and STAT2 heterodimerize and form a complex with IRF9. This heterotrimeric complex is termed ISGF3. This complex translocates to the nucleus and binds to IFN-stimulated response elements (ISRE) contained in the upstream regulatory sequences of most IFN- α/β inducible genes, thereby initiating their transcription (reviewed in Levy & Garcia-Sastre, 2001, Goodbourn *et al.*, 2000, Plataniias, 2005).

Interferon γ signalling

Figure 1.6 Representation of the IFN signalling pathway, known as the JAK-STAT pathway (from Plataniias, 2005).

Type I and type II IFNs bind to their cognate receptors on the surface of cells and activate the expression of numerous ISGs via the JAK-STAT pathway. Type I IFN signalling involves the activation and heterodimerisation of STAT 1 and STAT2 molecules. These heterodimers form a complex with IRF9 and translocate to the nucleus, activating any upstream regulatory regions of ISGs containing the ISRE sequence. Type II IFN signalling involves the activation and homodimerisation of STAT1. STAT1 homodimers translocate to the nucleus and activate any upstream regulatory regions of ISGs containing the GAS element.

Figure 1.6



from Plataniias, 2005

Type II IFN signalling follows a similar, but distinct, pattern. The cell-surface receptors are composed of at least two major subunits, IFNGR1 and IFNGR2 (reviewed in Bach *et al.*, 1997). In this case, IFNGR1 is associated with JAK1 and IFNGR2 is associated with JAK2. After activation by IFN- γ , JAK1 and JAK2 phosphorylate and activate STAT1 molecules, which in turn homodimerize and translocate to the nucleus. STAT1/STAT1 homodimers are also known as gamma-activated factor (GAF) and bind to the gamma-activated sites (GAS) elements present in upstream regulatory regions of some ISGs (reviewed in Goodbourn *et al.*, 2000). IFN- γ cannot induce the transcription of genes that only contain ISRE elements in their promoter.

Other important events

The tyrosine phosphorylation of STAT molecules is an essential step in IFN signalling. However, several other events involving STAT molecules are required for optimal IFN-regulated gene transcription. An essential factor for full transcriptional activation of ISGs is the phosphorylation of STAT1 on the serine residue at position 727, although this is not essential for translocation to the nucleus or binding to the promoters of ISGs. Additionally, when STAT molecules are in the nucleus, they interact with several co-activator proteins, such as p300 and CREB, which have important roles in the regulation of transcription (reviewed in Plataniias, 2005).

It is also important to refer to the attenuation of IFN signalling as a means to control the response to IFN and prevent ISG expression in the absence of IFN. Both types of IFN can induce IRF1, which can then serve to sustain expression of genes that contain ISREs. IFN signalling is generally down-regulated by the actions of IFN-induced IRF2 and IRF8 which are known to compete for binding to the ISRE regions and negatively regulate gene expression. Furthermore, other IFN-induced proteins can act in a negative feedback loop, inhibiting further expression of IFN-induced genes. Examples are the suppressor of cytokine signalling (SOCS) proteins which prevent STAT1 activation (Kubo *et al.*, 2003), and the protein inhibitor of activated STAT (PIAS) family members which inhibit the transcriptional activity of STATs (Shuai & Liu, 2005).

1.3.4.2 Alternative pathways

IFNs are associated with a number of biological activities and functions. It is thus easy to imagine not one unique signalling pathway, but multiple distinct pathways leading to the varied responses to IFN. The JAK-STAT pathway was the first to be discovered, but since then, there has been evidence that other pathways are also required for the generation of the plethora of responses to IFN. Some of the pathways incorporate STAT molecules, while others seem independent of the JAK-STAT pathway. These include the mitogen-activated protein kinase (MAPK) p38 and the phosphatidylinositol 3-kinase cascades. The MAPK pathways have a role in IFN signalling by phosphorylating serine residues of STAT1 and STAT3, and further enhancing their transcriptional activity. Apparently, both the JAK-STAT pathway and the p38 signalling pathway have to be activated to elicit antiviral responses (Platanias, 2003). Platanias has recently published an extensive review on the different pathways of IFN signalling (reviewed in Platanias, 2005).

1.3.5 Antiviral response induced by interferon

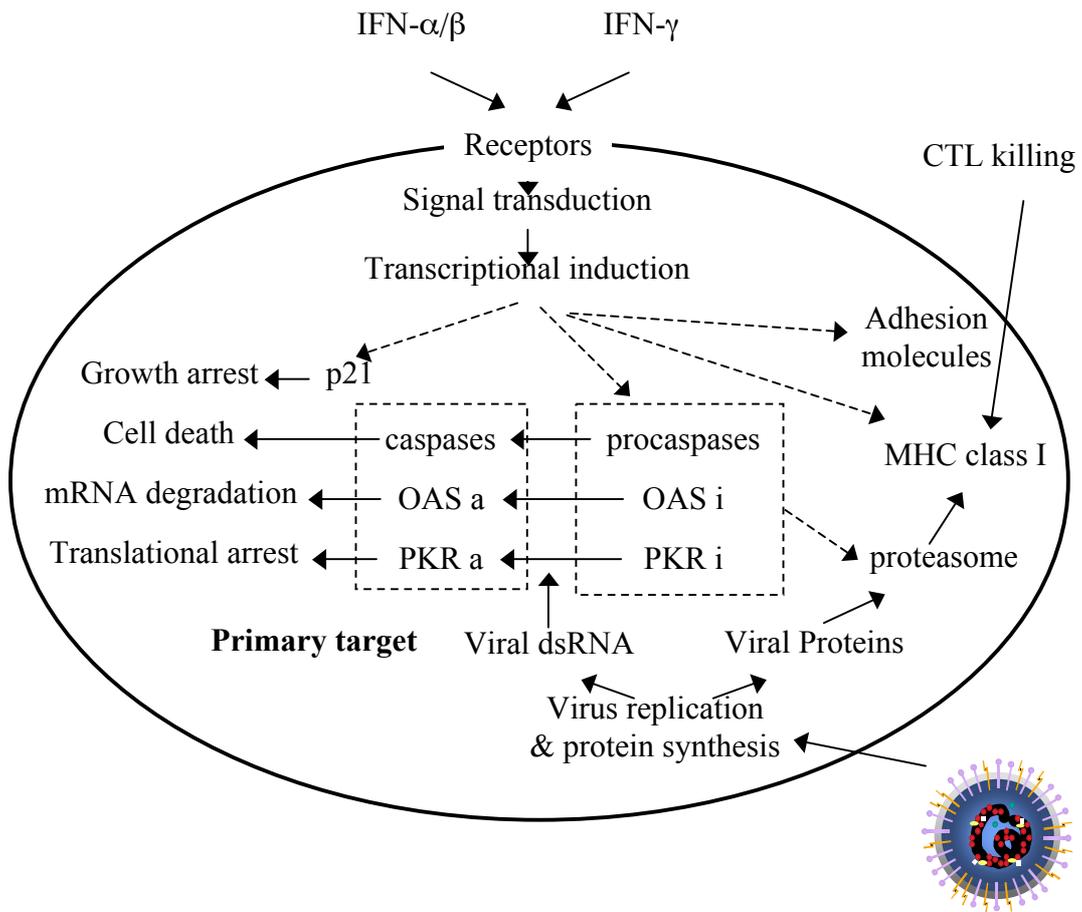
The fundamental property that allowed the discovery of IFNs is their ability to confer an antiviral state to cells. IFNs induce the expression of ISGs that will not only induce an antiviral state in infected cells, but also in neighbouring uninfected cells and cells of the adaptive immune system (figure 1.7; adapted from Goodbourn *et al.*, 2000). Before the development of the DNA microarray technique, it was thought that there were perhaps 30-40 ISGs. Nowadays, it is known that there are hundreds of IFN-regulated genes, many of which are repressed or down regulated by IFN. Type I IFNs alone activate the expression of more than 300 genes which have antiviral, antiproliferative and immunomodulatory functions (de Veer *et al.*, 2001, Der *et al.*, 1998).

Several IFN-induced antiviral pathways have evolved to fight different types of viruses. The best-characterized components of these pathways are the dsRNA-dependent protein kinase R (PKR), the 2'-5' oligoadenylate synthetases (OAS) and the myxovirus-resistance (Mx) protein(s). Other IFN-induced proteins have unknown roles, although it is thought that they will all function collectively to dampen down the activity of the host enzymatic machinery that viruses use to replicate.

Figure 1.7 Representation of the biological properties of IFN- α/β and IFN- γ (adapted from Goodbourn *et al.*, 2000).

When IFN molecules bind to their specific cell surface receptors they trigger the induction of many ISGs. In conjunction, the products of these ISGs establish an antiviral state in the cell. Among other products, PKR and 2-5 OAS are synthesised as inactive precursors (PKR_i and 2-5 OAS_i) and are further activated by viral dsRNA (PKR_a and 2-5 OAS_a). Activated PKR has the ability to shut down protein translation, while 2-5 OAS up-regulates mRNA degradation. IFNs also induce antiviral products that are involved in apoptosis, cell cycle arrest and the up-regulation of innate immune responses. In addition, IFNs induce the synthesis of proteins that are involved in the processing and presentation of virus proteins to cytotoxic T cells.

Figure 1.7



adapted from Goodbourn et al., 2000

1.3.5.1 dsRNA-dependent protein kinase R (PKR)

PKR is a serine-threonine kinase, which is normally present in cells in an inactive form. It is activated on binding to dsRNA (or other polyanions) or the cellular protein PACT (PKR-activating protein) which should be the most relevant activator in uninfected cells (George *et al.*, 1996, Katze *et al.*, 1991, Meurs *et al.*, 1992; reviewed in Sarkar *et al.*, 2005). Upon activation, PKR dimerizes and undergoes autophosphorylation. Once activated, this cellular protein plays multiple roles in control of transcription and translation in the cell. One of these roles is the phosphorylation of the eukaryotic translation initiation factor eIF2 α -subunit, which leads to its inactivation. This in turn leads to rapid inhibition of translation of cellular and viral mRNAs, thus preventing infected cells from producing viral proteins and creating infectious viral particles (reviewed in Clemens & Elia, 1997). PKR is also involved in signal transduction pathways in response to dsRNA and other ligands (reviewed in Williams, 1999). For example, PKR is involved in the induction of NF- κ B, which ultimately leads to induction of cytokines such as IFN- β and also MHC class I molecules. PKR may also exert its antiviral activity through the induction of apoptosis (reviewed in Goodbourn *et al.*, 2000) and it is thought to be involved in a large number of other cellular processes, such as differentiation, cell growth, and oncogenic transformation.

Despite being a central molecule in antiviral defence, PKR alone is not sufficient to mediate the full antiviral response.

1.3.5.2 The 2'-5' oligoadenylate synthetase (OAS)/ endoribonuclease L (RNase L) system

2'-5' oligoadenylate synthetase (OAS) proteins are IFN-inducible enzymes that are capable of synthesizing small 2'-5' linked oligomers of adenosine (2'-5'A) in the presence of dsRNA and ATP. These 2'-5'A oligomers bind and activate endoribonuclease L (RNase L) by mediating its dimerisation. RNase L is very important in cellular antiviral responses as it blocks protein synthesis by cleaving RNA, including mRNA and 28S ribosomal RNA (Iordanov *et al.*, 2000). Because 2'-5'A oligomers are

very labile, active RNase L is only found in close proximity to dsRNA, thus ensuring that viral RNA is degraded preferentially over cellular mRNAs (Nilsen & Baglioni, 1979). Active RNase L is also thought to be involved in the apoptosis of virally infected cells.

1.3.5.3 Myxovirus-resistance (Mx) proteins

Mx proteins are highly conserved, large dynamin-like GTPases, which have antiviral activity. They are induced upon virus infection in response to IFN and are capable of inhibiting the proliferation of a wide range of RNA viruses. In contrast to other ISGs, the Mx proteins are not constitutively expressed in cells, nor are they induced directly by viral infection or the presence of dsRNA. Mx proteins are only expressed when stimulated by IFN- α/β via the JAK-STAT signalling pathway, thus Mx has been considered as a marker for IFN action in clinical settings (Antonelli *et al.*, 1999, Deisenhammer *et al.*, 2004, Roers *et al.*, 1994). These proteins are found in all vertebrates, including mammals, birds and fish (reviewed in Staeheli *et al.*, 1993). Different Mx proteins have been shown to inhibit the replication of different RNA virus families (reviewed in Goodbourn *et al.*, 2000). As an example, the cytoplasmic human MxA protein interferes with RNA viruses that replicate in the cytoplasm, including members of the *Paramyxoviridae* family of viruses (Schneider-Schaulies *et al.*, 1994, Schnorr *et al.*, 1993, Zhao *et al.*, 1996). The mechanisms by which the Mx proteins inhibit proliferation of RNA viruses are not completely understood, but it has been proposed that Mx proteins might prevent trafficking or activity of virus polymerases (Stranden *et al.*, 1993).

1.3.5.4 Other antiviral pathways

From experiments on transgenic mice deficient in RNase L, PKR and Mx1, Zhou and colleagues (Zhou *et al.*, 1999) have shown that there must clearly be other pathways that are activated in response to IFNs. Additional proteins with potentially important antiviral activities are ISG20, p56, RNA-specific adenosine deaminase 1 (ADAR 1) and guanylate-binding protein 1 (GBP1). ISG20 is a protein with exonuclease activity that specifically degrades ssRNA and is likely to degrade viral RNA (Espert *et al.*, 2003). p56 is a product of the ISG56 gene that has been shown to inhibit initiation of translation by

interacting with a subunit of the eukaryotic translation initiation factor 3 (Guo *et al.*, 2000). ADAR1 is a dsRNA-editing enzyme, which is known to have a direct mutagenic effect on dsRNA (reviewed in Goodbourn *et al.*, 2000). GBP1 belongs to the dynamin superfamily of large GTPases like Mx and is induced predominantly by IFN- γ (Anderson *et al.*, 1999).

1.3.5.5 Antiproliferative action of interferon

IFNs can inhibit the propagation of certain viruses by slowing down cell growth and arresting cellular processes that are necessary for virus replication. This effect has been extensively studied because of its clinical importance, and it has been shown that it is very cell-type dependent. Experimental data has suggested that both PKR and RNase L are involved in the antiproliferative actions of IFNs. Additionally, IFNs up-regulate the levels of the cyclin-dependent kinase inhibitor p21, which results in a block to the cell cycle (reviewed in Goodbourn *et al.*, 2000). Moreover, IFNs down-regulate the expression of c-myc, an essential product that is required to drive cell cycle progression (Ramana *et al.*, 2000). Finally, IFNs also up-regulate a potent repressor of the cell cycle called p202, which has been shown to impair cell proliferation by inhibiting the functions of many cellular factors, including NF- κ B, p53 and c-myc (Sarkar & Sen, 2004).

1.3.5.6 Control of apoptosis by interferon

One of the antiviral actions of IFN is to ensure that when a cell is infected by a virus, it is also triggered to undergo apoptosis (Tanaka *et al.*, 1998). Besides triggering apoptosis in infected cells, IFN also induces a pro-apoptotic state in neighbouring uninfected cells (reviewed in Schindler, 1998), thereby limiting the spread of the infection. As mentioned before, when induced by IFN, both PKR and RNase L play major roles in the apoptosis response. IFN is also capable of inducing the expression of several caspases (Balachandran *et al.*, 2000, Chin *et al.*, 1997, Subramaniam *et al.*, 1998), which are enzymes that are known to play central roles in the apoptotic process.

1.3.5.7 Effects of interferon on the innate and adaptive immune responses

IFNs have many immunomodulatory functions which are integrated into almost all stages of the innate and adaptive immune responses. IFN- γ in particular is a very potent immune regulator.

In innate immunity, one of the roles of type I IFNs is to stimulate NK cell proliferation and to enhance NK cytotoxicity, which is achieved through up-regulation of perforin levels (Kaser *et al.*, 1999, Mori *et al.*, 1998). As to IFN- γ , its major function in innate immunity is to aid in the activation of macrophages which in turn use IFN- γ -induced mechanisms to kill targets.

All IFN family members are also involved in adaptive immunity. All members are capable of enhancing the expression of MHC class I molecules and thereby promoting CTL cell responses (reviewed in Boehm *et al.*, 1997). In contrast, TH cell responses are only promoted by IFN- γ which is capable of inducing the expression of MHC class II molecules. IFNs also enhance immunogenicity by increasing the range and quantity of antigenic peptides displayed to CTL cells. Proteasomes are modified by IFN- γ (reviewed in York & Rock, 1996) in a way to enhance the generation of peptides that bind to class I MHC proteins. Another function of IFN- γ in antigen presentation is to increase the expression of proteins that are involved in the transfer of proteasome-generated peptides from the cytoplasm into the endoplasmic reticulum to bind nascent MHC class I proteins. IFN- γ is also involved in the humoral arm of adaptive immune responses. It helps in the regulation of the balance between TH1 and TH2 cells, by increasing the synthesis of IL-12 and decreasing the synthesis of IL-4, which tips the balance towards the production of TH1 cells. IFNs also have direct effects on B cells for they regulate their development and proliferation and immunoglobulin secretion. Moreover, IFN- α/β regulates the production of IL-15, which stimulates memory T cell division and it is believed that IFN- α/β itself directly supports the survival of activated T cells (reviewed in Goodbourn *et al.*, 2000).

1.4 Virus countermeasures to the interferon response

The IFN response is a very powerful innate immune mechanism, possessing antiviral, anti-proliferative and immunoregulatory properties. How is it then that viruses are capable of surviving in the presence of such a vigorous response? The answer is that viruses have evolved to down-regulate or even completely block the IFN response by several means. Most (if not all) virus genomes contain information to express at least one IFN antagonist protein. These IFN antagonists are often non-structural proteins which are not essential for virus growth; they are also generally multifunctional proteins that interact with multiple host cell components and mediate many distinct functions. These antagonists can act to inhibit IFN production, bind and inactivate secreted IFN molecules, block IFN signalling, and/or disturb the action of IFN-induced antiviral proteins. Figure 1.8 (from Haller *et al.*, 2006) represents the array of activities mediated by IFN antagonists of viruses from various families. A single viral protein may inhibit different stages of the IFN response and a given virus may target different pathways by displaying more than one antagonist protein (figure 1.8, reviewed in Haller *et al.*, 2006).

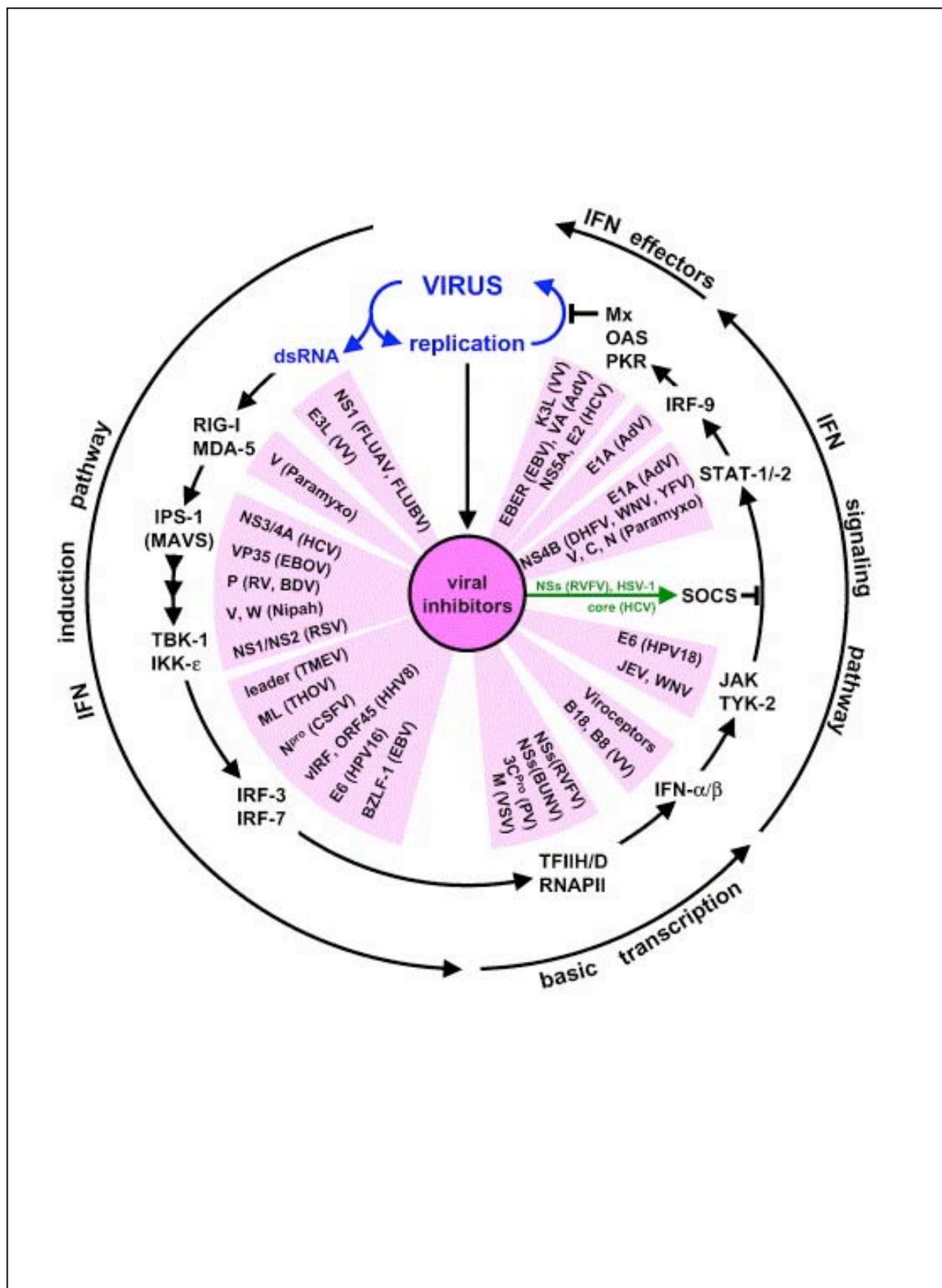
1.4.1 Viral shut-off of host cell protein synthesis

Many viruses with a lytic replication cycle have adopted the strategy to shut-off host gene transcription by affecting the basic cellular transcription or translation machinery. These viruses take over the protein synthesis machinery of the cell, resulting in a selective expression of viral proteins, whereas the host mRNA production or protein synthesis is down-regulated. This results in a higher availability of energy and cellular reagents to translate viral proteins. It also results in an inhibition of the IFN responses for this response depends on the translation of host factors. Examples of viruses that shut off host gene transcription are Poliovirus (PV, Yalamanchili *et al.*, 1996), Vesicular Stomatitis virus (VSV, Ahmed *et al.*, 2003, Yuan *et al.*, 1998) and some Bunyaviruses (BUNV, Billecocq *et al.*, 2004, Le May *et al.*, 2004, Thomas *et al.*, 2004, Weber *et al.*, 2002).

Figure 1.8 Representation of the range of activities mediated by an array of viral IFN antagonists (from Haller *et al.*, 2006).

Viral proteins have been discovered to cover the whole range of the IFN response in infected cells. Certain viruses may have more than one antagonist protein targeting different pathways. A single viral protein can target multiple components of the IFN induction and signalling cascade. The following viral IFN-antagonists are shown in clockwise order: NS1 of Influenza A virus, NS1 of Influenza B virus, E3L of VV (Vaccinia virus), V of Paramyxoviruses, NS3/4A of HCV, VP35 of Ebola virus, P of Rabies virus, P of Borna Disease virus, V and W of NiV, NS1/NS2 of RSV, leader protein of Theiler's Meningoencephalitis virus, ML of Thogoto Virus, Npro of Classical Swine Fever virus, vIRF and ORF45 of human Herpes virus 8, E6 of human Papilloma virus 16, BZLF-1 of Epstein-Barr virus, M of VSV, 3C^{pro} of PV, NSs of BUNV, NSs of Rift Valley Fever virus, B18 and B8 of VV, Japanese Encephalitis virus and West Nile virus, E6 of human Papilloma virus 18, core protein of HCV and Herpes Simplex virus (HSV)1, NSs of Rift Valley Fever virus, V, C and N of Paramyxoviruses, NS4B of Dengue Hemorrhagic Fever virus, West Nile virus and Yellow Fever virus, E1A of Adenovirus, NS5A and E2 of HCV, EBER of Epstein-Barr virus, VA of Adenovirus, K3L of VV.

Figure 1.8



from Haller et al, 2006

However, other viruses depend on a functional cellular environment and cannot afford to disturb the entire cellular metabolism. These viruses target specific components of the IFN system.

1.4.2 Viral interference with IFN induction processes

Some viruses interfere specifically with the mechanism of IFN induction. One of the key targets is dsRNA, for it is a trigger molecule that leads to expression of IFN genes. Some proteins expressed by viruses bind to and sequester dsRNA. These include the NS1 protein of most strains of Influenza A virus (Garcia-Sastre *et al.*, 1998, Li *et al.*, 2006, Talon *et al.*, 2000a), the NS1 protein of Influenza B virus (Dauber *et al.*, 2004, Wang & Krug, 1996) and the E3L proteins of Poxviruses (Xiang *et al.*, 2002), among others. Sequestering dsRNA prevents both the induction of IFN and the activation of dsRNA-dependent enzymes like PKR, OAS and ADAR and dsRNA-dependent apoptosis.

Another prominent target is IRF3, and proteins from many unrelated viruses inhibit its activation. Some proteins such as the VP35 protein of Ebola virus (Basler *et al.*, 2003) and the NS1/NS2 complex of RSV (Bossert *et al.*, 2003) prevent the initial phosphorylation of IRF3, thereby inhibiting its dimerisation and nuclear transport. In cells infected with bovine Viral Diarrhea virus (BVDV), the Npro protein blocks IRF3 from binding to DNA and targets IRF3 for degradation (Hilton *et al.*, 2006). More recently, it has been demonstrated that NS3/4A protease of HCV inhibits RIG-I signalling and also has the ability to disrupt TLR3 signalling by cleaving the TLR3 adaptor protein TRIF (Breiman *et al.*, 2005, Foy *et al.*, 2005, Kaukinen *et al.*, 2006, Li *et al.*, 2005), thereby inhibiting activation of IRF3. The immediate early protein ICP0 of Herpes Simplex virus (HSV) 1 can inhibit nuclear accumulation of IRF3 (Melroe *et al.*, 2004) while the ML protein of Thogoto virus interferes with IRF3 dimerisation and recruitment of the transcriptional coactivator CBP by IRF3 (Hagmaier *et al.*, 2004, Jennings *et al.*, 2005). Some viral proteins can also directly bind to and inactivate IRF3, such as the Rotavirus NSP1 protein (Graff *et al.*, 2002) and the human Papilloma virus 16 E6 protein (Ronco *et al.*, 1998). The V protein of PIV5 and other paramyxoviruses

prevents the action of MDA5 (but not RIG-I) by binding to it, thereby inhibiting its activation of the IFN- β promoter (Andrejeva *et al.*, 2004, Childs *et al.*, 2007). The NS1 protein of Influenza viruses inhibits the activity of RIG-I, but not MDA5, by binding to it (Hornung *et al.*, 2006, Mibayashi *et al.*, 2007, Pichlmair *et al.*, 2006). Weber and colleagues published a review containing an extensive list of viral antagonists that inhibit IFN induction (Weber *et al.*, 2004).

1.4.3 Viral interference with IFN signalling processes

Many viral antagonists interfere with the IFN signalling pathways to prevent ISG expression (reviewed in Weber *et al.*, 2004). This is not surprising because the IFN signalling pathways provide a positive feedback loop for enhanced IFN gene expression, meaning that viruses that block the IFN signalling pathways not only affect the establishment of an antiviral state in cells but also affect overall IFN production.

Several Poxviruses express soluble IFN-receptor homologues which compete with the cellular receptors for the binding of IFNs (Alcami *et al.*, 2000, Symons *et al.*, 1995). This prevents triggering of the IFN signalling cascades and also prevents the establishment of an antiviral state in non-infected cells surrounding the infected cells (reviewed in Goodbourn *et al.*, 2000 and Weber *et al.*, 2004).

Many viruses, including paramyxoviruses, inhibit IFN signal transduction by targeting cellular protein components of the JAK/STAT pathway (reviewed in Conzelmann, 2005, Horvath, 2004). For example, the V protein of PIV5 blocks IFN signalling by targeting STAT1 for proteasome-mediated degradation, thereby blocking type I and type II IFN signalling in infected cells (Didcock *et al.*, 1999b). The V protein of MuV targets both STAT1 and STAT3, and thereby also blocks type I and type II IFN signalling (Ulane *et al.*, 2003). However, the V protein of human Parainfluenza virus type 2 (hPIV2) only blocks type I IFN signalling because it targets only the STAT2 molecule (Young *et al.*, 2000). Infection of cells by MeV or Sendai virus (SeV) appears not to lead to STAT degradation. However, MeV C and V proteins are known to be involved in blocking the JAK/STAT signalling pathway (Palosaari *et al.*, 2003, Shaffer *et al.*, 2003), and the different C proteins of SeV block type I and type II IFN signalling by interfering with

STAT1 phosphorylation or stability (reviewed in Stock *et al.*, 2005 and Nagai & Kato, 2004). The V proteins of the newly emergent HeV and NiV subvert IFN responses by sequestering STAT1 and STAT2 into high-molecular-weight cytoplasmic complexes, which prevents STAT activation (Rodriguez *et al.*, 2002, Rodriguez *et al.*, 2003). Other virus families use comparable approaches to those used by paramyxoviruses. HCV for example, inhibits signal transduction through the JAK/STAT pathway for the core protein interacts with STAT1 to inhibit its phosphorylation and interaction with STAT2 (Heim *et al.*, 1999, Lin *et al.*, 2006, Francois *et al.*, 2000) and additionally, HCV has been reported to induce the normal cellular suppressor of cytokine signalling (SOCS-3) to down-regulate STAT and JAK phosphorylation (Bode *et al.*, 2003). The HCV core protein also interferes with IRF1 (Ciccaglione *et al.*, 2007). Another example of a multifunctional viral antagonist is the Adenovirus E1A protein, which targets STAT1 and IRF9, thus preventing formation of the ISGF3 complex (Leonard & Sen, 1996) and also sequesters the transcriptional coactivator CBP/p300 (Bhattacharya *et al.*, 1996). Other viruses use similar strategies to those just described.

1.4.4 Inhibition of IFN-induced antiviral proteins

Many viruses target the specific antiviral proteins that are induced by expression of IFNs and that mediate the antiviral state in cells (reviewed in Weber *et al.*, 2004).

Some of the effector proteins, such as PKR and OAS, depend on dsRNA to become activated, so viruses capable of sequestering dsRNA cannot only block IFN production (as described above) but also block the activation and activity of the effector proteins induced by IFN. An example is the NS1 protein of Influenza virus, which binds dsRNA and thereby inhibits the synthesis of IFN, and also inhibits dsRNA-mediated activation of PKR (Bergmann *et al.*, 2000, Lu *et al.*, 1995). Another example is the E3L protein of Vaccinia virus (VV), which by binding to dsRNA, prevents the activation of both PKR and OAS (Chang *et al.*, 1992). Additionally, E3L has been shown to interact directly with and inhibit PKR (Romano *et al.*, 1998, Sharp *et al.*, 1998). Likewise, other viruses also express proteins that either bind directly to or inactivate PKR. The E2 protein of HCV (Taylor *et al.*, 1999), the tat protein of HIV-1 (Brand *et al.*, 1997, Roy *et al.*, 1990) and

the K3L protein of VV (Davies *et al.*, 1992) act as substrates for PKR, which inhibit its kinase activity and block its inhibitory effect on protein synthesis and cell growth. Some viruses such as Adenovirus (Mathews & Shenk, 1991), HCV (Vyas *et al.*, 2003), Epstein-Barr virus (Elia *et al.*, 1996) and HIV-1 (Gunnery *et al.*, 1990) produce abundant short RNA molecules which compete with dsRNA for binding to PKR, thereby inhibiting PKR activation and activity (for a review on the viral inhibition of PKR, see Langland *et al.*, 2006).

Some viruses target the RNase L pathway to inhibit it, either by expressing dsRNA-binding proteins or by more direct ways. Viruses such as HIV-1 (Martinand *et al.*, 1999) and Encephalomyocarditis virus (EMCV, Martinand *et al.*, 1998) induce the expression of a cellular RNase L inhibitor, which antagonises binding of 2'-5'A oligomers to RNase L and hence prevents its activation. HSV-1 and HSV-2 viruses synthesise 2'-5'A derivatives which bind to and prevent RNase L activation (Cayley *et al.*, 1984). The NS1 protein of influenza A is a potent inhibitor of oligo A synthetase (Min & Krug, 2006).

Other viruses have the capacity to block other antiviral enzymes (reviewed in Weber *et al.*, 2004). It has been demonstrated that some other viruses can block the IFN response, although the mechanism by which they do this is unknown. An example is RSV, which uses the NS1 and NS2 proteins to block IFN (Schlender *et al.*, 2000).

1.5 Vaccines and Antiviral Drugs

The control of viruses and viral diseases is accomplished in two different ways: vaccines help in the prevention of viral infections whilst antiviral drugs provide treatment of diseases induced by viruses. In animals (including humans), immunisation with vaccines has been far more effective than the use of antiviral drugs, but vaccines against some viruses are less than ideal and for other viruses there is as yet no vaccine that gives any worthwhile protection. Vaccines have however been very successful in preventing some viral diseases. Nevertheless, vaccines are difficult to successfully deploy against rapidly mutating viruses, such as Influenza virus and HIV and in individuals who are already infected with a virus, they provide modest or no therapeutic effect. That is why antiviral drugs, that are capable of preventing an infection or stopping it once started, are important as a second arm of antiviral defence.

As mentioned before (section 1.1), new viruses and new virus strains sometimes arise due to their ability to jump the species barrier to infect hosts that are not their natural hosts, causing high virulence and lethal diseases in their new host. In light of the emergence of new zoonotic viruses, it is important to have methods to rapidly develop vaccines against them. The aim of this project was to develop a methodology that would allow the rapid selection and isolation of viruses attenuated in their ability to circumvent the host IFN response to be used as live attenuated vaccine candidates (see section 1.6).

1.5.1 Vaccines

A vaccine is remarkably valuable in controlling viruses and the diseases they cause, but it has to follow certain prerequisites to be effective. First of all, a vaccine must be safe: its side effects must be minimal and it should induce protective immunity in the population as a whole, evoking innate, cell-mediated and humoral responses. Not every individual in a population needs to be immunised to stop viral spread, but a sufficient number must become immune to prevent virus transmission. Protection provided by a vaccine should be long-term, meaning that more than one inoculation may be necessary in some cases. In

practical terms, an effective vaccine should be biologically stable: there should be no genetic reversion to virulence and it should be able to survive storage and use in different conditions. Vaccines should also be easy to administer at low cost.

When humans are the only natural host of a virus, appropriate use of a highly effective vaccine can help eradicate a major disease. A virus also has to surpass other criterion for worldwide eradication to be possible (table 1.3). Smallpox, which has been referred to as the most destructive disease in history, was globally eradicated in 1977 due to routine mass vaccination using the live VV vaccine. Other diseases, such as poliomyelitis (caused by PV) and measles (caused by MeV) have been similarly targeted by the WHO for eradication, and rapid and significant progress towards this goal has been made for both viral pathogens. Live virus vaccines (see below) have played and continue to play a central role in these current eradication efforts.

Table 1.3 Criteria for the worldwide eradication of human viruses (adapted from Dimmock *et al.*, 2001).

Criterion	Smallpox virus	Measles virus	Poliovirus types 1-3
There must be overt disease in every instance so infection can be recognised	+	+	-
The causal virus must not persist in the body after the initial infection	+	+	+
The must be no animal reservoir from which reinfection of humans can occur	+	+	+
Immunisation must provide effective and long-lasting immunity	+	+	+
Viral antigens must not change	+	+	+

Given the remarkable success of the smallpox and polio vaccines, it might seem reasonable to prepare vaccines against all viral diseases. Unfortunately, despite

considerable progress in research, it is difficult to predict with confidence the efficacy or side effects of vaccines.

1.5.1.1 Currently licensed viral vaccines

Since the introduction of VV, licensed vaccines have been developed for many other viral pathogens (table 1.4). Several of these vaccines have already helped to control the diseases against which they are directed, notably, poliomyelitis, yellow fever, mumps, measles and rubella.

Table 1.4 Viral vaccines licensed in the United States (adapted from Flint *et al.*, 2004).

Virus Vaccine	Type of vaccine	Target population
Adenovirus	Live attenuated, oral	Military recruits
Hepatitis A	Inactivated, whole virus	Travelers, health care workers
Hepatitis B	Yeast-produced recombinant surface protein	Universal childhood
Influenza A and B	Inactivated viral subunits	Elderly and other high-risk groups
Japanese encephalitis virus	Inactivated whole virus	Travelers to endemic region
Measles	Live attenuated	Universal childhood
Mumps	Live attenuated	Universal childhood
Poliovirus	Inactivated whole virus; live attenuated, oral	Universal childhood
Rabies	Inactivated whole virus	High-risk groups
Rotavirus	Live attenuated, oral	Universal childhood
Rubella	Live attenuated	Universal childhood
Smallpox	Live Vaccinia virus	Certain laboratory workers
Varicella	Live attenuated	Universal childhood
Yellow fever	Live attenuated	Travelers to endemic region

Viral vaccines can be classified broadly into two general groups: live attenuated virus vaccines and inactivated (killed) virus vaccines.

1.5.1.2 Inactivated or killed virus vaccines

Examples of effective killed virus vaccines administered to humans are the inactivated PV, Influenza virus, Hepatitis A virus and Rabies virus vaccines. There are also many inactivated virus vaccines used in veterinary medicine. The basis for the construction of an inactivated vaccine is the isolation of virions of the virus of interest and their subsequent inactivation by chemical or physical procedures. The infectivity of the virus is eliminated, but the viral antigenicity should not be compromised by these treatments. Common techniques include treatment with formalin (chemical) or disruption with a detergent (e.g. for influenza). The level of efficacy of these vaccines differs: inactivated PV is highly effective in preventing disease, whereas the Influenza virus vaccine is only partially protective.

An advantage of killed virus vaccines is that any other unknown contaminating viruses will probably be killed in the same process. Inactivated virus vaccines also offer the advantage of immunisation with little or no risk of infection of other people. However, there are some cases in which an inactivated virus vaccine has amplified disease rather than prevented it (Fulginiti *et al.*, 1967, Kim *et al.*, 1969). This was first observed with a formalin-inactivated MeV vaccine (Fulginiti *et al.*, 1967). This vaccine prevented measles initially, but after several years, vaccines lost their resistance to infection and when subsequently infected with naturally circulating MeV, patients developed an atypical illness with accentuated symptoms. A further disadvantage is that inactivated virus vaccines often do not induce CTL cells as efficiently as live attenuated viral vaccines. Furthermore, to induce the same immune response as live attenuated preparations, inactivated virions require mixing with adjuvants, which are substances that stimulate early processes in immune recognition, particularly the inflammatory response. Adjuvants can mimic or induce cellular damage, stress and release of heat shock proteins which directly stimulate the immune system. Another major disadvantage is that, as the killed virus cannot multiply, the immunising dose has to contain far more virus than a

dose of live vaccine and repeated doses may be required to induce adequate levels of immunity; this increases the overall cost of the vaccine.

1.5.1.3 Live attenuated viral vaccines

The process of producing a virus strain that causes a reduced amount of disease for use as a live vaccine is called attenuation. An attenuated virus is therefore a weakened, less virulent virus. A good candidate for generation of a vaccine would be a virus with low pathogenic potential that is, nevertheless, capable of inducing a long-lived, protective immune response.

Live virus vaccines allow the activation of all components of the immune system yielding both a balanced systemic and local immune response, and a balanced humoral and cell-mediated response. Furthermore, live viral vaccines also stimulate an immune response to each of the antigens of a virus, which is especially important for complex viruses encoding many antigens. As demonstrated in a study on Influenza A virus vaccines, immunity induced by live virus vaccines is generally more durable and more effective than that induced by inactivated virus vaccines (Johnson *et al.*, 1986). There are also practical advantages to many live viral vaccines such as low cost of production and ease of administration. However, there are also some major concerns regarding live virus vaccines. Firstly, they can contain foreign agents (contamination), although this has rarely been a problem. They can cause illness directly or lose their attenuation during manufacture or during replication in vaccinees by reversion or second-site compensatory mutations. Given the high rate of mutation associated with RNA virus replication, a reversion to virulence may occur quite frequently. Some live virus vaccines, such as the MeV, Rubella virus and Yellow Fever virus vaccines, retain a low level of residual virulence. Others, such as the PV vaccine, may restore a varying degree of virulence during infection by the vaccine virus, although this occurs at an extremely low frequency (Nkowane *et al.*, 1987). Another disadvantage is that live viral vaccines can lose infectivity during storage, transport or use. Also, naturally occurring wild-type viruses may interfere with infection by a live virus vaccine, resulting in a decrease in vaccine efficacy. Stability is also a serious problem with labile vaccine viruses such as MeV. The measles vaccine needs to be stored and transported at low temperature (4°C).

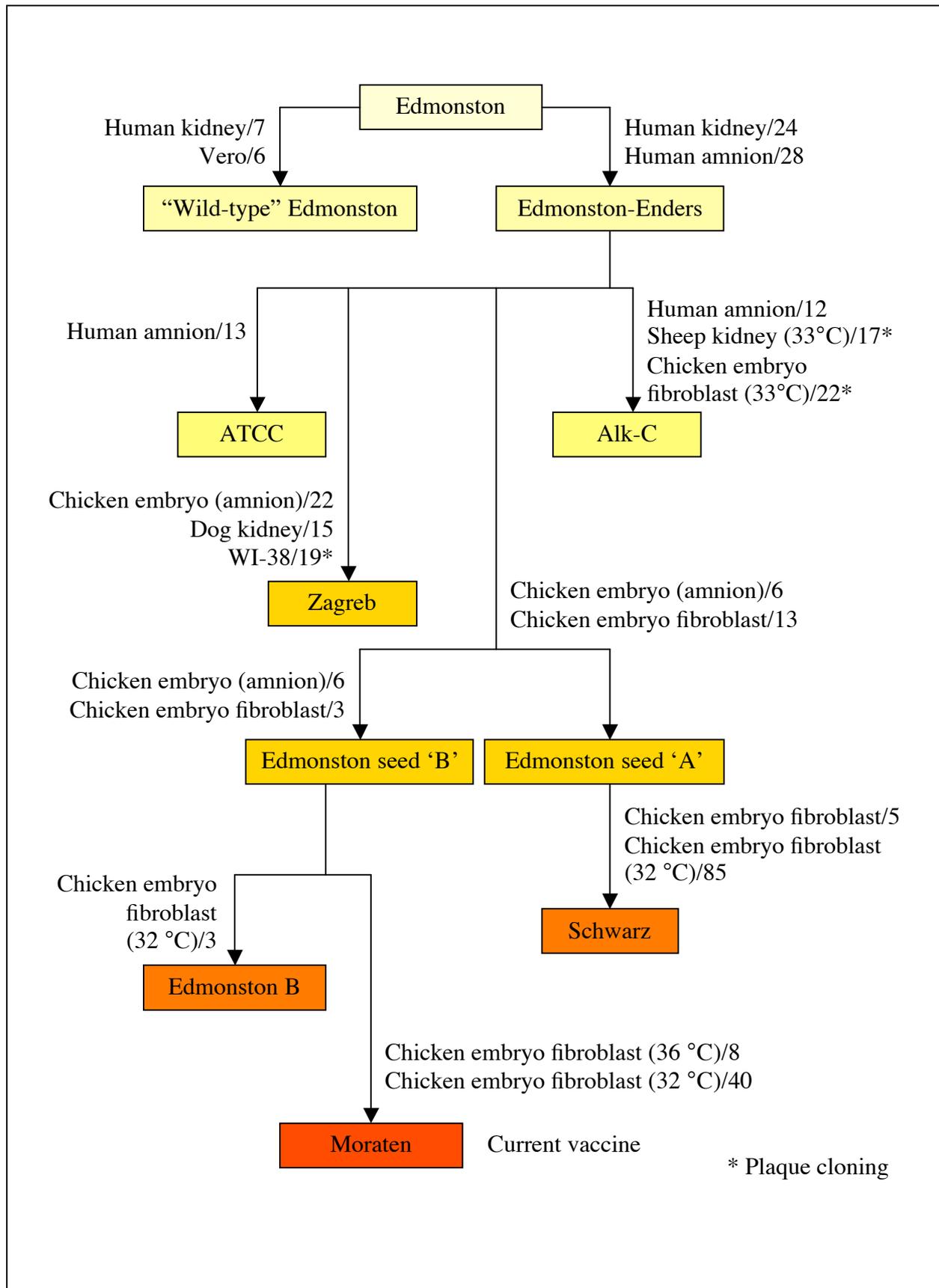
All of the licensed live vaccine viruses (table 1.4) have a clear lineage, except for VV. Many of them were derived from wild-type human viruses by serial passage in cells other than those of the natural host, leading to the emergence of mutants that were partially restricted in humans at the portal of entry and/or the target organ(s). Mutants of Rubella virus and types 1 and 3 PV (Sabin, 1957, Sabin & Boulger, 1973) were selected after passage in monkey kidney tissue culture; vaccine strains of Yellow Fever virus (Theiler & Smith, 1937) were generated by multiple passages in chicken embryo cell culture, while embryonated eggs were used for attenuation of MuV. The mutant of type 2 PV used in the current poliomyelitis vaccine was derived from a naturally occurring attenuated isolate. The current live attenuated Measles vaccine (Moraten strain) was obtained by initial propagation of the Edmonston strain in different cells (human, monkey, chick embryo), in addition to slight variations in the growth temperature of the cells (figure 1.9, adapted from Flint *et al.*, 2004).

In addition to serial passage in cells from an unnatural host, there are other traditional methods currently used to select and isolate attenuated virus mutants. An example is mutagenesis followed by selection of mutants with the desired phenotype. In a study by Blaney and colleagues, temperature-sensitive attenuated Dengue viruses were identified after the virus was grown in Vero cells in the presence of 5-fluorouracil, a chemical mutagen (Blaney *et al.*, 2001). Another way of selecting attenuated mutants is by propagating the virus at nonphysiological temperatures (temperature sensitive – ts – mutants). Many vaccine candidates are derived using a combination of these methods. An example is an RSV vaccine candidate that was derived from cold-passaged RSV by two sequential treatments with the mutagen 5-fluorouracil (Whitehead *et al.*, 1999). In paramyxoviruses, proteolytic cleavage of the F glycoprotein by trypsin-like enzymes (proteases) present in host tissues is required for activation of infectivity. Thus, it is possible to select protease activation (pa) attenuated mutants of SeV by growing the virus in the presence of another protease (Tashiro & Homma, 1983, Tashiro & Homma, 1985). Another option to obtain attenuated vaccine candidates is to select viruses that contain mutations in their IFN antagonist genes, so that they no longer interfere with the host IFN

Figure 1.9 Passage histories of live attenuated measles virus vaccines derived from John Enders' original isolate of Edmonston virus (top, pale yellow) (adapted from Flint *et al.*, 2004).

The current vaccine is called the Moraten strain (bottom, red). Attenuated mutants were selected through passage in different cell lines: human kidney, human amnion, Vero, chicken embryo intra-amniotic cavity, chicken embryo fibroblast, dog kidney, human diploid fibroblasts and sheep kidney. The temperature during growth is given in parentheses, and the number of passages of the virus in the particular cell line follows the slash. An asterisk indicates that a single plaque was picked for further propagation.

Figure 1.9



adapted from Flint et al, 2004

system. These viruses can be grown well in tissue culture in IFN non-responsive cells, but are highly attenuated in the host organism, due to a robust IFN and immune response. Most viral IFN antagonists of negative-strand RNA viruses appear to be accessory proteins, dispensable for viral infectivity but required for pathogenicity and efficient replication in the host (Garcia-Sastre, 2004, Goodbourn *et al.*, 2000).

Although all these mutants are in general the product of a process of genetic roulette, followed by selection of the desired phenotype, these conventional techniques used for the attenuation of viruses will continue to have a role in the development of live virus vaccines. They are generally straightforward and simple procedures and useful for new emergent viruses of which little genome information is known. However, alternatives to these conventional techniques based on reverse genetics and recombinant DNA technology can now be applied; alternatives to generating inactivated virus vaccines are also currently available.

1.5.1.4 New vaccine technologies – genetically engineered vaccines

Live, recombinant vaccines - attenuation by reverse genetics and recombinant DNA technology

Attenuation of a virus can be deliberately achieved by reverse genetics, the engineering of specific mutations into viral genomes. The genetic material of a virus, once isolated, is amenable to all the same genetic manipulation techniques as any other RNA or DNA molecule. DNA virus genomes may be cloned directly, while RNA virus genomes need to be cloned as complementary DNA (cDNA). The absence of a DNA intermediate in the replication cycle of RNA viruses has, until recently, limited research potential because there are no tools to modify RNA molecules in the same way that there are for DNA. However, for many RNA virus systems, it is now possible to generate DNA copies (cDNA) of virus genomes and convert these back into RNA. Viral genomes can be cloned and sequenced and genetic information can be analysed using standard laboratory organisms, cultured cells and animals. DNA/cDNA molecules can be modified so that they contain stable, defined, identifiable attenuating mutations, such as gene deletions or point mutations (e.g., ts and pa mutations). A recombinant virus must then be created by

rescuing these cloned, manipulated sequences into infectious particles. In essence, this process mirrors the traditional processes for the selection of attenuated viruses, but here attenuated virus is produced in a planned way rather than arising through chance genetic change. Multiple mutations or deletions are preferred to reduce or eliminate the probability of reversion to virulence, as demonstrated in a study to generate hPIV1 vaccine candidates by importation of attenuating mutations from other paramyxoviruses (Newman *et al.*, 2004). Vaccines made with such viruses could be directly monitored during all phases of development, manufacture and utilisation in humans because the genetic basis for attenuation would be known. However, this process for obtaining attenuated viruses is time consuming and technologically demanding. In addition, if entire genes are deleted, the resulting virus could be over-attenuated and not activate a full immune response.

In a 1999 publication, new well-characterized attenuating mutations of RSV were introduced into under-attenuated vaccine candidates by recombinant cDNA technology (Whitehead *et al.*, 1999). The researchers used the technique of reverse genetics with combinations of these well-characterized mutations obtained through conventional techniques to create novel vaccine candidates which were satisfactorily attenuated and immunogenic. The addition of more than one attenuating mutation can yield mutants that exhibit a stepwise increase in attenuation compared with the preceding mutant(s) in the series, as demonstrated with sequential introduction of several ts mutations into an attenuated strain of Influenza A by reverse genetics (Subbarao *et al.*, 1995).

More recently, viral IFN antagonists have been genetically manipulated for the development of novel candidate vaccines. Viruses with targeted deletions in genes known to code for IFN antagonists are promising candidates for live virus vaccines. This approach has been pioneered for influenza A (Ferko *et al.*, 2004, Fernandez-Sesma *et al.*, 2006, Talon *et al.*, 2000b), for paramyxoviruses (Teng *et al.*, 2000, Valarcher *et al.*, 2003, Wright *et al.*, 2006) and for bunyaviruses (Weber *et al.*, 2002), and may likewise apply to other viruses.

Thus, recombinant DNA technology offers new flexibility to add or subtract mutations in vaccine candidates to achieve the desired delicate balance between attenuation and immunogenicity that is required to produce a successful vaccine.

Subunit vaccines

Subunit vaccines are formulated with purified components of viruses, rather than the whole intact virion used in inactivated vaccines. DNA technology now allows the identification of the immunogenic proteins (proteins against which protective immunity is directed) of a virus. The viral genes that encode the immunogenic proteins can be cloned into an appropriate eukaryotic expression vector (viral DNA or a DNA copy if the virus has a RNA genome, can be excised and inserted into the vector) and expressed in cells of yeast, insect or mammalian origin. There can be no contamination of the resulting vaccine with the original virus because only a portion of the viral genome is required for production of the immunogenic protein. Also, viral proteins can be made inexpensively in large quantities under conditions that simplify purification and quality control. However, many candidate subunit vaccines do not induce an immune response sufficient to protect against infection. The immune response evoked by a live virus infection may only be partially represented in a response to a subunit vaccine.

Successful hepatitis B and influenza vaccines have been made using only a subset of viral proteins. The problem with making a hepatitis B vaccine was that the virus could not be grown in cell culture (and still grows only poorly today). This was solved by cloning and expressing the surface antigen HBsAg, which elicits protective immunity, in yeast. This was the first recombinant vaccine, and it has proved to be very effective.

Peptide vaccines

An extremely safe, well-defined vaccine may be obtained by using synthetic peptides representing immunologically important domains of viral surface antigens. However, peptide vaccines have had little commercial success, mainly because synthetic peptides are expensive to make in sufficient quantity, the antibody response they elicit is often weak and short-lived and it is difficult to generate peptides that will adopt the correct conformation of the epitope of pathogens. In addition, a critical prerequisite for peptide

vaccines is the need for the vaccine to contain peptides universally immunogenic when presented by the highly polymorphic MHC proteins found in human populations (Pier, 2004).

DNA vaccines

Another recent development is the DNA vaccine, where DNA encoding an antigenic protein is introduced through the skin rather than the antigenic protein itself. The DNA is usually a recombinant plasmid into which the gene for the antigen of interest has been inserted and placed under the control of a strong promoter that transcribes genes in mammalian cells. Injected DNA is taken up by host cells, the recombinant protein is produced and recognised as foreign by the immune system, and T- and B-cell responses are generated. The advantages of DNA vaccines are: they can potentially be produced inexpensively; they can be distributed widely without the need for equipment such as refrigerators; multivalent vaccines can easily be produced by incorporating multiple genes into the plasmid; they can be delivered via a 'gene gun', obviating the need for needles and syringes. Drawbacks include the fact that DNA vaccines are not very immunogenic in humans, the need to use strong mammalian promoters - these promoters could recombine with the host's DNA and activate undesirable genes - and the potential for the development of an autoimmune condition if the DNA itself is immunogenic.

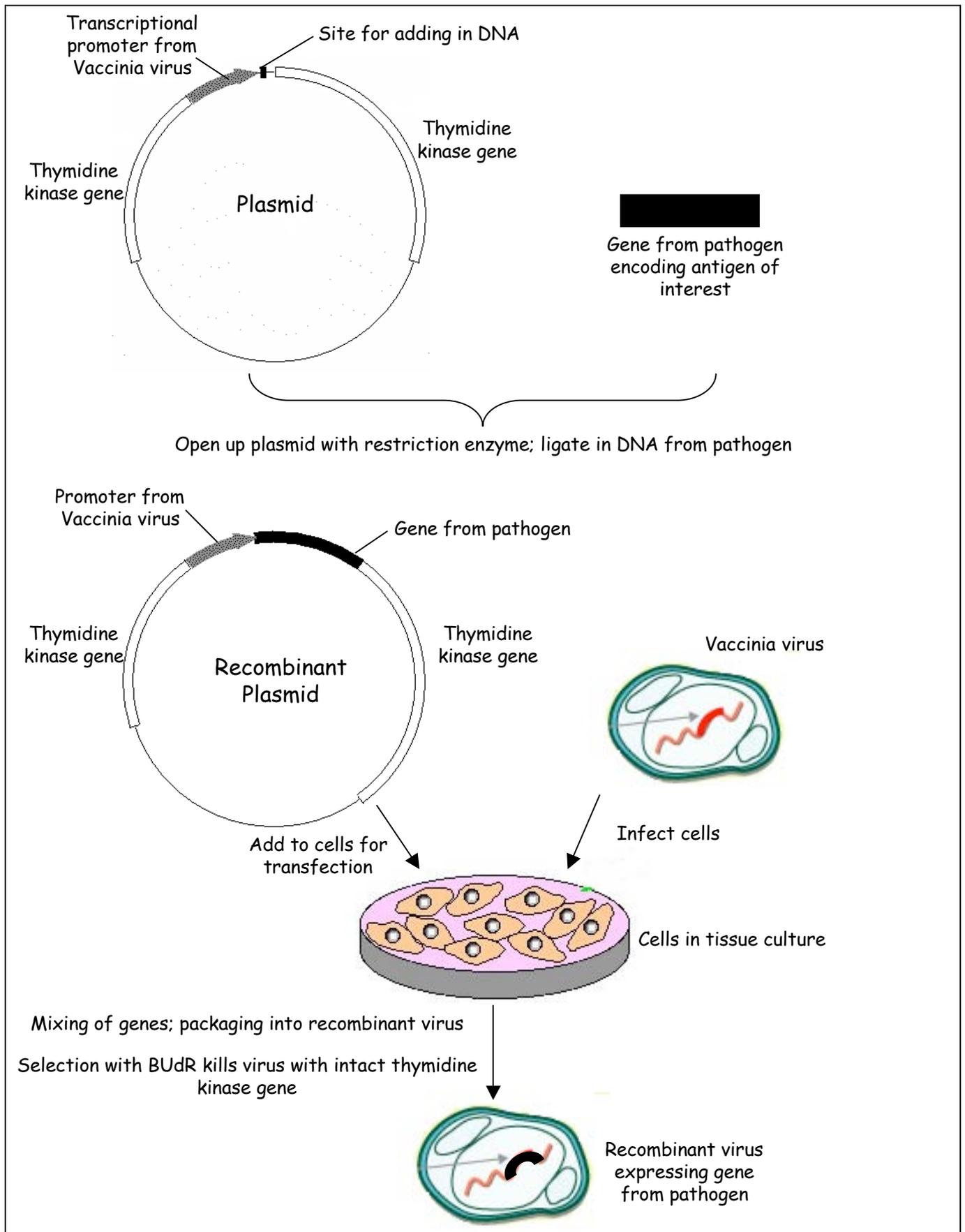
Live, recombinant vector vaccines

Live vaccines are very difficult to make in practice but evoke the most effective immunity and are the cheapest to produce. Thus, the idea of inserting the gene for the antigen of interest into a pre-existing live vaccine, so that it is expressed naturally as the virus multiplies, is particularly attractive. This has already been achieved experimentally for antigens of numerous viruses, including Influenza, Rabies, HSV-1 and HBV viruses, using VV as the live vaccine (figure 1.10, adapted from Pier, 2004).

Figure 1.10 Representation of the construction of a viral vector vaccine containing DNA encoding a protective antigen from a pathogen (adapted from Pier, 2004).

Plasmid DNA from a Poxvirus is engineered to contain a viral promoter (VV transcriptional promoter) in the middle of the non-essential thymidine kinase gene. The pathogen gene encoding the antigen of interest is inserted into the plasmid under the control of the VV promoter. When the resulting recombinant plasmid is mixed with live VV in cell culture, some of the viral particles will recombine with the plasmid DNA and incorporate the pathogen DNA and the interrupted thymidine kinase gene. Exposing cell cultures to bromodeoxyuridine (BUdR) kills the virus particles with intact thymidine kinase genes and selects for the virus particles with the pathogen antigen DNA interrupting the thymidine kinase gene.

Figure 1.10



adapted from Pier, 2004

1.5.2 Antiviral Drugs

Antiviral drugs are a class of medication used specifically for treating viral infections. The emergence of antiviral drugs is the product of a greatly expanded knowledge of the genetic and molecular function of viruses, major advances in the techniques for finding new drugs, and the intense pressure to deal with HIV. The general idea behind antiviral drug design is to identify target viral proteins, or parts of proteins, that can be disabled. These targets should be common across many strains of a virus, or even among different species of viruses in the same family, so a single drug will have a broad effectiveness. Once targets are identified, candidate drugs can be selected. High throughput screening (HTS) allows a researcher to effectively conduct millions of biochemical, genetic or pharmacological tests in a short period of time. Through this process one can rapidly identify active compounds, antibodies or genes which modulate a particular biomolecular pathway. The results of these experiments provide starting points for drug design and for understanding the interaction or role of a particular biochemical process in biology. Traditionally, the search for new antivirals has been by HTS, but this is moving successfully towards a combination of HTS, to initially identify target compounds, and computer design software, to alter the conformation of the compounds to improve their action.

There are two main difficulties with antiviral drugs. First there is the problem that by the time clinical signs and symptoms appear in acute infections, virus replication has reached such a peak that the antiviral has little therapeutic effect. The other problem is that virus multiplication is tied so intimately to certain cellular processes that most antivirals cannot discriminate between them. However, viruses do have unique features, so specific antivirals should be able to serve as effective chemotherapeutic agents. A further problem is the selection of drug-resistant mutants – any naturally-arising mutant that happens to have a selective advantage will rapidly outgrow wild-type virus and become dominant. The answer to this problem is to use two or more drugs simultaneously.

An antiviral would be effective if it inhibited any stage of virus multiplication: attachment, replication, transcription, assembly or release of progeny virus particles. The

current major antivirals act in one of these ways and examples are shown in table 1.5. Most of the antivirals now available are designed to help deal with HIV; Herpesvirus, which is best known for causing cold sores but actually covers a wide range of diseases; and HBV and HCV, which can cause liver cancer.

Table 1.5 Antivirals in clinical use or at an advanced stage of development (adapted from Dimmock *et al.*, 2001).

Antiviral drug	Mode of action	Usage
Inhibitors of virus replication: nucleoside analogues		
Zovirax (aciclovir)	DNA chain termination	HSV 1 and 2; also Varicella-Zoster virus but less sensitive
Ganciclovir	As above	Human Cytomegalovirus infection of the eye during AIDS
Zidovudine (AZT)	As above	HIV-1
Lamivudine	As above	HBV
Ribavirin	As above	HCV
Ion channel blockers		
Amantadine	Blocks the M2 proton channel	Type A Influenza viruses
Rimantadine	As above	Type A Influenza viruses
Interferons		
Interferons- α/β	Upregulate MHC class I	Although generically antiviral, are effective <i>in vivo</i> only against selected infections – chronic hepatitis B and C

before acyclovir can be used in DNA synthesis. Thus, the ultimate aim of creating a compound that is selectively toxic in infected cells has been achieved.

1.6 Aims

The overall aim of this project was to develop a universal cell-based methodology for rapidly selecting attenuated viruses from a wild-type or mutagenised virus population; more specifically, the goal was to select viruses attenuated in their ability to circumvent the host IFN response (IFN-sensitive viruses). In the first instance, the developed method would be tested on PIV5 (model paramyxovirus) and, when functioning correctly, the method would be extended and tested on other viruses, including viruses in other families. If such a method was developed, the attenuated viruses could be tested as candidates for the generation of live attenuated virus vaccines. In addition, the genomes of the selected IFN-sensitive viruses could be sequenced and the attenuating mutation(s) pinpointed: this would provide valuable background information about the location and how the mutation(s) led to the attenuation of the virus.

The second aim of this project was to further develop the cell-based methodology so that in addition to the selection of IFN-sensitive viruses, it could also be utilised to select revertant viruses that block the host's IFN system (IFN-resistant viruses) from an IFN-sensitive virus population. The selection of revertant viruses would again provide important background information as to the location of the mutation(s) that allowed the IFN-sensitive virus to become IFN-resistant. This method would be tested using CPI-, a strain of PIV5 that is IFN-sensitive.

Finally, after the development of the cell-based methodology, it became apparent that the generated cell lines (Mx GIPSE and Mx TIPSE) could also be useful in other contexts: in screening for compounds that inhibit IFN signalling, in screening for compounds that block the activity of viral IFN antagonists and in CPE reduction assays. The third and final aim was therefore to investigate and develop the potential of the generated cell lines in other contexts.

2. MATERIALS AND METHODS

2.1 DNA processing and analysis

2.1.1 Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify any DNA segments required for cloning into vectors and also to amplify cDNA fragments obtained from reverse transcription as described below. PCR products were obtained by DNA amplification using Taq polymerase (Promega Ltd., United Kingdom) or PfuI polymerase (produced in-house) according to the manufacturer's instructions, using a GeneAmp PCR System 2400 (Applied Biosystems). 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 PfuI polymerases respectively) for 90 sec. The PCR set (typically 30 cycles) was preceded by an initial step of melting at 95°C for 5 min followed by a final step of strand extension at 72°C or 68°C for 7 min.

2.1.2 Agarose gel electrophoresis

Amplified DNA was analysed by gel electrophoresis in horizontal mini-gels of 1% (w/v) agarose (Sigma-Aldrich Co Ltd., United Kingdom) in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA). DNA samples were mixed with the appropriate volume of DNA loading buffer (Promega Ltd., United Kingdom), prior to electrophoresis. Samples were run at 90 V in TBE buffer containing 1 µg/ml ethidium bromide, until bands were clearly resolved. Along with the samples, known DNA molecular weight markers were run (1kb ladder; Promega Ltd., United Kingdom). Electrophoresed DNA was exposed to UV light and photographed (Gel Doc 2000 UV transilluminator/photography system; Bio-Rad, United Kingdom).

2.1.3 Purification of DNA fragments from agarose gels

Following analysis of DNA by agarose gel electrophoresis, as described above, the DNA fragments of interest were subsequently purified by excision of the resolved bands from the gel and recovery on Qiaquick gel extraction columns (Qiagen Ltd., United Kingdom) according to the manufacturer's instructions. Typically, DNA was eluted in 30-50 μ l.

2.1.4 Treatment of DNA with enzymes

2.1.4.1 Digestion with restriction enzymes for cloning

Following purification of the PCR product, both backbone vector DNA and PCR product were mixed with 5U of (each of) the appropriate restriction enzyme(s) and correspondent buffer (Promega Ltd., United Kingdom and New England Biolabs Ltd., United Kingdom) in a total volume of 10-20 μ l. The reactions were incubated at the appropriate temperature (37°C for the majority of enzymes) for 2 to 4 hours (h) or overnight.

2.1.4.2 Dephosphorylation of linearized vector DNA

To prevent the backbone vector DNA from religation after restriction enzyme digest, linearized vectors were dephosphorylated using calf intestinal alkaline phosphatase (CIAP, Promega Ltd., United Kingdom). In general, 1 μ l of CIAP enzyme was added to the 10-20 μ l of digested plasmid DNA, and the solution was incubated at 37°C for a minimum of 30 minutes (min). DNA was cleaned up prior to ligation, using QIAquick PCR purification kit (Qiagen Ltd., United Kingdom), according to manufacturer's instructions.

2.1.4.3 Ligation of DNA

After the digestion of DNA with the appropriate restriction enzymes, insert and vector DNA were mixed with T4 DNA ligase and ligase buffer (New England Biolabs Ltd., United Kingdom) in a total reaction volume of 10-20 μ l. Reactions were incubated at 4°C overnight. Ligation of DNA into the pGEM-T easy vector (Promega Ltd., United

Kingdom) was carried out according to manufacturer's instructions. Ligation reaction products were used for heat-shock transformation of competent *Escherichia coli* DH5 α cells as described below.

2.1.4.4 Screening for positive clones

Following heat-shock transformation of ligation reactions into competent cells, amplification of growing colonies and purification of DNA using miniprep kits (described below), miniprep DNA samples were screened to find positive clones. 5 μ l of miniprep DNA was incubated with 2U appropriate restriction enzyme(s) and buffer in a total of 10-20 μ l and incubated at the appropriate temperature (37°C for the majority of enzymes) for 2 to 4 h. DNA samples were analysed by agarose gel electrophoresis.

2.1.5 Preparation of plasmid DNA

For small-scale preparations, bacterial cell cultures of 2-3 ml were grown overnight at 37°C in a shaking incubator. DNA was extracted from cells using the Qiagen DNA mini-prep kit (Qiagen Ltd., United Kingdom), according to the manufacturer's instructions. This extraction of DNA is based on alkaline lysis of bacterial cells followed by 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 37°C 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., United Kingdom), according to the manufacturer's instructions.

2.1.6 Plasmid DNA

Several vectors were used as backbone vectors or provided DNA sequences required for the construction of new plasmids (all the vectors described contain the gene that confers resistance to ampicillin):

- pdlNotI'MCS'F (provided by Dr. Yun-Hsiang Chen, University of St. Andrews, figure 2.1); this lentivirus vector was used as the backbone vector for the construction of the pdl Mx GIPSE vector described below;

- pHR-SIN-CSGWdlNotI (provided by Dr. Yun-Hsiang Chen, University of St. Andrews, figure 2.2); this lentivirus vector provided the eGFP sequence required for the cloning of the pdl Mx GIPSE vector;

- pGL3-Mx1P (provided by Dr. G. Kochs, University of Freiburg, figure 2.3); this plasmid vector was used to PCR amplify the murine Mx1 promoter sequence for cloning of the pdl Mx GIPSE lentivirus vector;

- pGEM-T easy (Promega Ltd., United Kingdom, figure 2.4); this plasmid was used for some intermediate cloning steps while preparing lentivirus vectors;

- LP2AT (provided by Dr. Pablo de Felipe, University of St. Andrews); this plasmid was used to PCR amplify the thymidine kinase (tk) gene for cloning of the pdl Mx TIPSE lentivirus vector;

- pCMV-Flag E3L (provided by Dr. Freidemann Weber, University of Freiburg); this plasmid was used to PCR amplify the E3L protein of VV containing a 5' terminal FLAG sequence for cloning of the pdl'Flag-E3L'puro lentivirus vector;

- pCMV-HA E3L (provided by Dr. Freidemann Weber, University of Freiburg); this plasmid was used to PCR amplify the E3L protein of VV containing a 5' terminal HA sequence for cloning of the pdl'HA-E3L'puro lentivirus vector.

Several lentivirus vectors were used or generated for transient transfections and generation of stable cell lines:

- pdl Mx GIPSE (figure 2.5); this lentivirus vector was generated to express both the eGFP and puromycin resistance genes under the control of the murine Mx1 promoter. It was used to generate HEp2 stable cell lines;

- pdl Mx TIPSE (figure 2.6); this lentivirus vector was generated to express both the tk and puromycin resistance gene under the control of the murine Mx1 promoter. It was used to generate HEp2 stable cell lines;

- pdl'PIV5V'bla (provided by Dr. Yun-Hsiang Chen, University of St. Andrews, figure 2.7); this vector expresses both the V gene of PIV5 and the blasticidin resistance

gene under the control of the constitutive SFFV promoter; it was used to generate the HEp2/Mx GIPSE/PIV5V cell line;

- pdl'HA-E3L'pac (figure 2.8); this lentivirus vector was generated to express both the HA-tagged E3L protein of VV and the puromycin resistance gene under the control of the constitutive SFFV promoter; the vector was used to generate the HEp2/HA-E3L cell line;

- pdl'Flag-E3L'pac (figure 2.9); this lentivirus vector was generated to express both the Flag-tagged E3L protein of VV and the puromycin resistance gene under the control of the constitutive SFFV promoter; the vector was used to generate the HEp2/Flag-E3L cell line.

All constructs that were generated were confirmed by automated DNA sequencing.

In addition, two helper vectors were used for generation of lentivirus stocks:

- pCMV;
- pVSV/G – expresses the Vesicular Stomatitis virus (VSV) glycoprotein G.

2.2 Bacterial transformations

2.2.1 Bacterial strains

Escherichia coli strain DH5 α [ϕ 80dlacZ Δ M15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (*rk[-] mk[+]*), *supE44*, *relA1*, *deoR*, Δ (*lacZYA-argF*)*U169*] was used in gene cloning. Bacterial cells were grown in liquid Luria-Bertani (LB) medium (10 g/l bacto-tryptone, 5 g/l yeast extract, 10 mM NaCl pH 7.5), or plated on solid LB medium supplemented with 1.5% (w/v) agar and 10M MgSO₄. To make use of antibiotic resistance markers, when required, media were supplemented with ampicillin (100 μ g/ml).

2.2.2 Preparation of competent bacterial cells

A single bacterial colony of *Escherichia coli* DH5 α was inoculated from an LB agar plate into 10 ml LB and incubated overnight at 37°C with shaking. The culture was

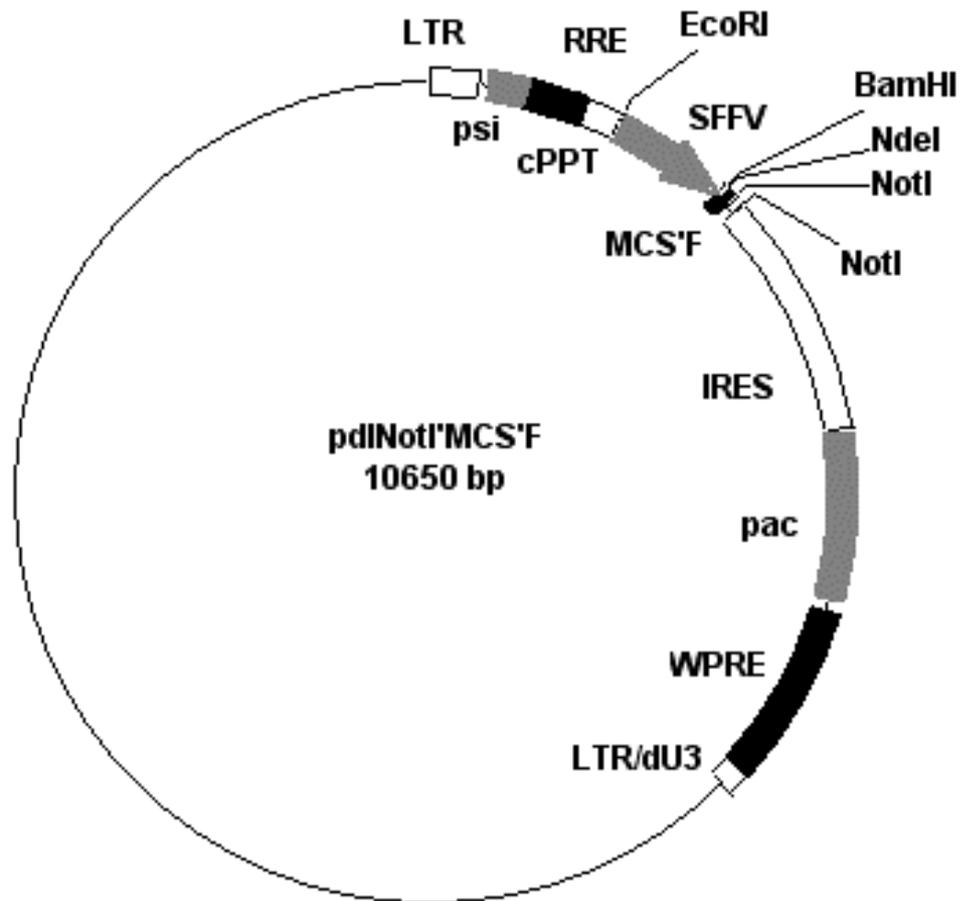


Figure 2.1 Schematic representation of the pdlNotI'MCS'F lentivirus vector.

Vector diagram of pdlNotI'MCS'F lentivirus vector showing approximate positions of the SFFV promoter, multiple cloning site, IRES and puromycin resistance genes (pac). Important restriction sites are shown. LTR – long terminal repeat; psi – Psi-sequence; RRE – Rev-responsive element; cPPT – HIV central polypurine tract; SFFV – Spleen focus forming virus promoter; MCS'F – Multiple cloning site; IRES – Internal ribosomal entry site; WPRE – woodchuck hepatitis virus post-transcriptional regulatory element.

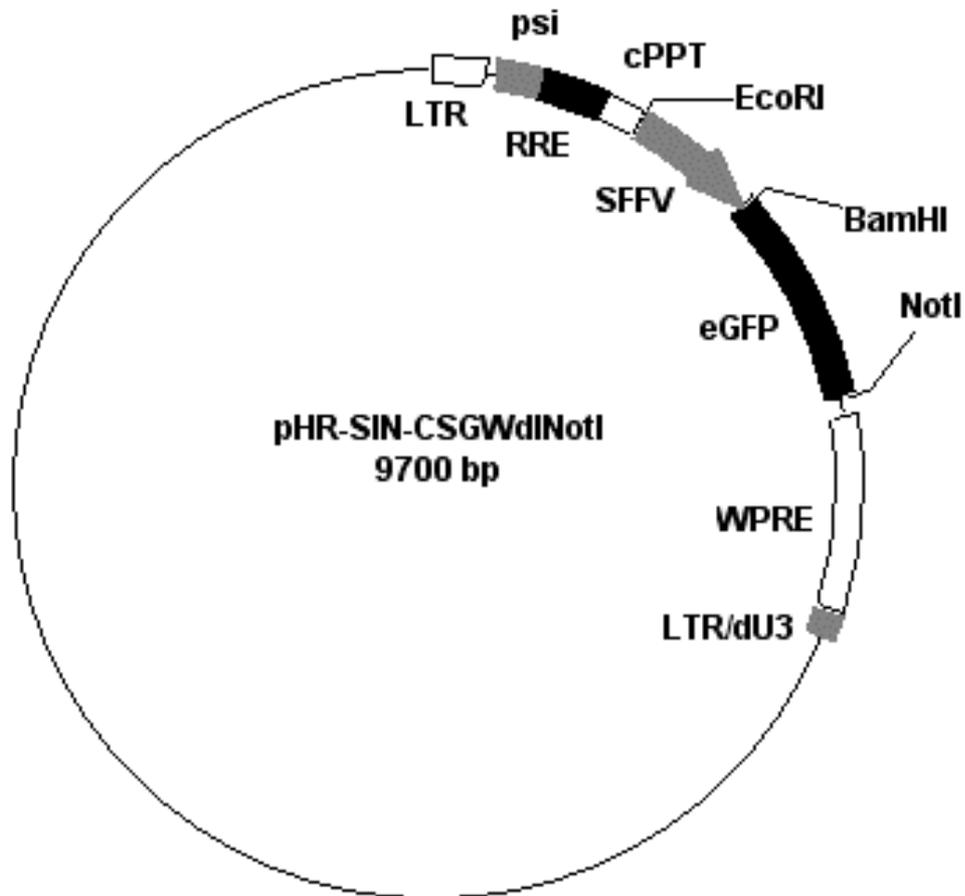


Figure 2.2 Schematic representation of the pHR-SIN-CSGWdINotI lentivirus vector.

Vector diagram of pHR-SIN-CSGWdINotI lentivirus vector showing approximate positions of the SFFV promoter and eGFP gene. Important restriction sites are shown. LTR – long terminal repeat; psi – Psi-sequence; RRE – Rev-responsive element; cPPT – HIV central polypurine tract; SFFV – Spleen focus forming virus promoter; eGFP – enhanced green fluorescent protein; WPRE – woodchuck hepatitis virus post-transcriptional regulatory element.

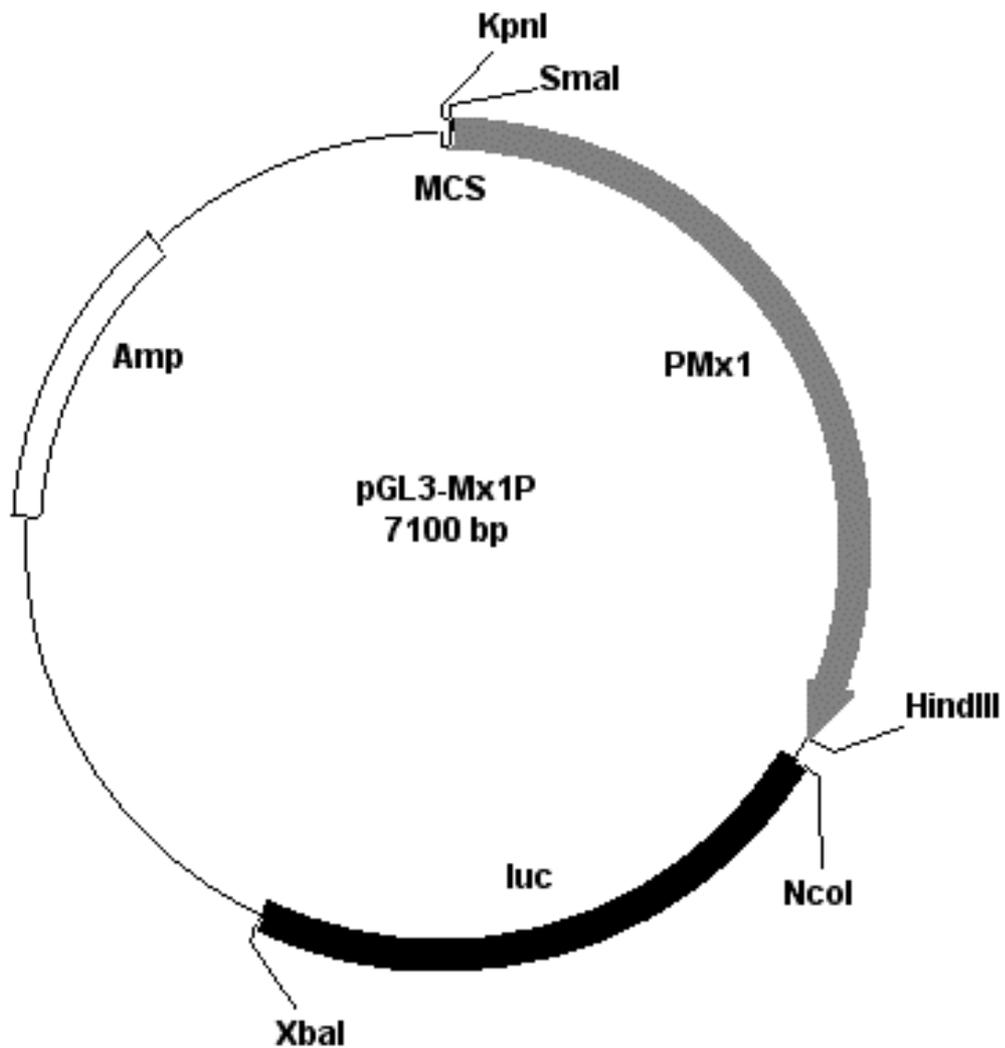


Figure 2.3 Schematic representation of the pGL3-Mx1P expression vector.

Vector diagram of pGL3-Mx1P vector showing approximate positions of the Mx1 promoter, luciferase and ampicillin resistance genes. Important restriction sites that were used in cloning strategies are shown. MCS – Multiple cloning site, PMx1 – Mx1 promoter; luc – luciferase gene; amp – ampicillin resistance gene.

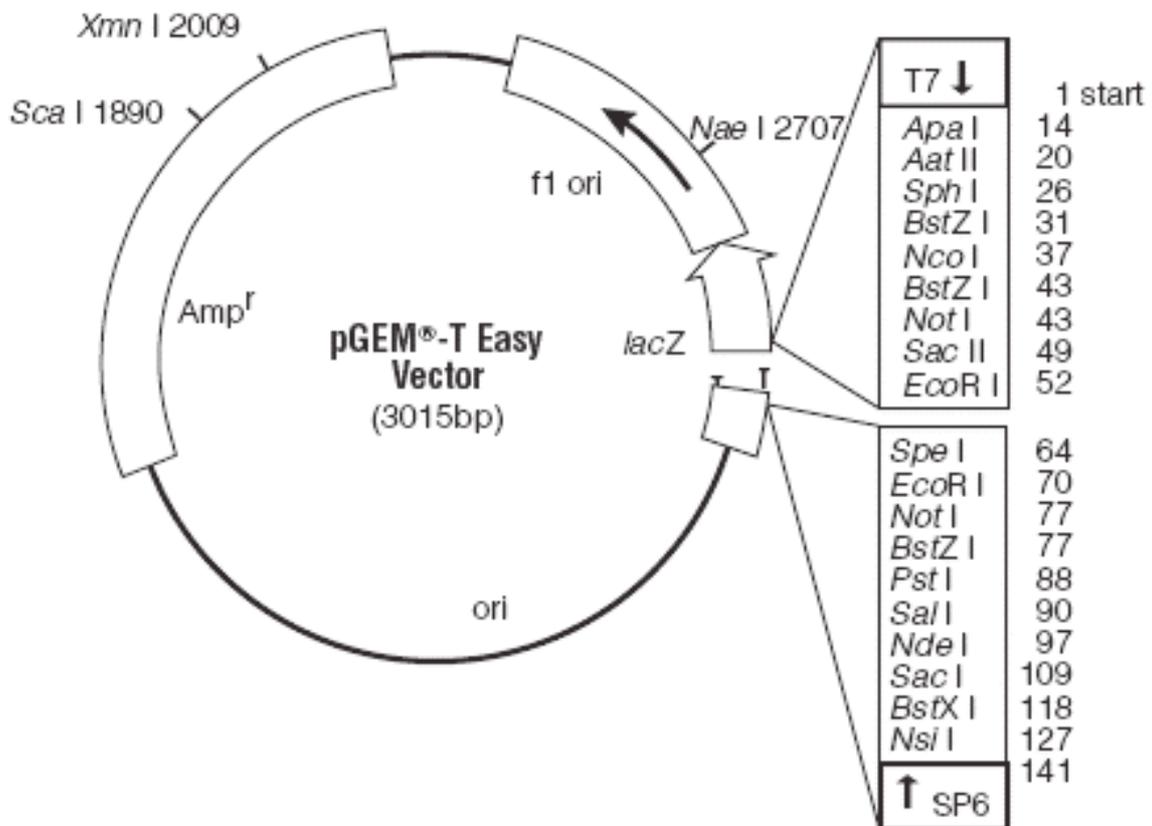


Figure 2.4 Schematic representation of the pGEM-T Easy expression vector (Promega Ltd, United Kingdom).

Vector diagram of pGEM-T Easy expression vector showing positions of the multiple cloning site and the ampicillin resistance gene. Important restriction sites are shown.

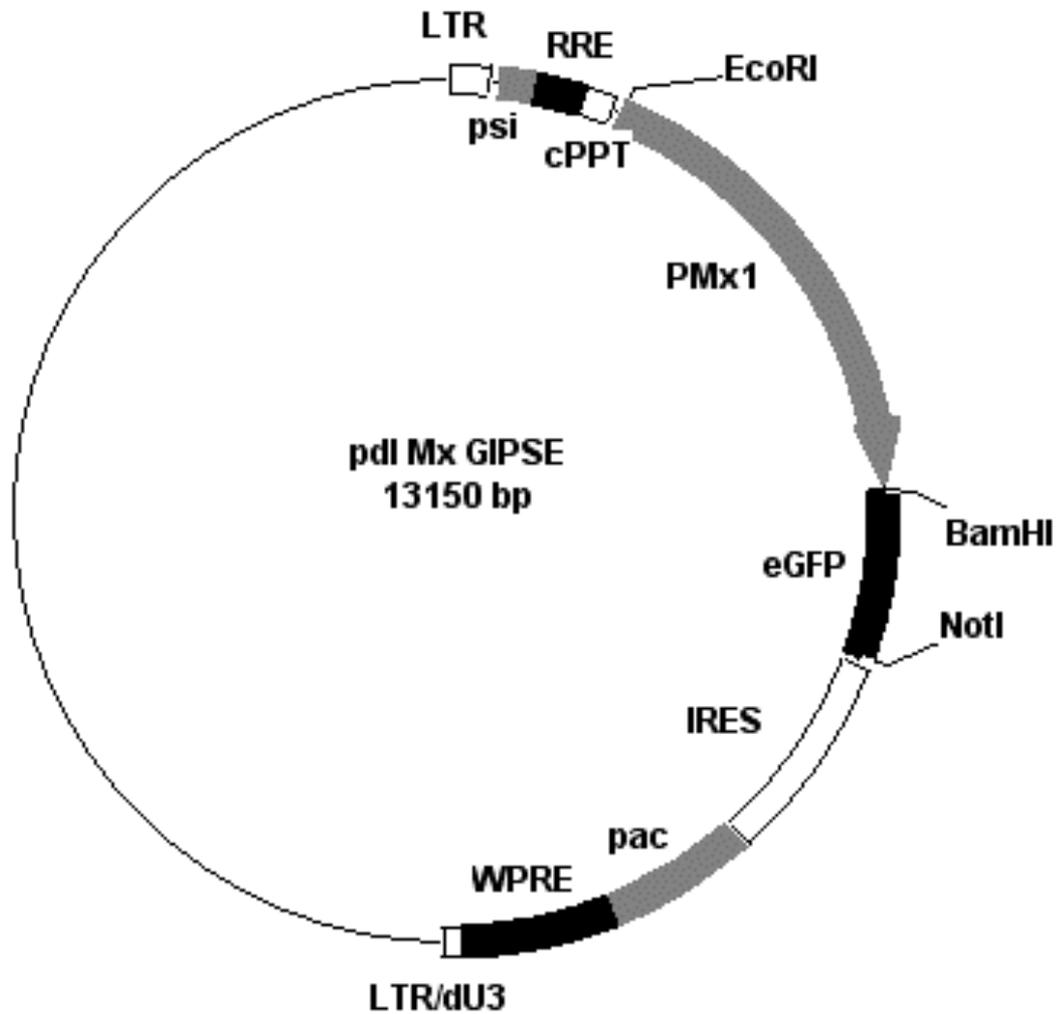


Figure 2.5 Schematic representation of the pdl Mx GIPSE lentivirus vector. Important restriction sites are shown.

The pdl Mx GIPSE vector contains both the eGFP and the puromycin resistance (pac) genes under the control of the IFN-inducible murine Mx1 promoter. The IRES element allows for the expression of both eGFP and pac in response to the Mx1 promoter. Mx GIPSE stands for Mx1 promoter, eGFP, IRES, puromycin resistance and IFN-selectable. LTR – long terminal repeat; psi – Psi-sequence; RRE – Rev-responsive element; cPPT – HIV central polypurine tract; PMx1 – Mx1 promoter; eGFP – enhanced green fluorescent protein; IRES – Internal ribosomal entry site; WPRE – woodchuck hepatitis virus post-transcriptional regulatory element.

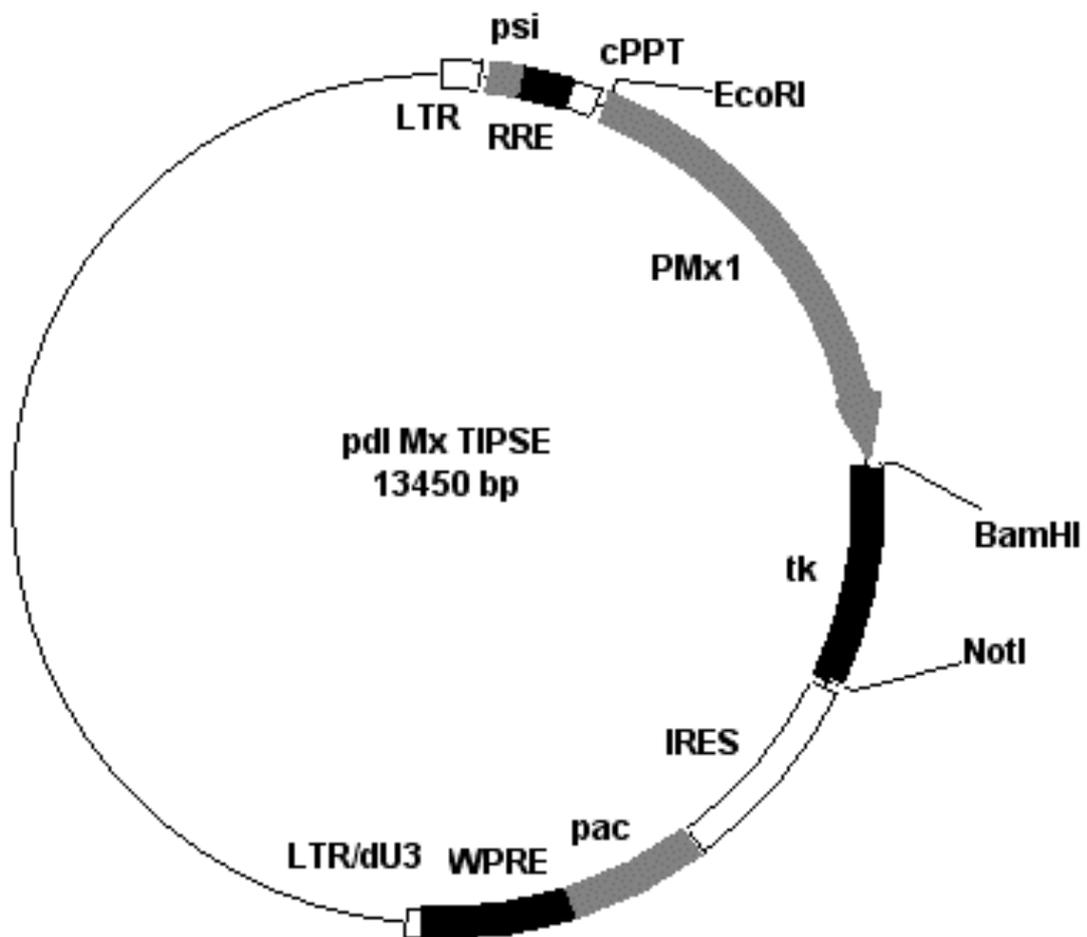


Figure 2.6 Schematic representation of the pdl Mx TIPSE lentivirus vector.

The pdl Mx TIPSE vector contains both the HSV-tk and the puromycin resistance (pac) genes under the control of the IFN-inducible murine Mx1 promoter. The IRES element allows for the expression of both HSV-tk and pac in response to the Mx1 promoter. Mx TIPSE stands for Mx1 promoter, tk, IRES, puromycin resistance and IFN-selectable. LTR – long terminal repeat; psi – Psi-sequence; RRE – Rev-responsive element; cPPT – HIV central polypurine tract; PMx1 – Mx1 promoter; tk – thymidine kinase gene; IRES – Internal ribosomal entry site; WPRE – woodchuck hepatitis virus post-transcriptional regulatory element.

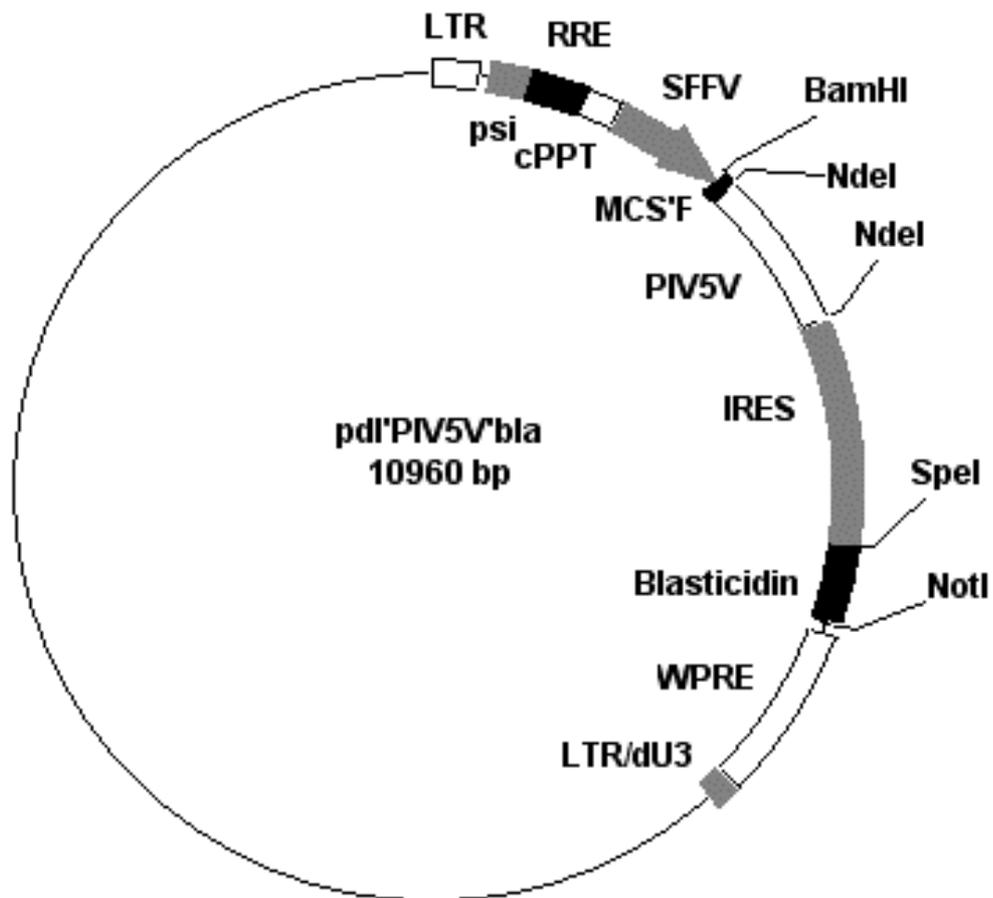


Figure 2.7 Schematic representation of the pdl'PIV5V'bla lentivirus vector.

Vector diagram of pdl'PIV5V'bla lentivirus vector showing approximate positions of the SFFV promoter, multiple cloning site, PIV5 V, IRES and blasticidin genes. Important restriction sites are shown. LTR – long terminal repeat; psi – Psi-sequence; RRE – Rev-responsive element; cPPT – HIV central polypurine tract; SFFV – Spleen focus forming virus promoter; MCS'F – Multiple cloning site; PIV5V – V gene of PIV5; IRES – Internal ribosomal entry site; blasticidin – blasticidin resistance gene; WPRE – woodchuck hepatitis virus post-transcriptional regulatory element.

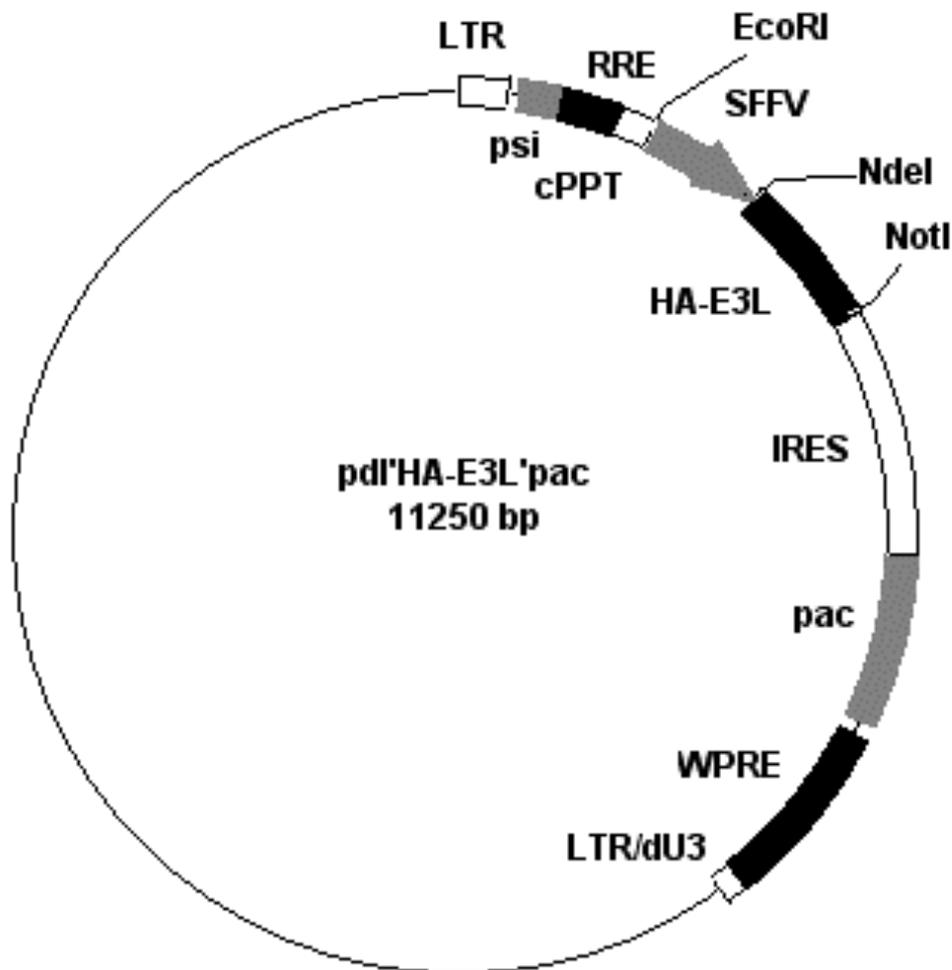


Figure 2.8 Schematic representation of the pdl'HA-E3L'pac lentivirus vector.

Vector diagram of pdl'HA-E3L'pac lentivirus vector showing approximate positions of the SFFV promoter, HA-tagged E3L, IRES and pac genes. Important restriction sites are shown. LTR – long terminal repeat; psi – Psi-sequence; RRE – Rev-responsive element; cPPT – HIV central polypurine tract; SFFV – Spleen focus forming virus promoter; HA-E3L – HA-tagged E3L gene of VV; IRES – Internal ribosomal entry site; pac – puromycin resistance gene; WPRE – woodchuck hepatitis virus post-transcriptional regulatory element.

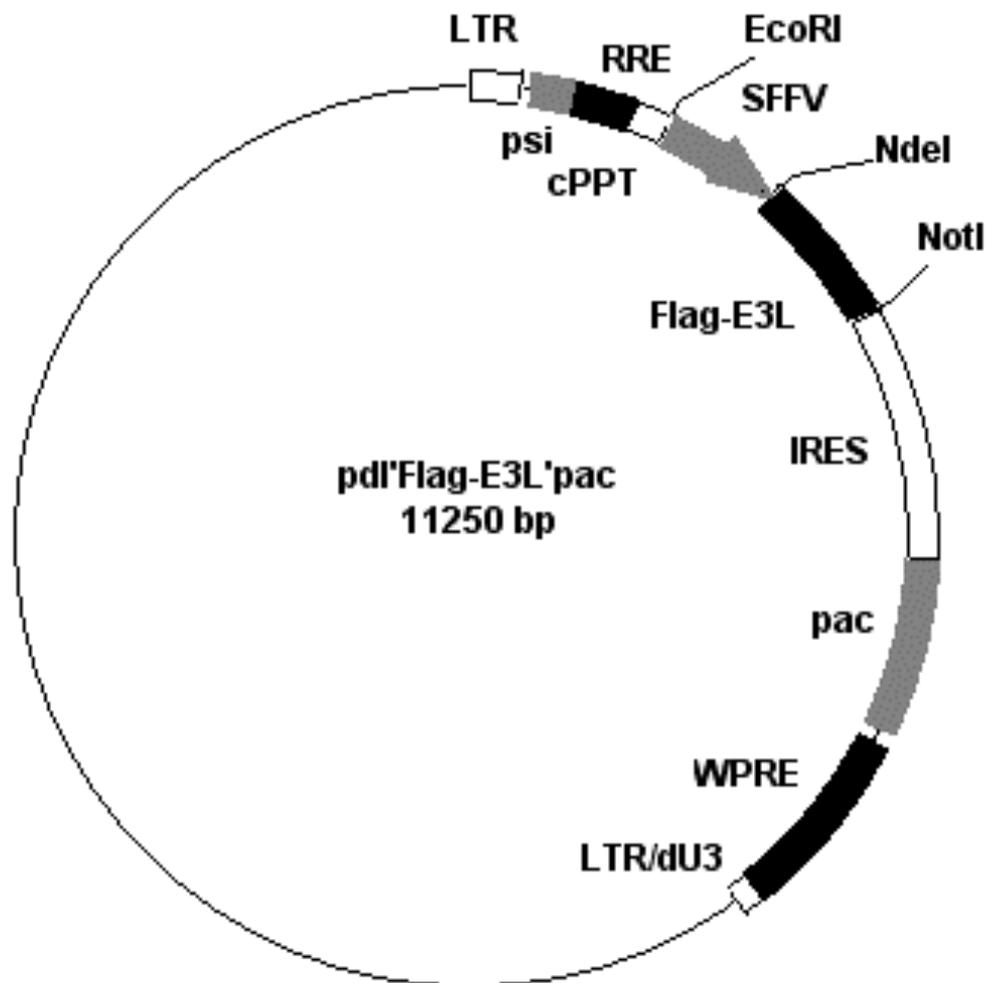


Figure 2.9 Schematic representation of the pdl'Flag-E3L'pac lentivirus vector.

Vector diagram of pdl'Flag-E3L'pac lentivirus vector showing approximate positions of the SFFV promoter, Flag-tagged E3L, IRES and pac genes. Important restriction sites are shown. LTR – long terminal repeat; psi – Psi-sequence; RRE – Rev-responsive element; cPPT – HIV central polypurine tract; SFFV – Spleen focus forming virus promoter; Flag-E3L – Flag-tagged E3L gene of VV; IRES – Internal ribosomal entry site; pac – puromycin resistance gene; WPRE – woodchuck hepatitis virus post-transcriptional regulatory element.

diluted 100 times in LB and grown until the OD_{600nm} reached 0.400 to 0.600 units. 50 ml cultures were then incubated on ice for 30 min and pelleted by centrifugation at 2800 rpm for 5 min at 4°C. Cells were resuspended in 12.5 ml of freshly mixed and pre-chilled filtered solutions of 100 mM $CaCl_2$ and 40 mM $MgSO_4$, incubated on ice for 30 min, re-centrifuged and finally resuspended in 2.5 ml of 100 mM $CaCl_2$ plus 2.5 ml of 40 mM $MgSO_4$. Pre-chilled glycerol was added to 10%, and the cell suspension was aliquoted (200 μ l per microfuge tube), frozen immediately in liquid nitrogen and stored at -70°C.

2.2.3 Transformation of competent bacterial cells

100 μ l of competent cells were incubated on ice until just thawed. DNA (in plasmid or ligated form) was added, mixed gently and incubated on ice for 30 min. Cells were then heat-shocked in a 42°C water bath for 60 sec and placed on ice for 2 min. 900 μ l of LB was added and cells were incubated for 1 h at 37°C. Cells were then plated onto L-Agar plates containing ampicillin (100 μ g/ml) and incubated at 37°C overnight. Bacterial mini-cultures were prepared the following day from selected colonies.

2.3 Cells and cell culture

2.3.1 Cell lines

This project required the use of basic cells of human and simian species origin:

- Vero cells; fibroblast-like cell line originating from kidney cells of the African green monkey (cell line obtained from ICN Pharmaceuticals Ltd., United Kingdom).

- 2fTGH cells (Pellegrini *et al.*, 1989); human diploid fibroblasts. The 2fTGH cell line was provided by Dr. Steve Goodbourn, St. George's Hospital Medical School, London.

- HEp2; human larynx carcinoma, epithelial cells, provided by ECACC.

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

- MRC5; human fetal lung fibroblasts, provided by ECACC.

In addition to the basic cell lines mentioned above, the following permanent cell lines were also used:

- 2fTGH/PIV5V; 2fTGH cells that express the V protein of PIV5 constitutively (produced by Dr. J. Andrejeva; Andrejeva *et al.*, 2002);
- MRC5/PIV5V; MRC5 cells that constitutively express the V protein of PIV5 (produced by Dan Young; Young *et al.*, 2003);
- HEp2/BVDVNpro; HEp2 cells that constitutively express the Npro protein of BVDV (produced by Dr. Yun-Hsiang Chen; Hilton *et al.*, 2006).

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

- HEp2/Mx GIPSE or Mx GIPSE; HEp2 cells that express eGFP and the puromycin resistance gene under the control of the murine Mx1 promoter;
- HEp2/Mx GIPSE/PIV5V or Mx GIPSE/V; HEp2/ Mx GIPSE cells that constitutively express the V protein of PIV5;
- HEp2/BVDVNpro/PIV5V; HEp2/BVDVNpro cells that constitutively express the V protein of PIV5;
- HEp2/Mx TIPSE or Mx TIPSE; HEp2 cells that express the tk and puromycin resistance genes under the control of the murine Mx1 promoter;
- HEp2/Flag-E3L; HEp2 cells that constitutively express the E3L protein of VV with an N-terminal Flag tag;
- HEp2/HA-E3L; HEp2 cells that constitutively express the E3L protein of VV with an N-terminal HA tag.

2.3.2 Cell culture

Cell cultures were maintained in 25 or 75 cm² tissue culture flasks (Greiner, United Kingdom) in Dulbecco's modified Eagle's medium (DMEM, Sigma United Kingdom), supplemented with 10% fetal calf serum (FCS; Invitrogen Ltd., United Kingdom) at 37°C

under 5% CO₂. Cells were routinely passaged, trypsinised (trypsin, EDTA; Becton Dickinson Ltd., United Kingdom) and diluted every three to five days, depending on the growth rate of the cell line. When required, media were supplemented with recombinant human IFN- α A/D (Rehberg *et al.*, 1982; PBL Biomedical Labs, New Brunswick, catalogue number 11200). For stimulation of IFN-responsive promoters, cells were incubated with IFN at a concentration of 10³ units per ml.

2.3.3 Transient transfection of mammalian cells with DNA

Cells were transfected with DNA using FuGENE 6 transfection reagent (Roche Diagnostics Co., United Kingdom), according to the manufacturer's instructions. Cells were incubated with the lipid:DNA mix overnight.

2.3.4 Preparation of stable mammalian cell lines

2.3.4.1 Production of lentivirus

Figure 2.10 is a representation of how lentivirus was produced. 293T cells contained in 75 cm² tissue culture flasks were co-transfected with three plasmids: pCMV, pVSV/G and the p Δ l vector (containing the gene(s) of interest) using FuGENE™ 6 transfection reagent, in a total volume of 8 ml per flask. The next day, the medium was changed to 5 ml of 10% FCS in DMEM. 48 and 72h after transfection, the supernatant was harvested, centrifuged to remove cell debris, filtered through 0.4 μ m filters and used to infect the mammalian cell line(s) of interest. The remaining supernatant was stored at -70°C.

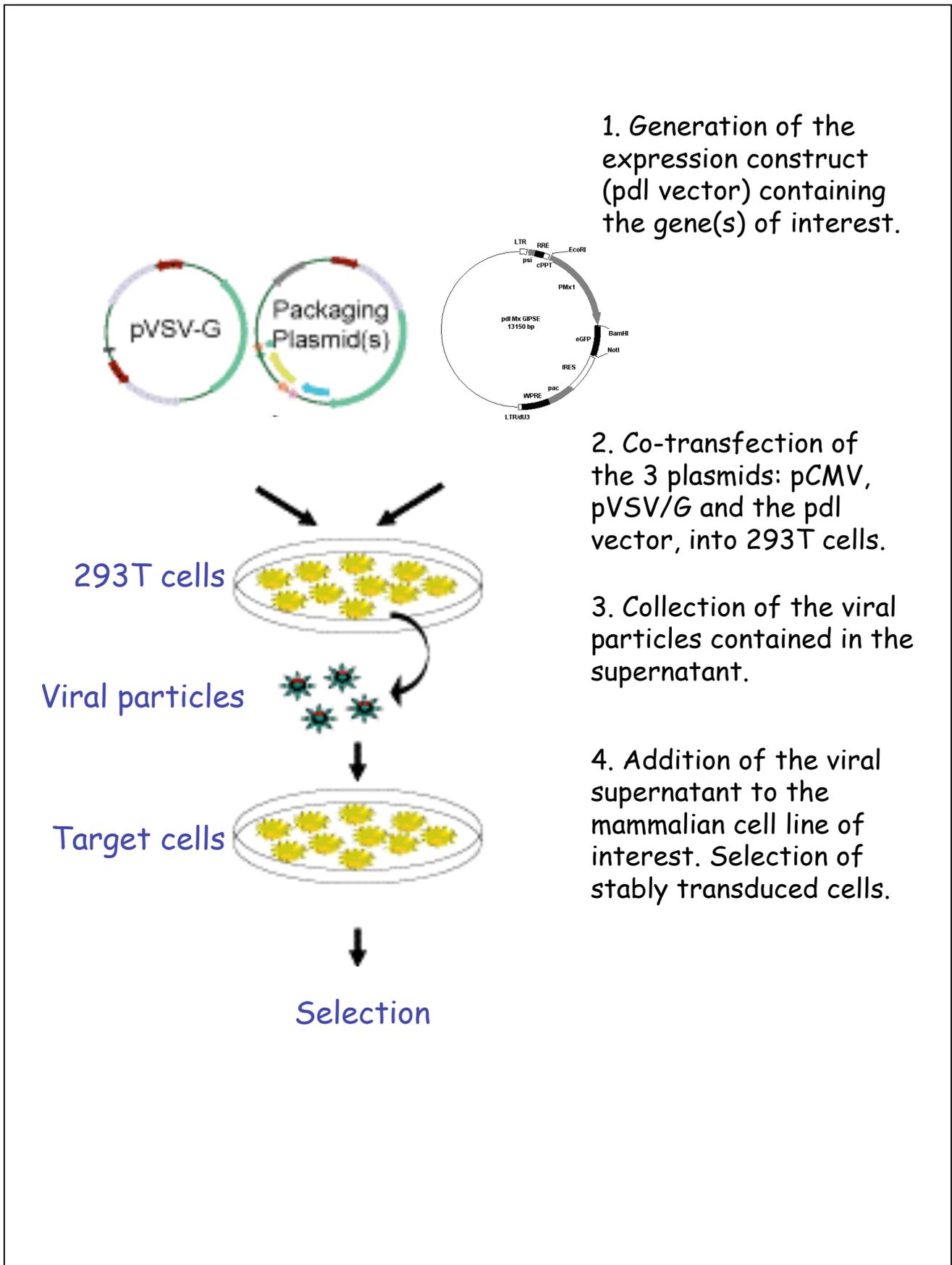
2.3.4.2 Subcloning

Following the appropriate selection of the lentivirus-infected mammalian cells with antibiotic (and IFN, if necessary), the cell lines were subjected to subcloning. Cells were counted using a haemocytometer, and plated into 96-well microtitre plates at an average of 1 cell/well. After three weeks, wells containing a single colony of cells were trypsinized and transferred into a separate well of a 24-well plate and grown as a

Figure 2.10 Representation of the general steps required to produce lentivirus particles containing a gene of interest and to then use these to infect mammalian cell lines.

The first step is to clone the gene(s) of interest into the expression construct (in this case, the pdl vector). The second step is to co-transfect 293T cells with three plasmids: pCMV, pVSV/G and the pdl vector. The third step is to harvest the supernatant at 48 to 72h after the transfection. This supernatant must then be centrifuged to remove cell debris and filtered. The final step is to use the viral supernatant to infect the mammalian cell line(s) of interest or target cells.

Figure 2.10



monolayer. The cells were subsequently passed and tested by immunofluorescence to confirm that they expressed the protein of interest. The cell clones that showed the highest protein expression levels were selected and stored at -70°C for future reference.

2.4 Viruses and virus infection of cells

2.4.1 Virus isolates and virus infections

In this study, the following viruses were used to infect cells:

- Parainfluenza virus type 5, strain W3 (Choppin, 1964, Hull *et al.*, 1956);
- Two closely related canine isolates of Parainfluenza virus type 5, namely CPI+ (formerly called 78-238) and CPI- (Baumgartner *et al.*, 1991, Baumgartner *et al.*, 1987, Baumgartner *et al.*, 1982, Baumgartner *et al.*, 1981). As described in section 1.1.1.3, CPI+ was isolated from the cerebrospinal fluid of a dog with temporary posterior paralysis, and CPI- was isolated from the brain of a dog that had been infected experimentally with CPI+;
- Bunyamwera virus, BUN;
- Bunyamwera Δ NSs, BUN Δ NSs; Bunyamwera virus containing a deletion of the NSs accessory gene.

Mammalian cells were infected as monolayers at different multiplicities of infection (m.o.i.; plaque-forming-units per cell), in DMEM containing 2% FCS (maintenance medium). After an adsorption period of 1 to 2 h on a rocking platform at 37°C, fresh maintenance medium was added to the virus inoculum (or plain growth medium, where mock infections were required as control treatments). When necessary, media were supplemented with recombinant human interferon- α A/D (rHuIFN- α A/D) (Rehberg *et al.*, 1982; PBL Biomedical Labs, New Brunswick, catalogue number 11200).

2.4.2 Preparation of virus stocks

When required, virus stocks were prepared to obtain sufficient amounts of virus. Monolayers of Vero cells (25cm² flasks) were initially infected with the virus of interest using the appropriate master stock (prepared by D. Young) and then incubated at 37°C until plaques began to form in the monolayer. The supernatant was subsequently collected and centrifuged at 4000 rpm for 5 min to precipitate cell debris. A portion of the purified supernatant was then used to infect cells at a larger scale (cells grown as monolayers in roller bottles; Scientific Laboratory Supplies Ltd., United Kingdom). Cells were incubated with the inoculum on a rolling platform for 1 to 2 h at 37°C to allow virus adsorption onto the cells and the medium was then replaced with fresh maintenance medium and cells reincubated on rolling platforms at 37°C. When fusion was evident in cell monolayers (usually after two to five days), the supernatant was harvested, centrifuged for 5 min to remove cell debris, and the virus stock was aliquoted and stored at -70°C. The virus titre was subsequently determined by plaque assay method, as described below.

2.4.3 Titration of virus preparations

Monolayers of Vero cells were grown in 6-well plates (Greiner, United Kingdom) until 80 to 90% confluence. The virus preparation was diluted in a series of ten-fold dilutions in DMEM supplemented with 2% FCS. Duplicate wells of cells were set up for each dilution, and cells were inoculated with each virus stock dilution (1ml/well). The cells with the virus inoculum were incubated for 2 h (37°C, 5% CO₂) on a rocking platform to allow adsorption of virus onto the cells. The virus inoculum was then removed and 10 ml of overlay, which consists of 0.5% of carboxy methyl cellulose (Methocel MC; Sigma-Aldrich Co Ltd., United Kingdom) and 2% FCS in DMEM, were added to each well. The cells were incubated (at 37°C, 5% CO₂) for 8 to 10 days without any mechanical agitation. When plaques were distinctly formed, the medium was aspirated off and the monolayers were fixed in 5% formaldehyde and 2% sucrose in phosphate-buffered saline

(PBS) for 30 min, after which the fixative solution was washed away with water. Areas of cell lysis and therefore sites of virus infection or plaques were visualised by crystal violet staining, by incubating the fixed cells with a solution of 0.1% crystal violet for 10 to 20 min, on a rocking platform at room temperature. After briefly washing the monolayers with water, plaques were observed as "holes" or unstained areas of the monolayer. Plaques were counted and the titre was estimated (plaque-forming-units per ml of virus preparation; pfu/ml), taking into account the dilutions made.

2.5 Protein analysis

2.5.1 Antibodies

Antibodies were used in immunofluorescence, immunoblotting or immune-staining of plaque assays, as described in the following sections. The primary antibodies specific for viral and cellular proteins used in this study are shown in Table 2.1.

The following antibodies were used as secondary antibodies to detect primary antibodies:

- Anti-mouse Ig Texas Red conjugated Ab (Oxford Biotechnology Ltd., United Kingdom);
- Anti-mouse Ig FITC conjugated Ab (Oxford Biotechnology Ltd., United Kingdom);
- Goat anti-rabbit IgG-AP alkaline phosphatase conjugated (Santa Cruz Biotechnology, United Kingdom, sc-2007);
- Goat anti-mouse IgG-AP alkaline phosphatase conjugated (Santa Cruz Biotechnology, United Kingdom, sc-2008);
- Anti-mouse IgG HRP linked Ab (Amersham Bioscience, United Kingdom).

Table 2.1 Primary antibodies and target proteins

Antibody	Target Protein	Source
PIV5-NP-a (mAb)	PIV5 NP	Randall <i>et al.</i> , 1987
PIV5-P-e (mAb)	PIV5 P (unique C domain)	Randall <i>et al.</i> , 1987
PIV5-P-f (mAb)	PIV5 P (unique C domain)	Randall <i>et al.</i> , 1987
PIV5-P-k (mAb)	PIV5 V and P (common N domain)	Randall <i>et al.</i> , 1987
PIV5-HN-4a (mAb)	PIV5 HN	Randall <i>et al.</i> , 1987
PIV5-HN-4b (mAb)	PIV5 HN	Randall <i>et al.</i> , 1987
PIV5-M-f (mAb)	PIV5 M	Randall <i>et al.</i> , 1987
PIV5-M-h (mAb)	PIV5 M	Randall <i>et al.</i> , 1987
Bun 592 Rabbit polyclonal	Bun N	Kindly provided by Prof. Richard Elliott (University of St. Andrews)
Anti-Flag (mAb)	Flag-tagged proteins	SIGMA
Anti-HA mouse polyclonal	HA-tagged proteins	SIGMA
eIF2- α -P (mAb)	Phosphorylated eIF2- α	Cell Signalling Technology

2.5.2 Immunofluorescence

For immunofluorescence analysis, cells were grown on 10 mm-diameter coverslips (General Scientific Co. Ltd., Redhill, United Kingdom) in individual wells of 6 well or 24 well plates. Cells were treated as necessary for different experiments, including treatment by stimulation with IFN. Monolayers were first incubated in fixing solution (5% formaldehyde and 2% sucrose in PBS) for 15 minutes at room temperature, then permeabilized (5% Nonidet-P40 and 10% sucrose in PBS) for 5 min, and washed three times in PBS containing 1% FCS and 0.1% Azide. To detect the proteins of interest, cell monolayers were incubated with 20 μ l of appropriately diluted antibody for 1 h (primary antibody). Cells were subsequently washed in PBS containing 1% FCS and 0.1% Azide several times, and the antibody-antigen interactions were detected by indirect immunofluorescence (1 h incubation) with a secondary antibody, depending on the

primary antibody used. In addition, cells were stained with the DNA-binding fluorochrome 4', 6'-diamidino-2-phenylindole (DAPI, 0.5 µg/ml; Sigma-Aldrich Co Ltd., United Kingdom) for nuclear staining. All reactions were performed at room temperature and antibody treatments were allowed to progress in a humidified chamber in order to prevent desiccation of the monolayers. Following staining, monolayers were washed with PBS, mounted with coverslips using Citifluor AF-1 mounting solution (Citifluor Ltd., United Kingdom) and examined under a Nikon Microphot-FXA immunofluorescence microscope.

2.5.3. Immunostaining

When crystal violet staining was not sufficient for the visualization of sites of virus infection due to small plaques, immunostaining was carried out instead. For immunostaining analysis, MRC5 and MRC5/PIV5V cells were grown in 6-well plates and infected with virus preparations as mentioned above. At 3 to 10 days pi depending on the virus, the medium was aspirated off and the monolayers were fixed in 5% formaldehyde and 2% sucrose in phosphate-buffered saline (PBS) for 60 min. Monolayers were then permeabilized (5% Nonidet-P40 and 10% sucrose in PBS) for 15 min and washed in PBS containing 1% FCS. Areas of cell lysis and therefore sites of virus infection or plaques were visualised by immunostaining, by detecting the viral proteins of interest. Cell monolayers were incubated with 500 µl (per well) of appropriately diluted antibody for 90 min (primary antibody) on a rocking platform at room temperature. Cells were subsequently washed with PBS and monolayers were incubated with 500 µl (per well) of appropriately diluted secondary alkaline phosphatase-conjugated antibody, depending on the primary antibody used, for 90 min on a rocking platform. Monolayers were subsequently washed with water and incubated for 30 min with 500 µl (per well) of alkaline phosphatase substrate (Sigma Ltd., United Kingdom, catalogue number B56555) until sites of virus infection were easily visualised.

2.5.4 SDS polyacrylamide gel electrophoresis

Protein samples were prepared in gel electrophoresis sample buffer (0.05 M Tris-HCl pH 7.0, 0.2% SDS, 5% 2-mercaptoethanol, and 5% glycerol) and heated at 100°C for 5 min prior to electrophoresis analysis. Polypeptides were separated through SDS-PAGE (7 to 12% bis-acrylamide) in thin (0.75 mm) mini-slab gels of the Bio-Rad mini-protean II electrophoresis system, by electrophoresis at 150 to 180 V until maximum resolution of polypeptide bands. More recently, polypeptides were also separated through 4-12% polyacrylamide gradient gels (Invitrogen), by electrophoresis at 160 V.

2.5.5 Immunoblotting

Cells were washed twice in PBS prior to harvesting, and subsequently disrupted in SDS gel electrophoresis loading buffer. Cell lysates were then sonicated and heated at 100°C for 5 min. Polypeptides in samples were separated by SDS-PAGE as described above, and transferred to nitrocellulose membrane using a Trans-Blot Cell (Bio-Rad, United Kingdom), assembled according to the manufacturer's instructions, in standard transfer buffer (39 mM Glycine, 48 mM Tris base and 20% (v/v) Methanol) for 2 hours at constant current of 200 mAmp. Following electroblotting, any unoccupied protein binding sites on the membrane were blocked by incubation of the membrane in 10% (w/v) skimmed milk powder (Marvel) and 0.1% polyoxyethylene sorbitan monolaurate (Tween 20) in PBS (blocking buffer) for 1 hour. The proteins in study were detected by incubating the membrane with the appropriate monoclonal antibodies (see Table 2.1) in antibody suspension (1/100 to 1/1000 dilution in blocking buffer) for 1 h or longer, depending on the antibody. The membrane was then washed carefully (three washes, each involving 15 to 30 min incubation on a rocking platform) to remove unbound primary antibody. The protein-antibody interactions were detected by incubation of the membrane with a secondary peroxidase-conjugated antibody suspension (1/1000 dilution in blocking buffer) for a maximum of 1 h on a rocking platform. The membrane was then washed as described above, and the protein bands were visualised by enhanced chemiluminescence according to the manufacturer's recommendations (Amersham Biosciences Ltd., United Kingdom).

3. RESULTS

IFN-sensitive viruses are viruses that are attenuated in their ability to circumvent the host IFN response and would therefore be potential candidates for the generation of live attenuated viral vaccines (Didcock *et al.*, 1999a, Garcia-Sastre *et al.*, 1998, Young *et al.*, 2003). IFN-sensitive viruses could be generated “artificially” using reverse genetics and DNA technology techniques by deletion or truncation of the IFN antagonist gene from the viral genome or by site-directed mutagenesis, if an attenuating mutation was previously known and described. An alternative to reverse genetics is to attempt to select naturally-occurring IFN-sensitive viruses from an IFN-resistant virus population. Section 3.2 describes the design and development of a model system with the aim of selecting IFN-sensitive viruses from an IFN-resistant virus population by use of a cell-based assay. Whichever method is used to obtain IFN-sensitive viruses, there is always the need to amplify and grow them to high titres to allow their analysis and sequencing of the viral genome to identify the attenuating mutation(s). The attenuated viruses also need to be grown to high titres if they are to be tested and used as live attenuated vaccines. Section 3.1 describes the development of IFN-permissive cell lines for this purpose.

3.1 Generation of IFN-permissive cell lines for the growth and amplification of IFN-sensitive viruses

Vero cells (monkey kidney cells) are an IFN-permissive cell line for they do not produce IFN due to a spontaneous gene deletion (IFN- α/β genes; Desmyter *et al.*, 1968; Mosca & Pitha, 1986), but can respond to IFN if it is added exogenously. As part of a group effort, it was of interest to specifically engineer IFN-permissive cell lines expressing viral IFN antagonists (Young *et al.*, 2003), with the aims of comparing viral growth and spread in the different generated cell lines and Vero cells, and amplifying any IFN-sensitive viruses selected from given virus populations. To decide on the best cell line(s), it was important to compare the generated cell lines regarding their ability to support the growth of IFN-sensitive viruses. The constitutive expression of viral IFN antagonists in cells should allow the growth and spread of IFN-sensitive viruses, due to a block of the host's IFN response. The host's IFN response can be targeted by blocking the IFN induction pathways, the IFN signalling pathways and/or the action of the IFN-induced antiviral proteins, depending on which antagonist(s) is being stably expressed in the mammalian cells.

My part in this project was to generate a mammalian stable cell line expressing a tagged version of the E3L protein of Vaccinia virus (VV). The E3L protein is one of the VV IFN antagonists: it specifically binds to and sequesters dsRNA and therefore blocks the IFN production pathway (Xiang *et al.*, 2002) and antiviral proteins that depend upon dsRNA for activation, such as PKR and OAS (Chang *et al.*, 1992).

A lentivirus-based vector system was used to establish the IFN-permissive stable cell lines expressing the E3L protein of VV. Lentiviral vectors can accommodate long sequences, the products of which are stably expressed due to integration into the cell chromosome. The lentiviral system widely used in our laboratory to express foreign proteins relies on the simultaneous transfection of three plasmids containing the necessary elements for the production of replication-defective lentivirus particles, into

293T producer cells. The gene(s) of interest is cloned into one of the plasmids, generally under the control of the constitutively-expressing SFFV (Spleen Focus-Forming Virus) promoter. The lentivirus particles produced are released into the medium, which is then harvested and used to directly infect the mammalian cell line(s) of interest. If necessary, transduced cells can be selected by treatment with the selection antibiotic (generally puromycin or blasticidin).

To generate the lentivirus vectors, the E3L gene was amplified by PCR from two independent plasmids, pCMV-Flag E3L and pCMV-HA E3L, using the following primers: E3L-HAfw, E3L-HA/FLAGrev and E3L-FLAGfw. The pCMV-Flag E3L vector contains an N-terminal Flag tagged E3L, while the pCMV-HA E3L contains an N-terminal HA tagged E3L. Following PCR, the PCR products were digested with NdeI and NotI enzymes and cloned into the same sites present in the pdlNotI'MCS'F vector, thus generating two independent vectors: pdl'HA-E3L'puro and pdl'Flag-E3L'puro (figures 3.1 and 3.2). The pdlNotI'MCS'F vector already contained the puromycin resistance gene termed pac under the control of the constitutive-expressing SFFV promoter. These two vectors were used to generate lentivirus stocks that were further used to infect HEp2 cells. The HEp2 cell line was the chosen cell line to express the IFN antagonists as HEp2 cells (human epithelial cells established from a laryngeal carcinoma) are easy to grow and maintain in cell culture, can be passaged indefinitely and had previously been used in our laboratory to establish stable cell lines using the lentiviral vector. Vero cells would have offered an advantage in relation to HEp2 cells, because they do not produce IFN due to a spontaneous gene deletion (IFN- α/β genes; Desmyter *et al.*, 1968; Mosca & Pitha, 1986) and thus would have better supported the amplification of attenuated viruses. However, the presence of an inhibitory cellular factor in simian cells restricts lentivirus replication at an early post-entry step, thereby blocking the transduction of simian cells using lentivirus-based vectors (Kootstra *et al.*, 2003). Consequently, it would not have been possible to generate Vero cell lines using the lentivirus-based vector system.

The lentivirus-infected HEp2 cells were subjected to puromycin selection, which was maintained for approximately 10 days after which time, total cell extracts were harvested

Figure 3.1 Schematic diagram of the cloning procedure of the HA-tagged E3L gene of VV into a lentivirus vector.

The HA-tagged E3L gene of VV was obtained by PCR from the pCMV HA-E3L vector using the E3L-HA_{fwd} (containing an NdeI restriction site) and E3L-HA/FLAG_{rev} (containing a NotI restriction site) primers. The DNA gel represents the PCR fragment obtained (600 bp). Both PCR fragment and backbone lentivirus vector (pdlNotI'MCS'F) were digested with NdeI and NotI and ligated overnight. The resulting vector, pdl'HA-E3L'pac, contains the HA-tagged E3L and pac genes under the control of the constitutive SFFV promoter.

Figure 3.1

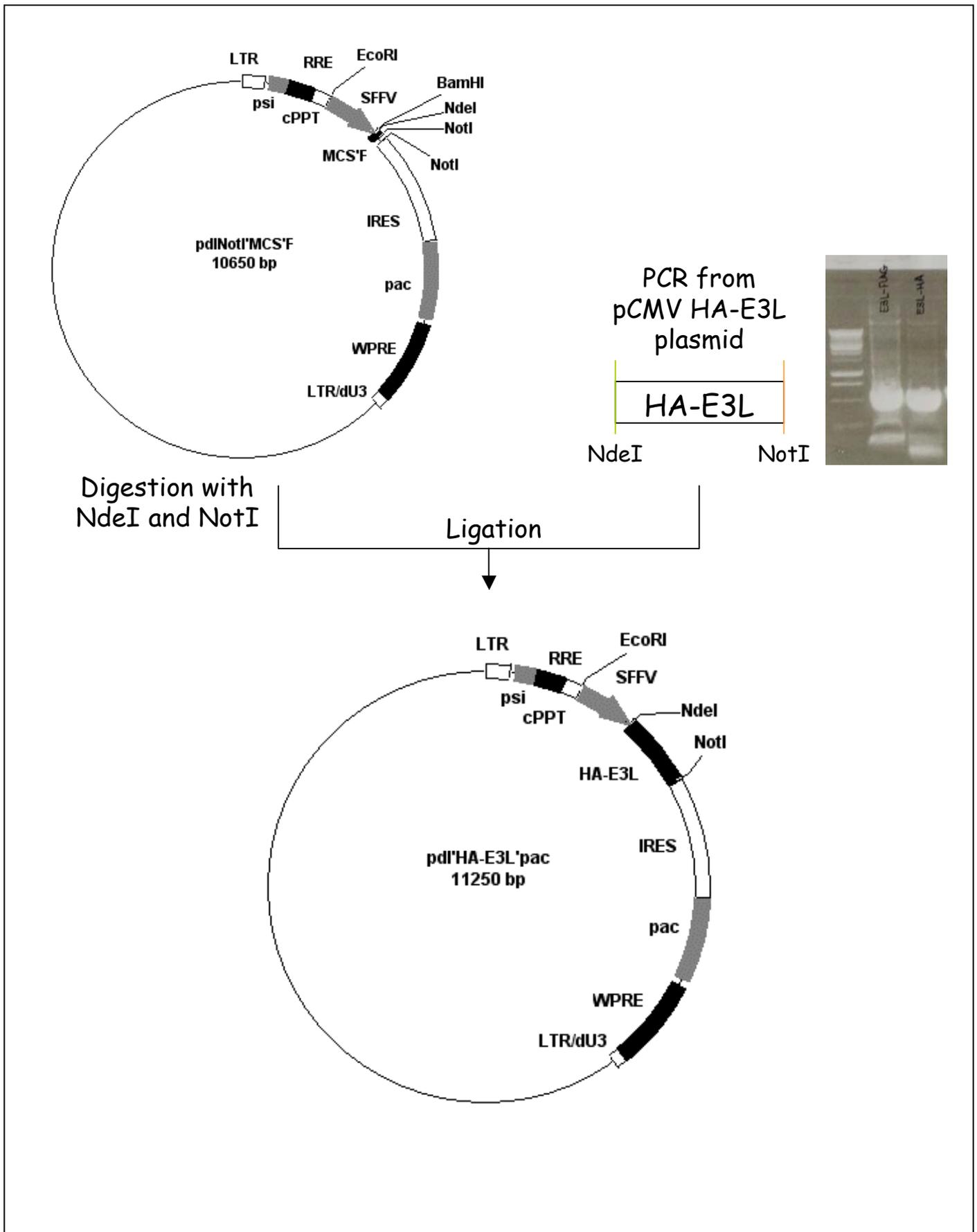
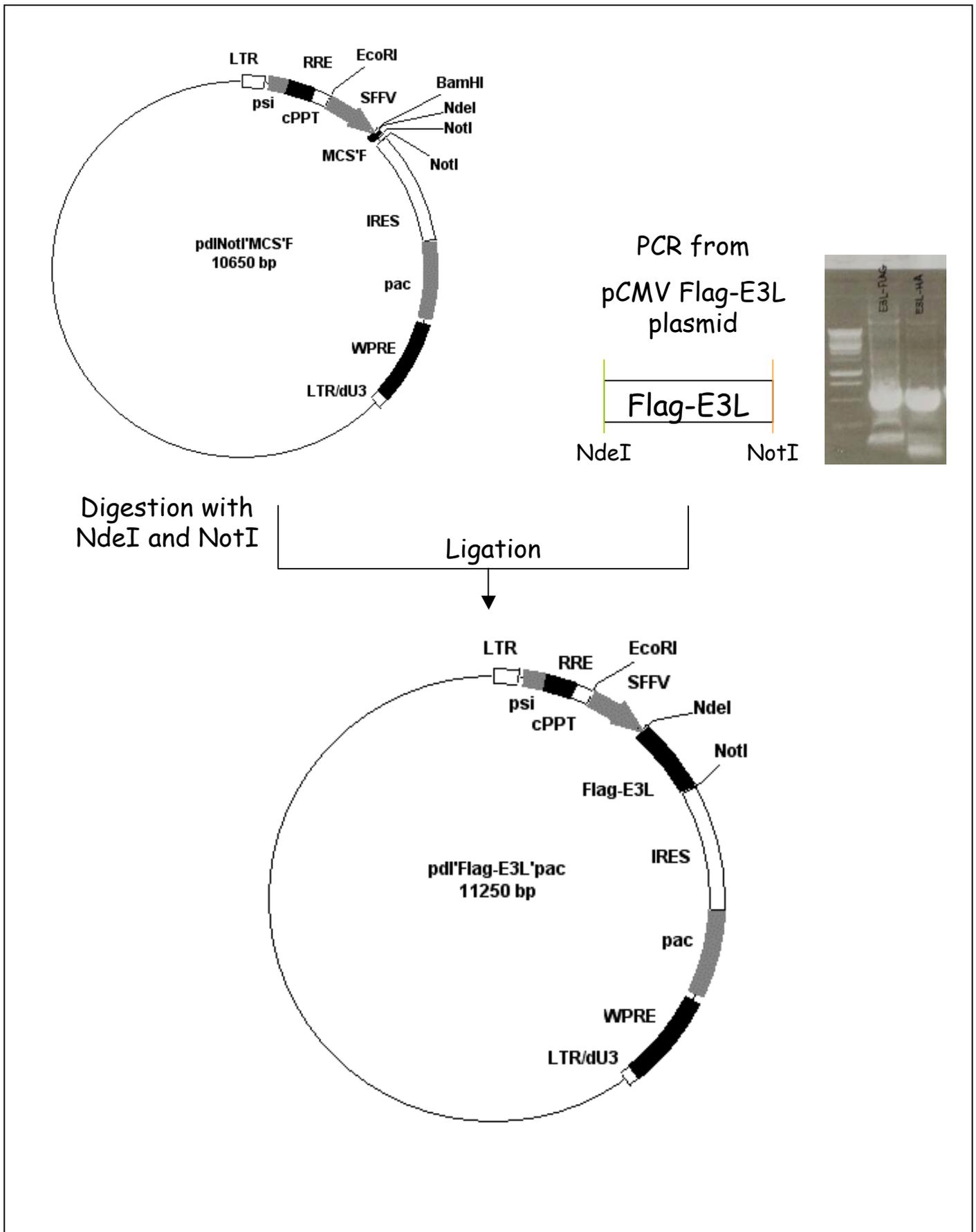


Figure 3.2 Schematic diagram of the cloning procedure of the Flag-tagged E3L gene of VV into a lentivirus vector.

The Flag-tagged E3L gene of VV was obtained by PCR from the pCMV Flag-E3L vector using the E3L-FLAGfwd (containing an NdeI restriction site) and E3L-HA/FLAGrev (containing a NotI restriction site) primers. The DNA gel represents the PCR fragment obtained (approximately 600 bp). Both PCR fragment and backbone lentivirus vector (pdlNotI'MCS'F) were digested with NdeI and NotI and ligated overnight. The resulting vector, pdl'Flag-E3L'pac, contains the Flag-tagged E3L and pac genes under the control of the constitutive SFFV promoter.

Figure 3.2



and analysed by western blot using primary antibodies against Flag and HA (figure 3.3). Immunoblotting results indicated that E3L was being expressed in both cell lines (HEp2/Flag-E3L and HEp2/HA-E3L). Expression of E3L was also detected by immunofluorescence analysis (figure 3.4), again using primary antibodies against the Flag and HA tags. As observed, E3L is expressed in both cell lines as a nuclear protein.

After detecting the expression of E3L, it was necessary to verify if the protein was functional. As mentioned before, E3L is an IFN antagonist that blocks the activation of PKR by preventing PKR binding to dsRNA (Chang *et al.*, 1992, Xiang *et al.*, 2002). However, if PKR is activated by dsRNA, it will phosphorylate the α -subunit of the eukaryotic translation initiation factor eIF2. This in turn will lead to rapid inhibition of the translation of cellular and viral mRNAs (reviewed in Clemens & Elia, 1997). Thus, to determine if E3L was functional in the HEp2/Flag-E3L cell line, naïve HEp2 and HEp2/Flag-E3L cell lines were infected with either CPI- or mock infected and stimulated with exogenous IFN (or untreated) at 12h pi. Total cell extracts were obtained at 18h pi. Samples were analysed by immunoblotting using a primary antibody against the phosphorylated form of eIF2- α (figure 3.5). Naïve HEp2 cells were used as the control and, as observed, levels of phosphorylated eIF2- α were very low in HEp2 uninfected cells, while there was an increase for HEp2 CPI- infected cells, indicating that virus infection triggered the IFN system, leading to activation of PKR and consequently, phosphorylation of eIF2- α . However, for HEp2/Flag-E3L cells, the levels of phosphorylated eIF2- α did not increase when cells were infected with CPI- indicating that expression of E3L protein was indeed blocking the phosphorylation of eIF2- α by PKR. Therefore, the E3L protein expressed in the HEp2/Flag-E3L cell line was inhibiting protein synthesis. This immunoblotting analysis to show the functionality of E3L in the HEp2/Flag-E3L cell line was carried out by Dr. Teresa Sequeira Carlos.

After the generation and characterisation of HEp2 cell lines expressing other viral IFN antagonists, such as the V protein of PIV5 (HEp2/PIV5V cells) and the Npro protein of BVDV (HEp2/BVDVNpro cells), by other members of our group, the cell lines' ability to support the replication and growth of attenuated viruses was tested (the assays were

Figure 3.3 Immunoblotting analysis indicated that the E3L protein was being expressed in both HEp2/HA-E3L and HEp2/Flag-E3L cell lines.

Total cell extracts were obtained from naïve HEp2, HEp2/HA-E3L and HEp2/Flag-E3L cell lines. Cell extracts were analysed by immunoblotting using primary antibodies against the HA and Flag tags, and secondary anti-mouse HRP-conjugated antibodies. Results clearly indicated that the tagged E3L protein (approximately 30 kDa) was being expressed in both HEp2/HA-E3L and HEp2/Flag-E3L cell lines.

Figure 3.3

1. HEp2 cells
2. HEp2/HA-E3L cells
3. HEp2/Flag-E3L cells

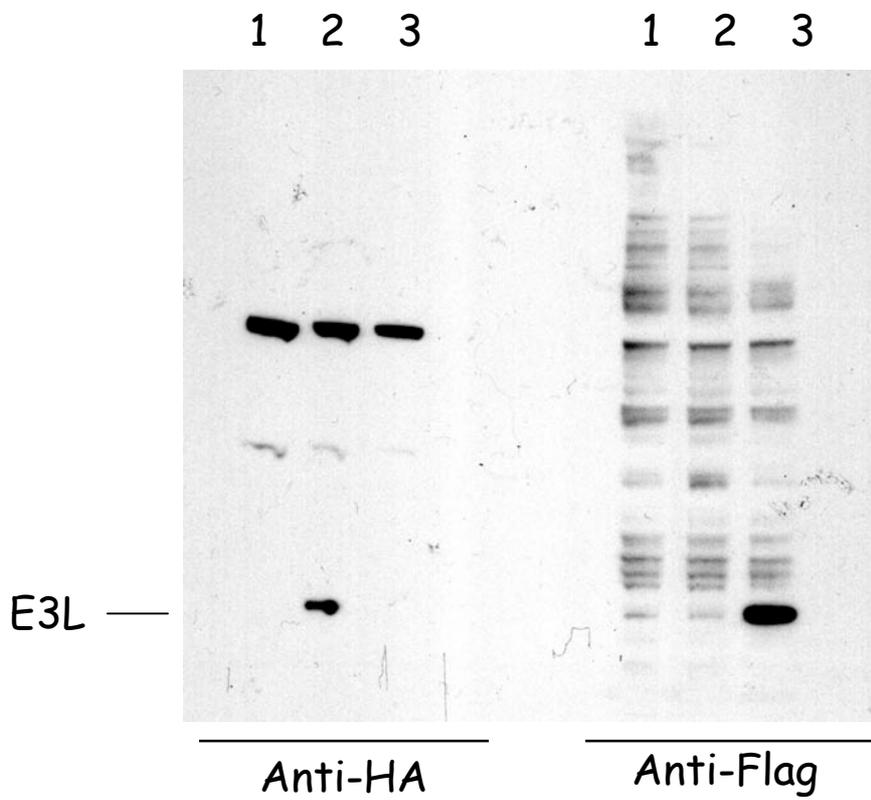


Figure 3.4 Expression of E3L in HEp2/HA-E3L and HEp2/Flag-E3L cell lines was verified by immunofluorescence analysis.

Naïve HEp2, HEp2/HA-E3L and HEp2/Flag-E3L cells were setup as monolayers on coverslips. After fixing cells with formaldehyde solution, monolayers were stained with primary antibodies against HA or Flag tags and secondary anti-mouse FITC-conjugated secondary antibody. Results clearly indicated that E3L was expressed as a nuclear protein in both HEp2/HA-E3L and HEp2/Flag-E3L cell lines.

Figure 3.4

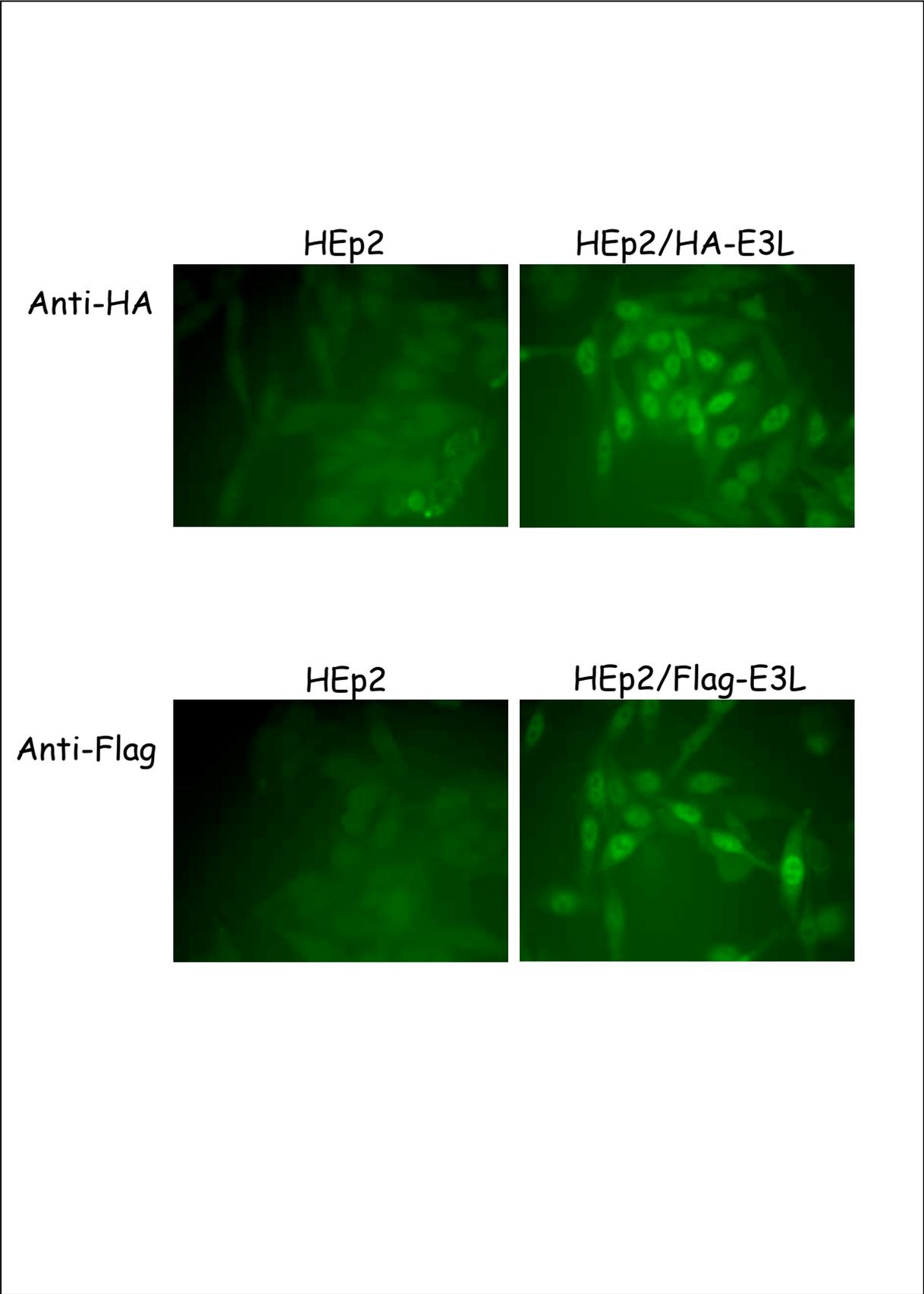
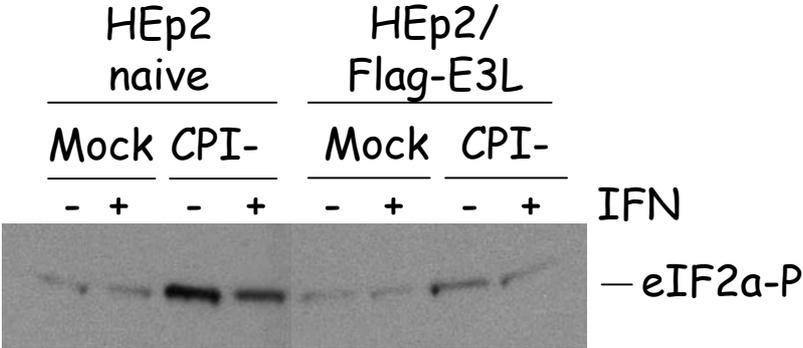


Figure 3.5 Immunoblotting analysis against phosphorylated eIF2- α indicated that the E3L protein was functional in HEp2/Flag-E3L cells.

HEp2 and HEp2/Flag-E3L cells were infected with CPI- or mock infected and stimulated with exogenous IFN (or untreated) at 12h pi. Total cell extracts were harvested at 18h pi. Samples were run on SDS-polyacrylamide gels and immunoblotting analysis was carried out using a primary antibody against the phosphorylated form of eIF2- α and an anti-mouse HRP-conjugated secondary antibody. If the E3L protein was functional, the levels of phosphorylated eIF2- α would be similar in infected and uninfected cells because E3L indirectly blocks the phosphorylation of eIF2- α that occurs after viral infection. As observed, the levels of phosphorylated eIF2- α were the same for HEp2/Flag-E3L cells, but increased in CPI- infected naïve HEp2 cells when compared to uninfected, indicating that E3L is functional in the HEp2/Flag-E3L cell line.

Figure 3.5



carried out by Dr. Monica Galiano). Naïve HEp2, HEp2/BVDVNpro, HEp2/PIV5V and HEp2/Flag-E3L cells were infected with the Enders strain of MuV (an attenuated virus). Supernatant was harvested from the cells at 2, 4, 6 and 8 days post-infection and was then used to carry out plaque assays on Vero cells to titrate the amount of virus present in the supernatant. The results (figure 3.6) clearly showed that the cell lines expressing the IFN antagonists are better at supporting the replication of the attenuated MuV than the naïve HEp2 cells; however, the cell line that best supports the replication of the attenuated virus is the HEp2/BVDVNpro cell line. Later on, another member of the group, Yun Hsiang Chen, generated a HEp2 cell line expressing both the Npro protein of BVDV and the V protein of PIV5 - HEp2/BVDVNpro/PIV5V cells. When expressed independently in HEp2 cells, both these proteins allowed the efficient amplification of the attenuated MuV (figure 3.6). However, these two proteins block different pathways in the host's IFN response and thus, when expressed together, should allow for even better amplification of attenuated viruses than when expressed independently. It was therefore decided to use the HEp2/BVDVNpro/PIV5V cell line to amplify and grow any selected IFN-sensitive viruses.

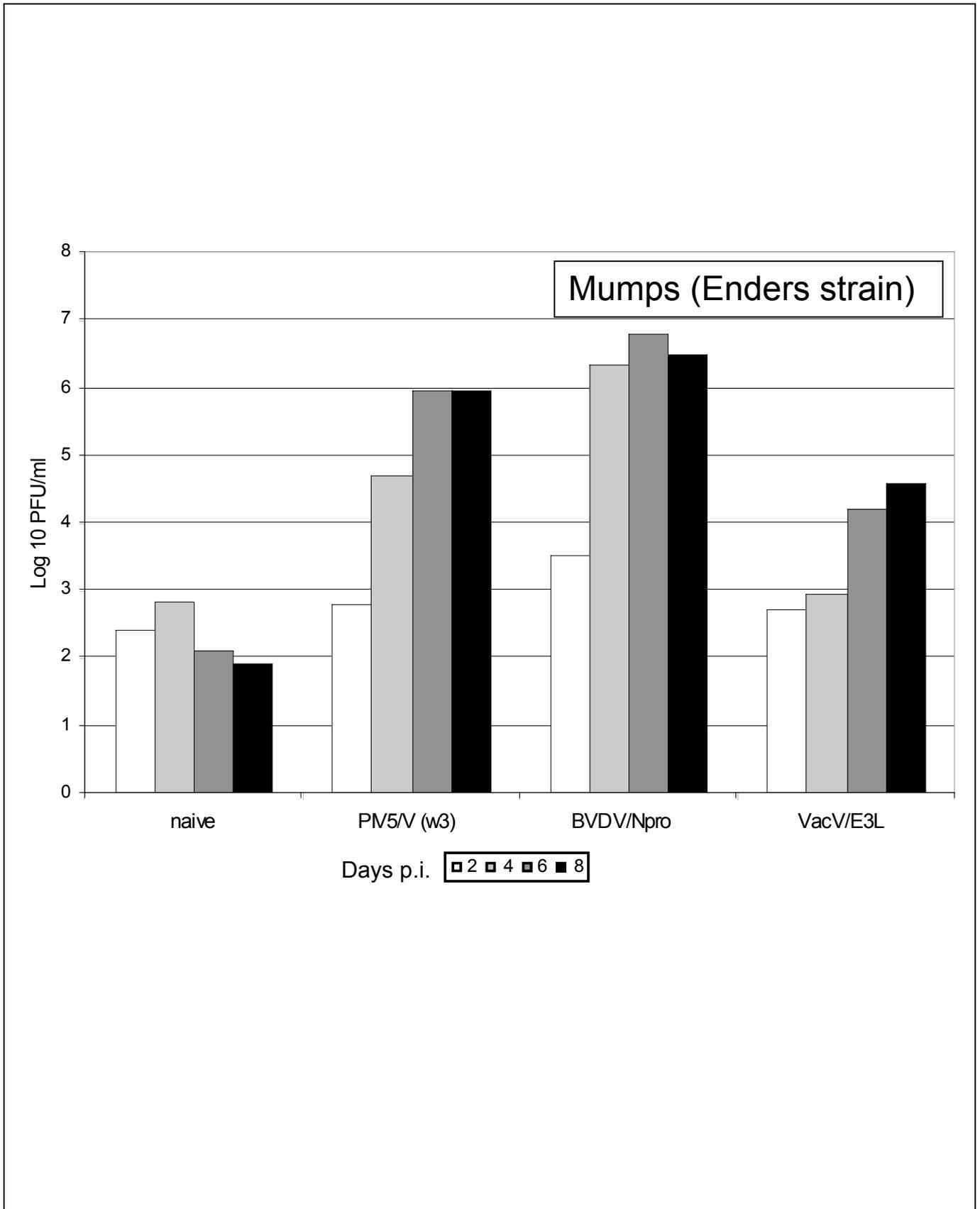
The next section (section 3.2) describes the development of methodologies for the selection of naturally-occurring IFN-sensitive viruses from given virus populations.

Figure 3.6 Comparison of the ability of different HEp2 cell lines, naïve or expressing viral IFN antagonists, to support the growth of the attenuated Enders strain of Mumps virus.

HEp2 cells were previously engineered to express the Npro protein of BVDV (HEp2/BVDVNpro), the V protein of PIV5 (HEp2/PIV5V) or the E3L protein of VV (HEp2/Flag-E3L) using a lentivirus vector expression system.

Naïve HEp2, HEp2/BVDVNpro, HEp2/PIV5V and HEp2/Flag-E3L cells were infected with the attenuated Enders strain of Mumps virus. Supernatant was harvested from the cells at 2, 4, 6 and 8 days post-infection and was then used to carry out plaque assays on Vero cells to titrate the amount of virus present in the supernatant. As observed, all the three cell lines expressing viral IFN antagonist proteins are better at supporting the replication and growth of attenuated Mumps virus than naïve HEp2 cells. This assay demonstrates that, out of the four cell lines tested, the best one to amplify the attenuated Mumps virus is the HEp2/BVDVNpro cell line, although the HEp2/PIV5V cell line also demonstrates potential to amplify the attenuated virus.

Figure 3.6



3.2 General principles for the development of a methodology to select IFN-sensitive attenuated viruses and IFN-resistant revertant viruses

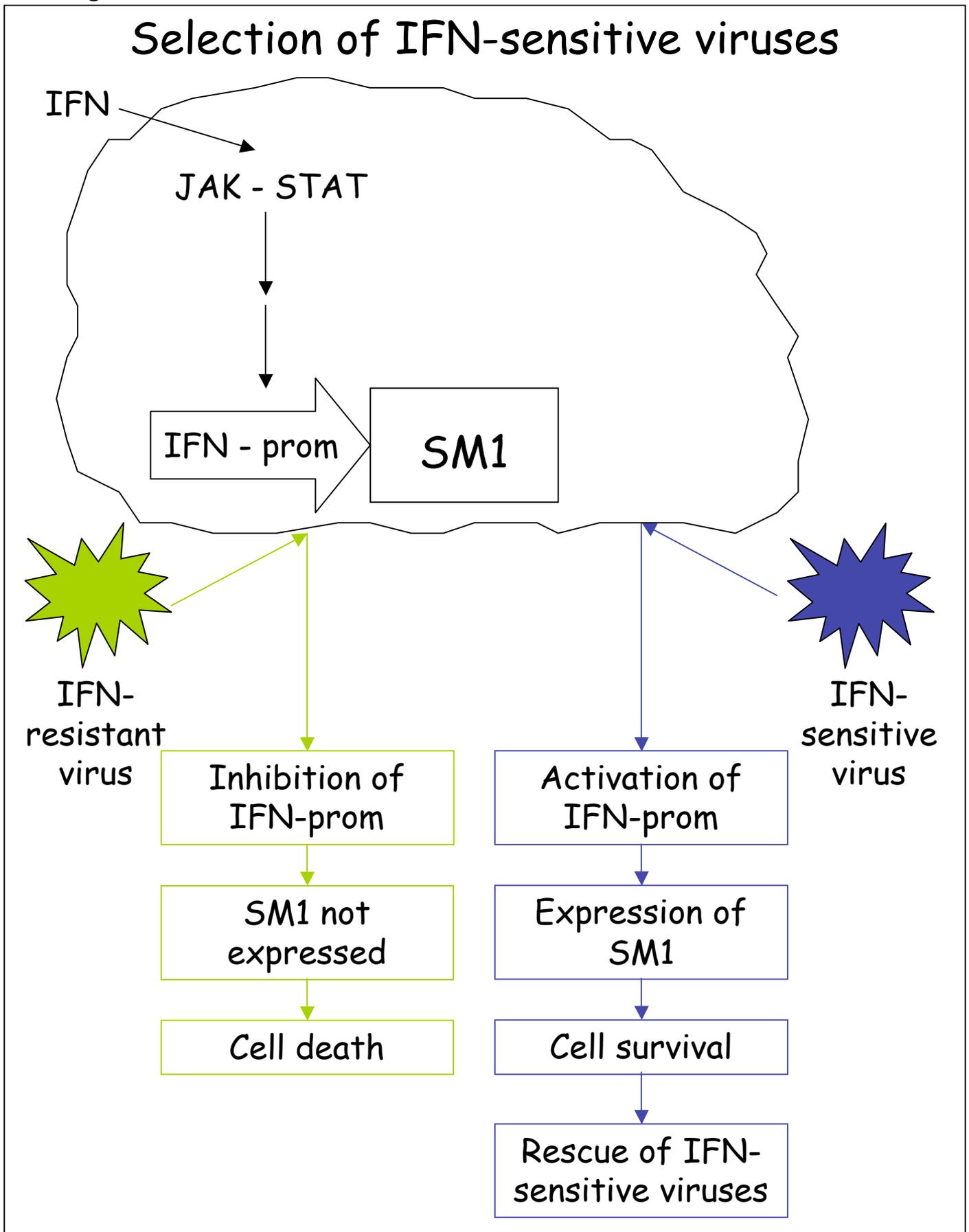
As mentioned previously, newly identified IFN-sensitive viruses may potentially be developed as live attenuated viral vaccines. To address this question and to further characterise the wild-type IFN-resistant viruses in the way they circumvent the IFN response and understand which mutations could render them sensitive to IFN, a model system in which IFN-sensitive viruses could be selected from a given virus population by using a cell-based assay was designed.

The aim was to select viruses that had lost their ability to block the IFN signalling pathway and consequently the expression of the IFN-induced antiviral proteins. To select for IFN-sensitive viruses, an essential requirement was to use a cell line that, upon infection with a mixed virus population and subsequent selection process, would depend on a functioning IFN response for cell survival. Therefore, it seemed appropriate to specifically engineer a cell line that would require a functioning IFN-responsive promoter for cell survival under a selection process. When cells undergo an infection, they respond by producing IFNs that trigger signalling pathways in neighbouring uninfected and infected cells which lead to the activation of IFN-inducible promoters, which ultimately lead to the expression of antiviral proteins. Thus, cells infected with IFN-resistant viruses should not survive the selection process because the viral IFN antagonist protein(s) would block the IFN response (more specifically, the viral antagonist would block IFN signalling). However cells infected with viruses unable to inhibit the IFN signalling pathway should survive because cell signalling would remain intact, thereby ensuring cell survival. Subsequently, these IFN-sensitive viruses would need to be rescued from the surviving cells and grown in cells containing a defect in the IFN response (it was previously reported that HEp2/BVDVNpro/PIV5V cells would be the best cell line to do so; refer to section 3.1). Figure 3.7 is a representation of the positive selection process using a cell line containing a selectable marker (SM1, which cells would need to be

Figure 3.7 Schematic representation of the cell line of interest and the positive selection process that should ultimately lead to the selection of IFN-sensitive viruses from an IFN-resistant virus population.

‘SM1’ represents a selectable marker which cells would need to be expressed in order to survive the selection process; ‘IFN-prom’ represents an IFN-inducible promoter that would lead to the expression of SM1 when activated by IFN. The green section demonstrates what would happen if a cell was infected with an IFN-resistant virus and the blue section demonstrates what would happen if a cell was infected with an IFN-sensitive virus. If a cell was infected with an IFN-resistant virus, it would block the host’s IFN response and would consequently block the induction of the IFN-inducible promoter. This would lead to cell death, because the SM1 protein would not be produced in this cell. If a cell was infected with an IFN-sensitive virus that could no longer block the hosts’ IFN response, the IFN-inducible promoter would be induced, leading to the expression of the SM1 protein which would allow this particular cell to survive the selection process.

Figure 3.7



expressed in order to survive) under the control of an IFN-inducible promoter. It illustrates what would happen if a cell was to be infected with an IFN-resistant virus (that would block the IFN signalling process or JAK-STAT pathway) or with an IFN-sensitive virus.

In addition to the selection of IFN-sensitive viruses, it was also of interest to determine if any IFN-resistant revertant viruses would spontaneously arise from an IFN-sensitive virus population after a certain number of passages in tissue culture cells, and what the time scale for this process would be. IFN-resistant revertant viruses could be useful in pinpointing specific amino acids in the viral proteins that might be essential regarding viral IFN sensitivity. For the selection of IFN-resistant revertant viruses, the chosen cell line would have to contain a second selectable marker (SM2, which would lead to cell death if expressed) under the control of the same IFN-inducible promoter. Figure 3.8 is a representation of the negative selection process and what would occur if a cell was to be infected with an IFN-sensitive virus or with an IFN-resistant revertant virus.

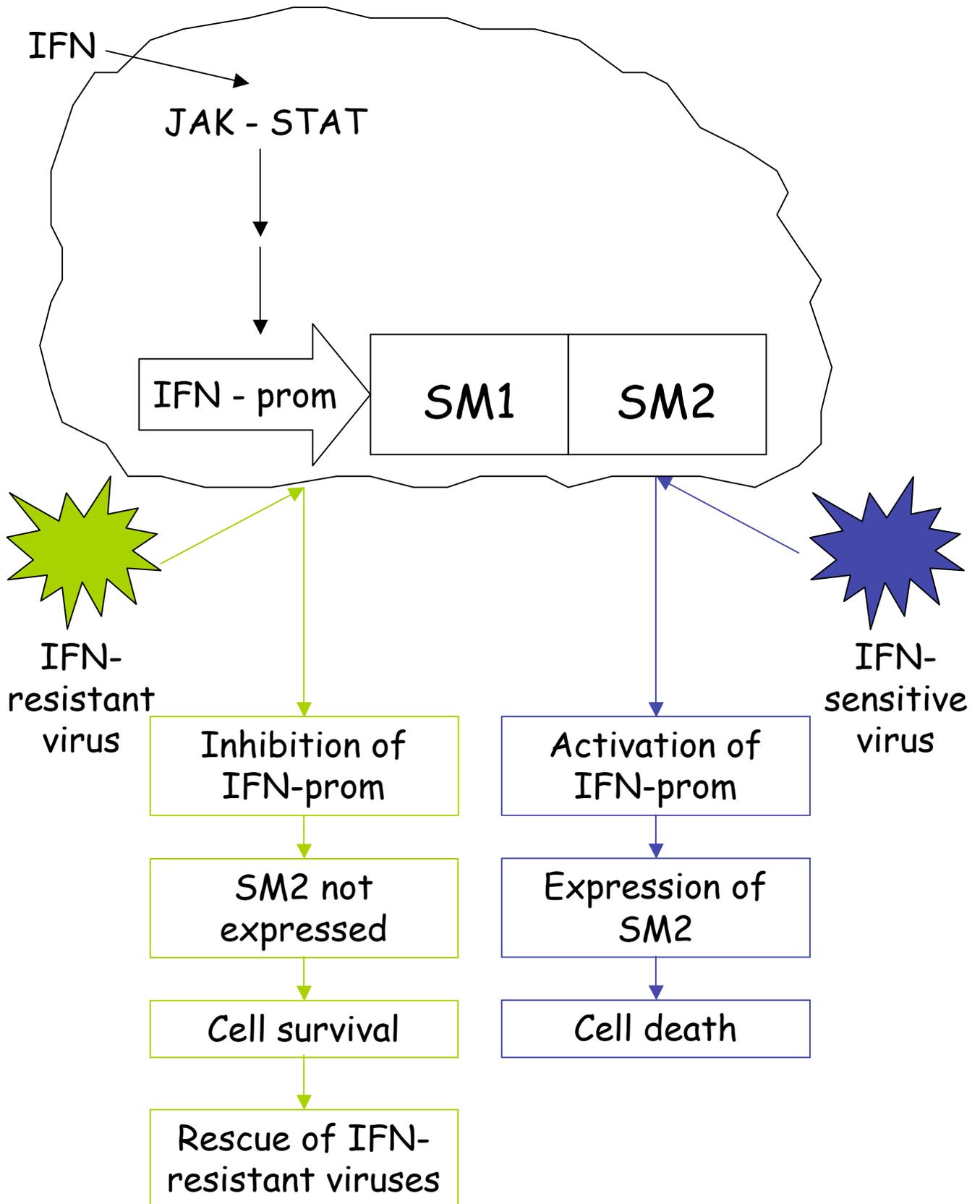
IFN-sensitive viruses will most probably contain the attenuating mutation(s) in the gene(s) that encodes the IFN antagonist protein(s). Viral anti-IFN proteins are usually dispensable for virus replication in cell culture and, in the case of negative strand RNA viruses, most IFN antagonists appear to be accessory proteins, dispensable for viral infectivity but required for pathogenicity and efficient replication in the host (Garcia-Sastre, 2004, Goodbourn *et al.*, 2000). Therefore, such attenuated IFN-sensitive viruses would be suitable candidates for the generation of live attenuated viral vaccines. Additionally, RNA virus replication is associated with a high rate of mutation because the viral polymerase has no proof-reading or error-correcting abilities. Thus, it seemed adequate to test the developed methodology using negative strand RNA viruses. In addition, the virus used in this system should not kill cells immediately after infection, or it would not allow enough time for the selection process to work. PIV5 is a negative-strand RNA virus and it has been reported to establish persistent infections in human cells (Young *et al.*, 2007); it is also extensively used in the laboratory as a model paramyxovirus and was therefore the chosen virus to test the system on.

Figure 3.8 Schematic representation of the cell line of interest and the negative selection process that should ultimately lead to the selection of IFN-resistant revertant viruses from an IFN-sensitive virus population.

‘SM2’ represents a selectable marker which would lead to cell death when expressed; ‘IFN-prom’ represents an IFN-inducible promoter that would lead to the expression of SM1 and SM2. The green section demonstrates what would happen if a cell was infected with an IFN-resistant virus and the blue section demonstrates what would happen if a cell was infected with an IFN-sensitive virus. If a cell was infected with an IFN-resistant virus, it would block the host IFN response and would consequently block the induction of the IFN-inducible promoter. This would lead to cell survival, because the SM2 protein would not be produced in this cell. If a cell was infected with an IFN-sensitive virus that could no longer block the hosts’ IFN response, the IFN-inducible promoter would be induced, leading to the expression of the SM2 protein which would lead to cell death. This cell line could still be used to select IFN-sensitive viruses using the positive selection process via SM1 (see figure 3.7).

Figure 3.8

Selection of IFN-resistant revertant viruses



An additional requirement for the system to work would be the infection of the cells with a multiplicity of infection (m.o.i.) of approximately 1 plaque-forming-unit (pfu) per cell. Ideally, to allow the selection of IFN-sensitive viruses, the maximum number of cells should be infected but each cell should only be infected with one virus particle. If a cell was infected with for example, both an IFN-sensitive and an IFN-resistant virus, the IFN response would be blocked by the resistant virus and consequently, the cell would die and the IFN-sensitive virus would be lost.

3.2.1 Investigation of 2fTGH cells as a potential cell line for the selection of IFN-sensitive viruses

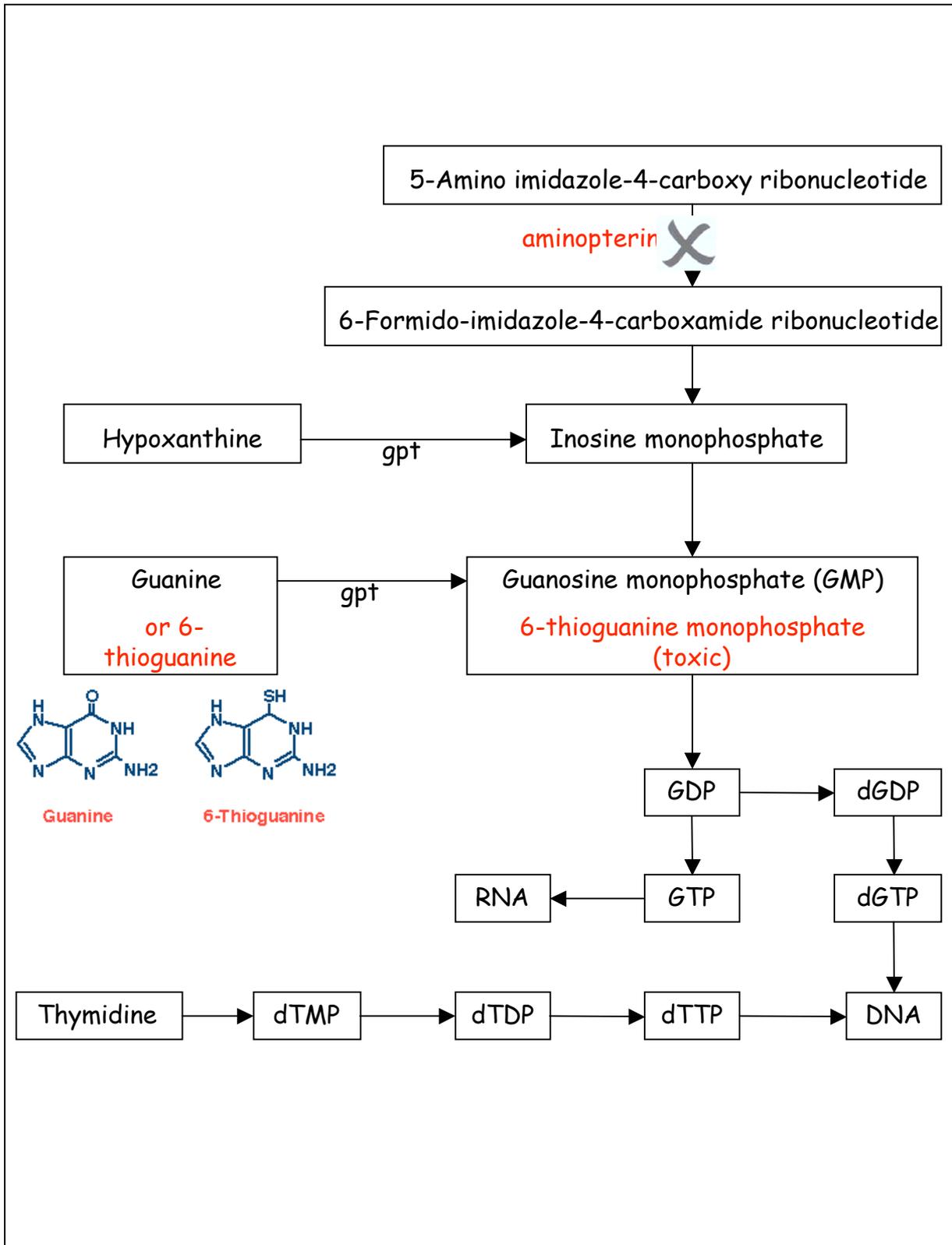
Initially, it seemed reasonable to try selecting IFN-sensitive viruses using an already established cell line termed 2fTGH cells. 2fTGH cells are human diploid fibroblasts that were first described in 1989 (Pellegrini *et al.*, 1989). The 2fTGH cell line was generated by transfection of human gpt (guanine-phosphoribosyl transferase gene) negative cells with a plasmid containing the *Escherichia coli* gpt gene under the control of the IFN-inducible 6-16 promoter. Gpt is an enzyme that exists naturally in cells and is part of the salvage biosynthetic pathway for guanosine. It is only used when the main pathway, termed *de novo* pathway, is blocked (figure 3.9). Thus, in 2fTGH cells, when the main biosynthetic pathway for guanosine is blocked, cell survival depends on a functioning IFN response for induction of the 6-16 promoter and subsequent production of the gpt enzyme which in turn, can rescue guanosine production through the salvage pathway.

HAT-supplemented growth medium contains hypoxanthine, aminopterin and thymidine. Aminopterin blocks the *de novo* biosynthetic pathway for guanosine; however, hypoxanthine, thymidine and a functional gpt enzyme allow for guanosine production through the salvage pathway (Goding, 1986; figure 3.9). Consequently, if stimulated with exogenous IFN (to mimic virus infection) and HAT-supplemented growth medium,

Figure 3.9 Metabolic pathways that lead to the synthesis of DNA and RNA.

When the main biosynthetic pathways are blocked with the folic acid analogue aminopterin, the cells depend on the presence of hypoxanthine, thymidine and the “salvage” enzyme gpt to survive. Gpt converts hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate. If 6-thioguanine is present in the medium, induction of gpt will lead to the synthesis of 6-thioguanine monophosphate, which will be incorporated into DNA. This incorporation is deadly to cells because single strand DNA breaks occur on the DNA strand on which guanine has been replaced by thioguanine (Pan & Nelson, 1990).

Figure 3.9



2fTGH cells should survive (as opposed to IFN untreated cells in the presence of HAT supplement).

Infection of 2fTGH cells with a given virus population at a m.o.i. of 1 to 5 pfu/cell and subsequent selection with IFN and HAT supplement should only consent the survival of uninfected cells or cells infected with IFN-sensitive viruses existent in the initial population (figure 3.10). This assay would therefore function as a model system for the enrichment of the starting virus population in IFN-sensitive attenuated viruses.

2fTGH cells were grown in HAT-supplemented medium in the presence or absence of exogenous IFN- α . The cells that were grown in the presence of exogenous IFN survived and grew to confluency whilst, when no exogenous IFN was added to the growth medium, approximately 30% of the starting population of cells had survived after 10 days incubation. However, 100% cell death should have been observed in this case, as both the main and salvage pathways for the production of guanosine were blocked by the presence of aminopterin and by the absence of gpt enzyme respectively. One possible explanation is that this selection process is very slow and would take more than 10 days to kill the cells: if guanosine production is blocked, nucleoside analogues will be incorporated into DNA in its place, which will lead to an accumulation of mutations, which will finally lead to cell death. Therefore, the 2fTGH cells seemed unsuitable for the selection of IFN-sensitive viruses as the HAT/IFN selection process wasn't working as expected.

3.2.2 Investigation of 2fTGH cells as a potential cell line for the selection of IFN-resistant revertant viruses

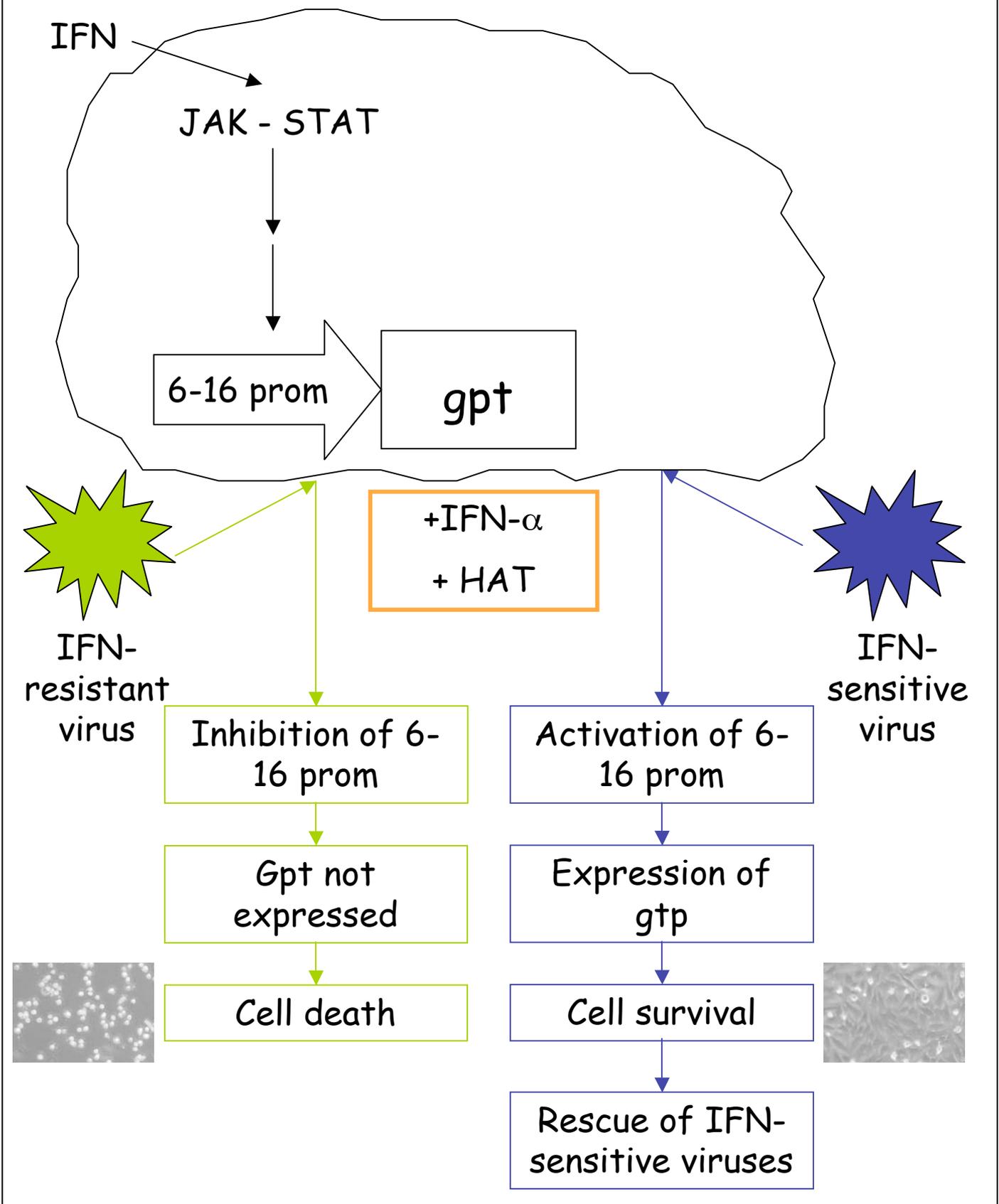
As mentioned earlier, it was also of interest to try selecting IFN-resistant viruses from an IFN-sensitive virus population. 6-thioguanine (6-TG) was used in this set of experiments as the selection drug. 6-TG is a non-toxic reagent when added to 2fTGH cells in the absence of IFN. However, gpt enzyme converts 6-TG into 6-TG monophosphate, which functions as an analogue of guanosine monophosphate and can therefore be incorporated

Figure 3.10 Representation of the selection of IFN-sensitive viruses using 2fTGH cells and the IFN/HAT selection procedure.

2fTGH cells contain the gpt enzyme under the control of the IFN-inducible 6-16 promoter. When HAT supplement is added to the growth medium, 2fTGH cells require the expression of the gpt enzyme to survive the selection process. Therefore, if a 2fTGH cell is infected with an IFN-resistant virus in the presence of exogenous IFN and HAT supplement, the virus will block the IFN response and consequently, will also block the induction of the 6-16 promoter. This will lead to cell death because of the necessity of the expression of gpt to survive the HAT toxicity. However, if a 2fTGH cell is infected with an IFN-sensitive virus that can no longer block the host IFN response, the 6-16 promoter will be activated thus leading to the expression of gpt and consequent cell survival.

Figure 3.10

Selection of IFN-sensitive viruses using 2fTGH cells (gpt⁻)



into DNA (figure 3.9). Thus, when cells are stimulated with IFN (either added exogenously or induced by virus infection), the 6-16 promoter is activated and gpt is expressed; gpt then converts 6-TG into 6-TG monophosphate, which gets incorporated into DNA. Single strand DNA breaks occur on the DNA strand on which guanine has been replaced by thioguanine (Pan & Nelson, 1990) probably due to blockage of strand extension because of its poor ability to act as a substrate for polymerase and DNA ligase (Ling *et al.*, 1992). This leads to cell death.

To test whether the selection using 2fTGH cells, 6-TG and IFN was working, 2fTGH and 2fTGH/PIV5V cells (2fTGH cells constitutively expressing the V protein of PIV5) were treated with IFN- α alone, 6-TG alone, IFN- α and 6-TG or untreated during 3 days. When IFN- α and 6-TG were added simultaneously to 2fTGH cells, cell death was observed 3-4 days after stimulation (figure 3.11). No cell death was observed in unstimulated cells or cells stimulated with either IFN or 6-TG alone, indicating that the selection process was functioning correctly. No cell death was observed in any case when using 2fTGH/PIV5V cells, because the V protein of PIV5 was blocking the JAK-STAT signalling pathway, thereby blocking the induction of the 6-16 IFN-inducible promoter. This experiment proved that the selection process was working correctly and consequently lead to an investigation of 2fTGH cells as a potential cell line for the selection of IFN-resistant revertant viruses (figure 3.12).

Besides PIV5 W3 (which is considered the wild-type virus), two closely related canine PIV5 strains are frequently used in the laboratory. These isolates are termed CPI+ and CPI- and they differ in their ability to circumvent the IFN response. CPI+ blocks IFN signalling in human cells by targeting STAT1 for proteasome-mediated degradation, whilst CPI- fails to degrade STAT1 and thus does not block IFN signalling, and is therefore an IFN-sensitive virus (Chatziandreu *et al.*, 2002). To test whether CPI+ or CPI- infection would kill 2fTGH cells, 2fTGH cells were grown to 70% confluency in 25 cm² flasks and infected with CPI+ or CPI- at a m.o.i. of 1 pfu/cell or uninfected. The infection of the cells was confirmed through immunofluorescence assays using PIV5-P-e antibody that stains the P protein of PIV5. Infection of 2fTGH cells with either CPI- or

Figure 3.11 2fTGH cells do not survive when both 6-thioguanine and IFN are added to the culture medium.

2fTGH and 2fTGH/PIV5V cells were setup on 6-well plates 48h prior to the addition of IFN or 6-TG or both. Photos were taken 3 days after stimulation with IFN and/or 6-TG. As observed, 2fTGH cells died when both 6-TG and IFN were added to the culture medium, but survived when they were added individually. As the V protein of PIV5 blocks IFN signalling, in 2fTGH/PIV5V cells, the gpt enzyme is not induced, which allows for cell survival in the presence of both IFN and 6-TG. This result demonstrates that 2fTGH might potentially be useful when used with IFN and 6-TG to select IFN-resistant revertant viruses from an IFN-sensitive population.

Figure 3.11

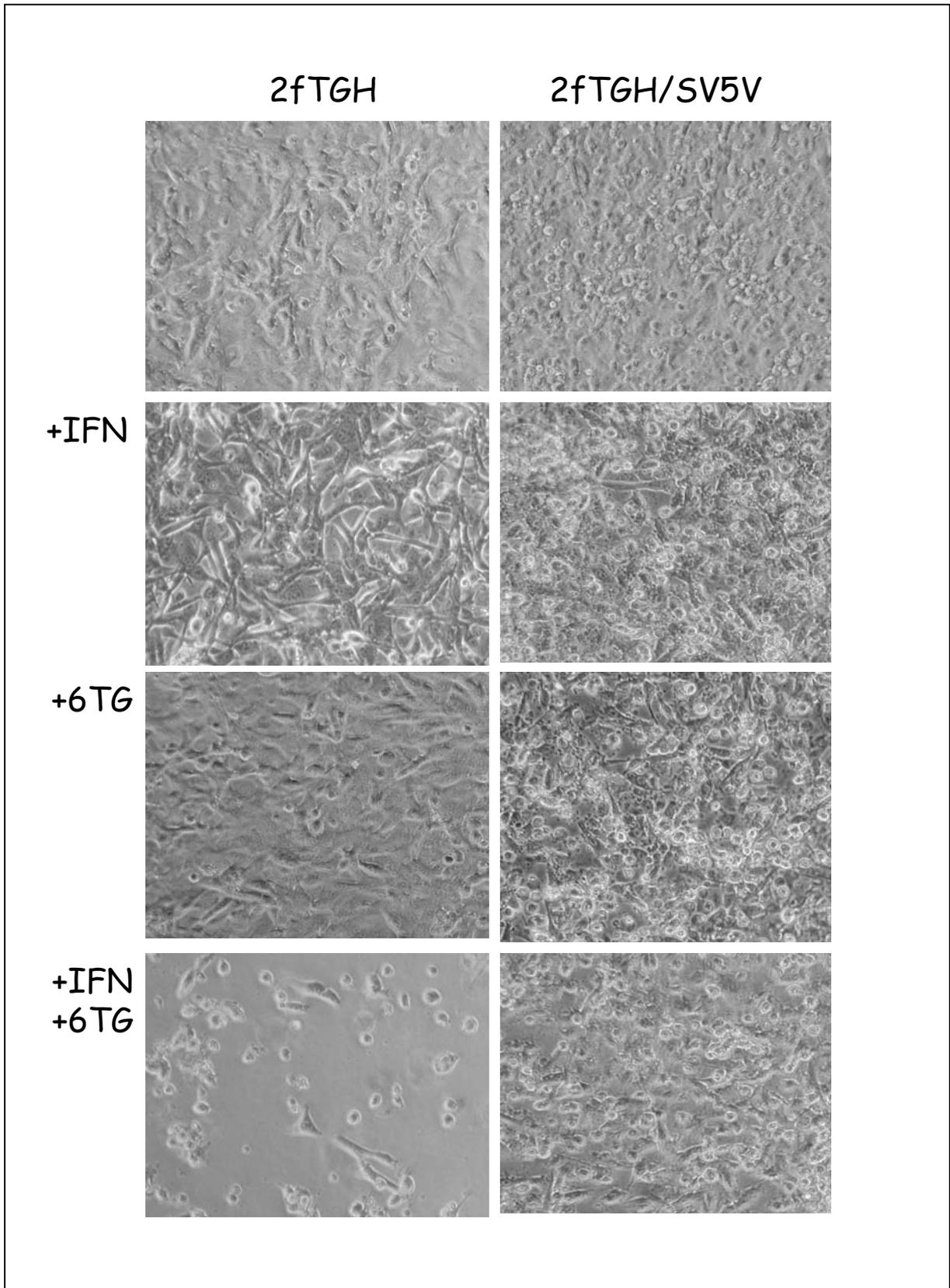
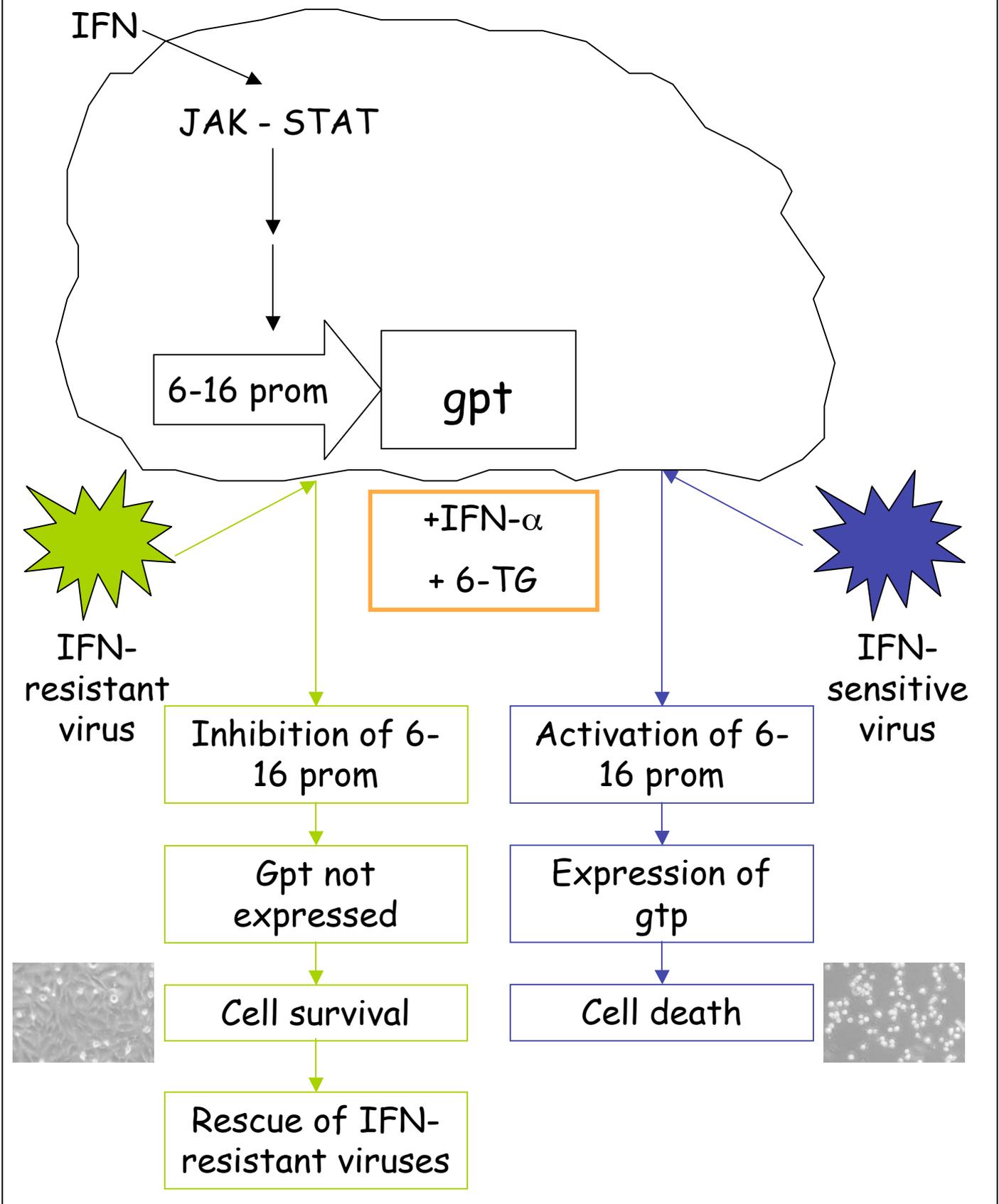


Figure 3.12 Representation of the selection of IFN-resistant revertant viruses using 2fTGH cells and the IFN/6-TG selection procedure.

2fTGH cells contain the *gpt* enzyme under the control of the IFN-inducible 6-16 promoter. When 6-TG is added to the growth medium, if *gpt* is expressed, it will convert 6-TG into a toxic product to the cells. Therefore, if a 2fTGH cell is infected with an IFN-sensitive virus that can not block the hosts' IFN response, the 6-16 promoter will be activated thus leading to the expression of *gpt* and consequent cell death. However, if a 2fTGH cell is infected with an IFN-resistant virus in the presence of exogenous IFN and 6-TG, the virus will block the IFN response and consequently, will also block the induction of the 6-16 promoter. This will lead to cell survival because *gpt* will not be expressed and will not convert 6-TG into a toxic product. Cells infected with IFN-resistant revertant viruses can thus be selected from the cell population.

Figure 3.12

Selection of IFN-resistant viruses using 2fTGH cells (gpt⁻)



CPI+ did not kill the cells. Consequently, if infected 2fTGH cells do not survive the selection process using IFN- α and 6-TG, it is not due to the viral infection, but due to the selection process itself and the toxicity of the reagents generated. Thus, to test the selection procedure, CPI+ and CPI- were used to infect 2fTGH cells.

2fTGH cells were grown to 70% confluency in 25 cm² flasks and infected with CPI- at a m.o.i. of 1 pfu/cell or uninfected. 24h after infection, cells were treated with exogenous IFN- α , 6-TG, IFN- α and 6-TG or untreated. At 4 days post-infection, 100% of CPI-infected 2fTGH cells treated with exogenous IFN- α and 6-TG had died, whilst CPI-infected cells treated with IFN- α , 6-TG or untreated had survived and grown to confluency. A possible explanation for the 100% cell death observed with added IFN- α and 6-TG is that IFN-resistant revertant viruses are present in a very small proportion or completely absent from the PIV5 CPI- population. It might have been necessary to adapt the experiment to a larger scale or to carry out more than one round of selection to select IFN-resistant revertant viruses from the CPI- population. Although the 2fTGH system using the 6-TG and IFN selection procedure seems suitable (with some adjustments) for the selection of IFN-resistant revertant viruses from an IFN-sensitive virus population, it was decided to return to the main focus of this project: the attainment of IFN-sensitive viruses.

3.2.3 Development of alternative cell lines for the selection of IFN-sensitive viruses

The 2fTGH cell line was inappropriate to select IFN-sensitive viruses from an IFN-resistant virus population; thus, it was necessary to specifically engineer a new cell line that would require a functioning IFN-responsive promoter for cell survival under a selection process. The generation of a new cell line first implied the cloning of a new vector containing all the necessary elements.

The results from the 2fTGH experiments demonstrated that the selection process of incorporating mutations in the cells' DNA through the action of the gpt enzyme was too slow at inducing cell death; therefore, the new cell line would require a faster selection process. Puromycin is a drug that induces cell death by inhibition of protein synthesis – puromycin inhibits both the synthesis of cellular and of viral proteins. Cell death is induced more rapidly through inhibition of protein synthesis than through the process of incorporation of mutations in DNA. Resistance to puromycin can be conferred to cells by the action of the puromycin N-acetyltransferase (pac) protein. The puromycin/pac system was therefore chosen as the new positive selection process, and the pac gene included in the design of the new vector.

In addition, there was some uncertainty regarding the strength of the 6-16 promoter (how quickly the 6-16 promoter responded to IFN and how strong it was at inducing gene expression); it was therefore decided to use another IFN-inducible promoter - the murine Mx1 promoter. The murine Mx1 promoter was chosen because it is a strong IFN-inducible promoter, has been characterized (Hug *et al.*, 1988) and has been previously used in cell-based assays for the quantification of type I IFNs (Canosi *et al.*, 1996, Bollati-Fogolin & Muller, 2005). In addition, it is tightly regulated by IFN and shows no basal gene expression in the absence of IFN.

Additionally, it was considered that it would be advantageous to have an immunofluorescent gene included in the system under the control of the Mx1 promoter, rather than to have to rely on antibodies to detect expression of the IFN-inducible protein(s). eGFP is a green colour variant of the green fluorescent protein from the jellyfish *Aequoria Victoria* that has been extensively used as an *in vivo* reporter (Patterson *et al.*, 2001), and was therefore included in the design of the new vector.

3.2.3.1 Generation of the pdl Mx GIPSE vector

The lentivirus-based vector system previously described was used to establish the IFN-selectable stable cell line. To generate the vector of interest, the IFN-inducible murine Mx1 promoter and the eGFP (enhanced green fluorescent protein) gene were cloned into

a backbone lentivirus vector (pdlNotI'MCS'F) thereby generating the pdl Mx GIPSE vector (figure 3.13). It was decided to use the full-length Mx1 promoter (2.3 kb) containing not only the core promoter sequence (the minimal promoter sequence required to properly initiate transcription) but also the proximal and distal promoter sequences which contain regulatory elements such as enhancers and silencers that are important to direct the level of transcription of the gene.

The pdlNotI'MCS'F backbone vector already contained the puromycin resistance gene termed *pac* under the control of the constitutive-expressing SFFV promoter. To generate the pdl Mx GIPSE vector, the Mx1 promoter was amplified by PCR from the pGL3-Mx1P vector using the fwdMx1Pseq and revMx1Pseq primers and cloned into the unique EcoRI and BamHI sites in pdlNotI'MCS'F to replace the SFFV promoter. Secondly, the eGFP gene was digested from the pHR-SIN-CSGWdlNot lentivirus vector and directly cloned into the unique BamHI and NotI sites present in the pdlNotI'MCS'F vector, to finally generate the pdl Mx GIPSE vector. The IRES (internal ribosomal entry site of EMCV) was already present in the initial vector; in the pdl Mx GIPSE vector, IRES allows simultaneous expression of the eGFP gene and the puromycin resistance gene from the Mx1 promoter. Primers were designed (termed lentiPMx1seqfwd, lentiPMx1seqrev and PMx1fwd) to sequence the Mx1 promoter and the eGFP gene from the pdl Mx GIPSE vector. Sequencing results confirmed that the cloning strategy was successful.

To verify if the vector was expressing eGFP under the control of the Mx1 IFN-inducible promoter, transient transfections were carried out in 293T cells using the pdl Mx GIPSE plasmid. At 4h after transfection, cells were either stimulated with human exogenous IFN- α (added at a working concentration of 10^3 units/ml) or unstimulated and were fixed 24h after the initial transfection. Expression of eGFP was verified by fluorescence in transfected cells both in the presence and absence of exogenous IFN (figure 3.14).

Figure 3.13 Representation of the cloning process to generate the pdl Mx GIPSE vector.

To generate the pdl Mx GIPSE vector, the first step was to amplify the Mx1 promoter from the pGL3-Mx1P vector by PCR, using the following primers: fwdMx1Pseq and revMx1Pseq (containing a BamHI restriction site). From prior sequencing of the Mx1 promoter from the pGL3-Mx1P vector, an EcoRI site was detected at the start of the promoter sequence, for which reason it was not necessary to include an EcoRI restriction site in the design of the fwdMx1Pseq primer. The DNA gel labelled 'A' represents the PCR fragment obtained (2.3 kb). Both the PCR fragment and the backbone lentivirus vector (pdlNotI'MCS'F) were digested with EcoRI and BamHI (the SFFV promoter was thus cut out of the pdlNotI'MCS'F vector) and ligated overnight, generating the intermediate vector pdl'Mx'MCS'F.

The second step in the process was to digest the pHR-SIN-CSGWdlNotI vector with BamHI and NotI to obtain the eGFP gene (DNA gel 'B'). The intermediate vector pdl'Mx'MCS'F was also digested with BamHI and NotI, after which ligation of this vector with the eGFP fragment was performed overnight. This finally generated the pdl Mx GIPSE vector.

Figure 3.13

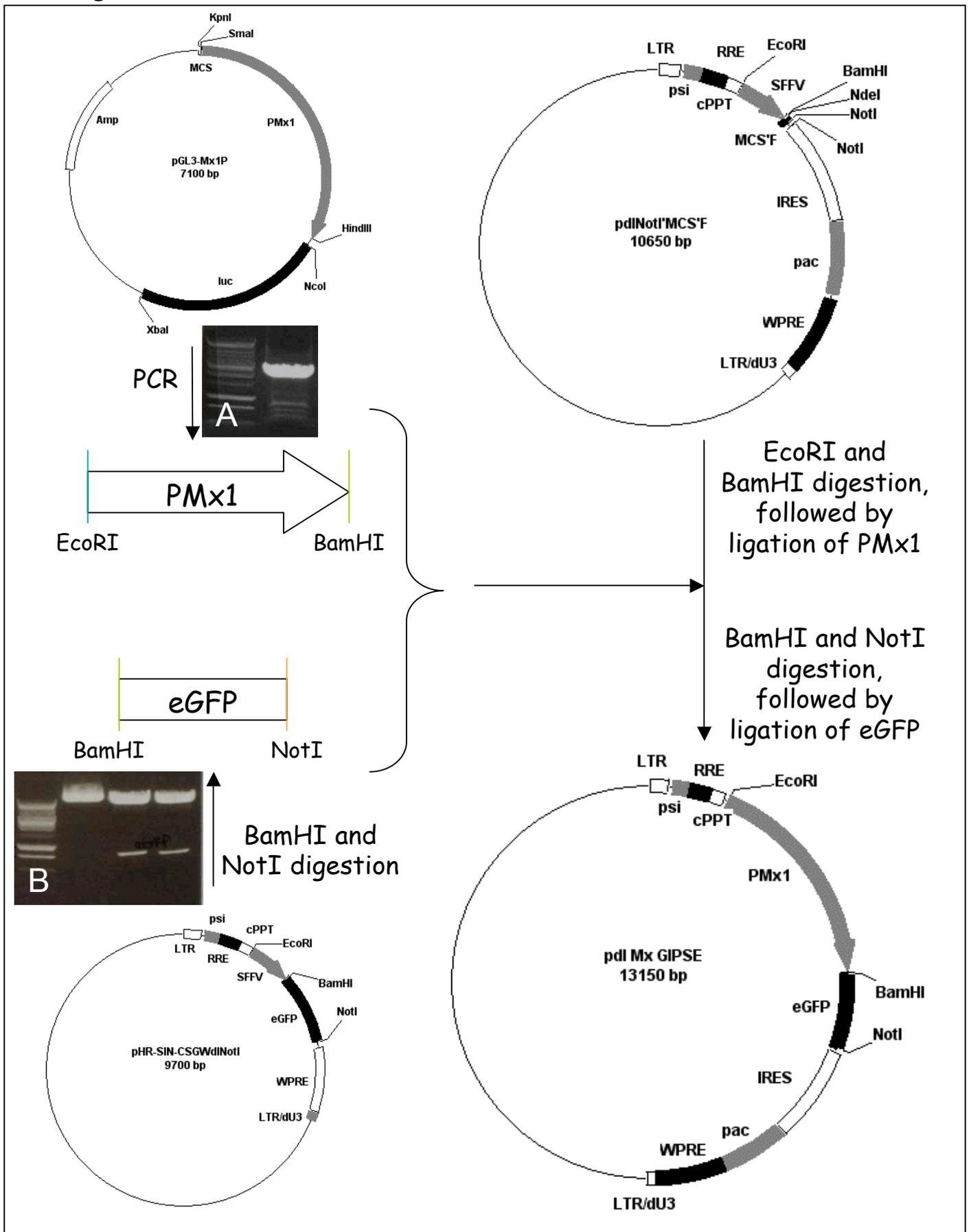
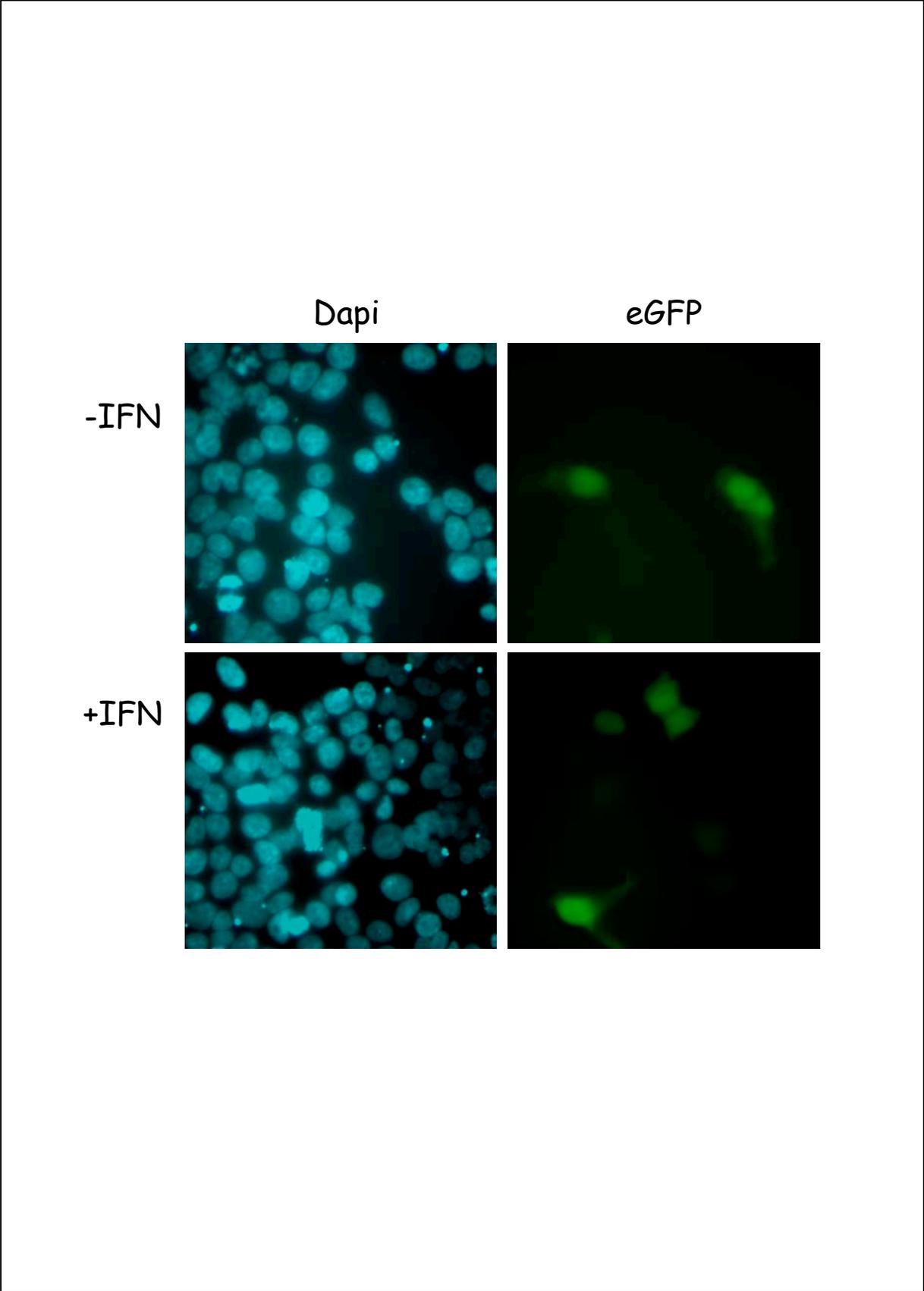


Figure 3.14 Fluorescence analysis showed that eGFP is expressed in transient transfections using the pdl Mx GIPSE vector in 293 cells.

293 cells were transfected using the pdl Mx GIPSE lentivirus vector. IFN (or mock) was added at 24h after the transfection and cells were fixed and stained with Dapi at 48h after the transfection. Expression of eGFP was observed in both the absence and presence of exogenous IFN.

Figure 3.14



3.2.3.2 Generation of the Mx GIPSE cell line

It is believed that transfection might induce the production of IFN in cells and this may be the reason why, in the previous experiment, eGFP expression was observed in both unstimulated and IFN-stimulated cells. If cells were made to constitutively express the lentivirus vector, there should be no IFN induction as there would be no need to transfect the cells. Thus, it was decided to generate a human stable cell line using the pdl Mx GIPSE lentivirus vector. The chosen cells were again HEp2 cells, which had previously been used in our laboratory to establish stable cell lines using the lentiviral vector system referred to above. To produce lentivirus stocks, 293T cells were co-transfected with the pdl Mx GIPSE, the pCMV and the pVSV/G vectors. The supernatant was harvested at 48 and 72h post-transfection, filtered and used to infect HEp2 cells. For the selection process, exogenous human IFN- α (10^3 units/ml) was added to infected HEp2 cells at 2 days pi, and puromycin (2 μ g/ml) was added 4h after stimulation with IFN to select for the cells that would respond quickly to IFN treatment by producing the puromycin resistance protein. HEp2 cells were grown in the presence of puromycin for 2 days, after which time the culture medium was replaced with normal growth medium containing 10% FCS. Approximately 10% of the starting cell population survived the selection process, and these cells were grown to confluency. Fluorescence assays to check eGFP expression indicated that many cells were expressing eGFP both in the presence and absence of added exogenous IFN, which made it necessary to subclone the cells. The subcloning resulted in three positive, IFN-inducible cell lines, named HEp2/Mx GIPSE 4, 8, and 24 cell lines, which were further characterized by fluorescence analysis in the absence and presence of exogenous IFN (figure 3.15). The best cell line obtained was the HEp2/Mx GIPSE 24 cell line which will be referred to as Mx GIPSE cell line for simplicity. The Mx GIPSE cell line was used in subsequent experiments.

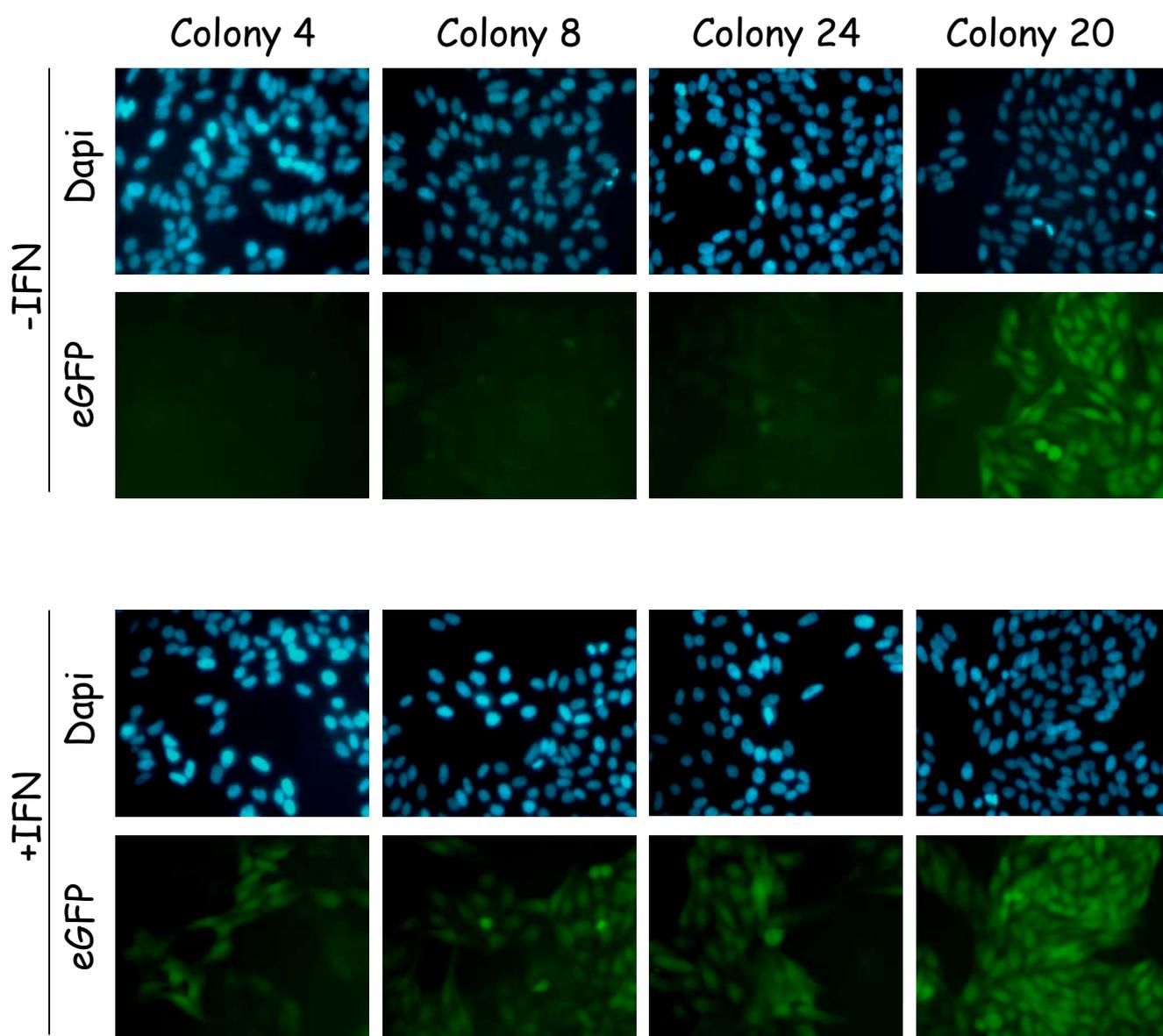
3.2.3.3 Characterization of the Mx GIPSE cell line

Fluorescence assays were carried out on Mx GIPSE cells to assay the conditions required for the induction of eGFP expression. Cells were plated out 2 days before the addition of exogenous IFN- α to the culture medium, and fixed at different time-points after stimulation with IFN (24, 48 and 72h), after which Dapi staining was carried out. Results

Figure 3.15 eGFP expression was IFN-inducible in colonies 4, 8 and 24 of the Mx GIPSE subcloned cells.

The Mx GIPSE cell line was subjected to subcloning to obtain a more homogenous cell population, where all cells would express eGFP in an inducible manner. Subcloned cells were setup on coverslips in duplicate and 48h after setup, exogenous IFN was or was not added to cells. Coverslips were fixed and stained with Dapi 48h after addition of exogenous IFN. Three of the tested colonies (colonies 4, 8 and 24) resulted in inducible expression of eGFP: eGFP was only expressed in the presence of exogenous IFN. Colony 20 is representative of cells where expression of eGFP was constitutive (eGFP was expressed both in the presence and absence of added IFN). Out of the three positive colonies, it was observed that colony 24 exhibited a stronger expression of eGFP in the presence of IFN; thus, colony 24 was chosen for further experiments.

Figure 3.15



confirmed that eGFP expression was dependant upon IFN treatment (figure 3.16), although some cells seemed to be expressing low levels of eGFP in the absence of exogenous IFN. To quantify the induction of eGFP fluorescence in the Mx GIPSE cells in the absence and presence of IFN, a fluorometer was used. The excitation peak of eGFP is 489 nm while the emission peak is 508 nm (Patterson *et al.*, 2001) The best available filters in the laboratory were an excitation filter of 480 ± 5 nm and an emission filter of 520 ± 5 nm which were used to measure eGFP fluorescence. Mx GIPSE cells were plated in 96-well microtitre plates 2 days before the stimulation with exogenous IFN- α (at 10^3 or 10^4 units/ml). Plates were fixed at 5, 24 or 48h after IFN treatment and the intensity of eGFP fluorescence was measured (figure 3.17). It was observed that the longer the Mx GIPSE cells were stimulated with IFN (up to 48h), the more intense eGFP fluorescence. In addition, it was also observed that eGFP intensity increased with IFN concentration. At 48h after stimulation, 10^3 units of IFN- α /ml were sufficient to induce approximately a 10-fold increase of eGFP fluorescence when compared to unstimulated cells. Thus, it was decided that 10^3 units of IFN- α /ml would be the working concentration in subsequent experiments. The results also indicated again that Mx GIPSE cells expressed very low levels of eGFP in the absence of exogenous IFN- α .

Given the fact that the selection process would depend on the expression of the puromycin resistance gene *pac*, it was important to determine the conditions required for its induction and consequent prevention of cell death in the presence of a toxic concentration of puromycin. It was thus necessary to determine the working concentration of puromycin and at what time puromycin should be added to the culture medium after IFN stimulation. To generate the Mx GIPSE polyclonal cell line, puromycin was added to the culture medium 4h after treatment with IFN at a concentration of 2 μ g/ml, and so these conditions were used as general guide lines in the assays. Mx GIPSE cells were plated out into 96-well microtitre plates and in the first plate, different concentrations of puromycin were added 4h after treatment with exogenous IFN- α ; in the second plate, IFN was added to cells at different time-points before addition of 2 μ g/ml of puromycin to the culture medium (figure 3.18). As observed in panel A, working concentrations between 1.25 and 2 μ g of puromycin per ml

Figure 3.16 Fluorescence analysis revealed that eGFP is expressed in Mx GIPSE cells when these are treated with IFN for at least 24h.

Mx GIPSE cells were setup 48h prior to the stimulation with exogenous IFN- α . Coverslips were fixed at different time-points (0, 24, 48 and 72h) after stimulation with exogenous IFN. Cells were stained with Dapi and visualised using the fluorescence microscope. As observed, eGFP is expressed only when IFN has been present in the culture medium for 24h or more. The longer the Mx GIPSE cells are in the presence of IFN, the stronger the eGFP fluorescence.

Figure 3.16

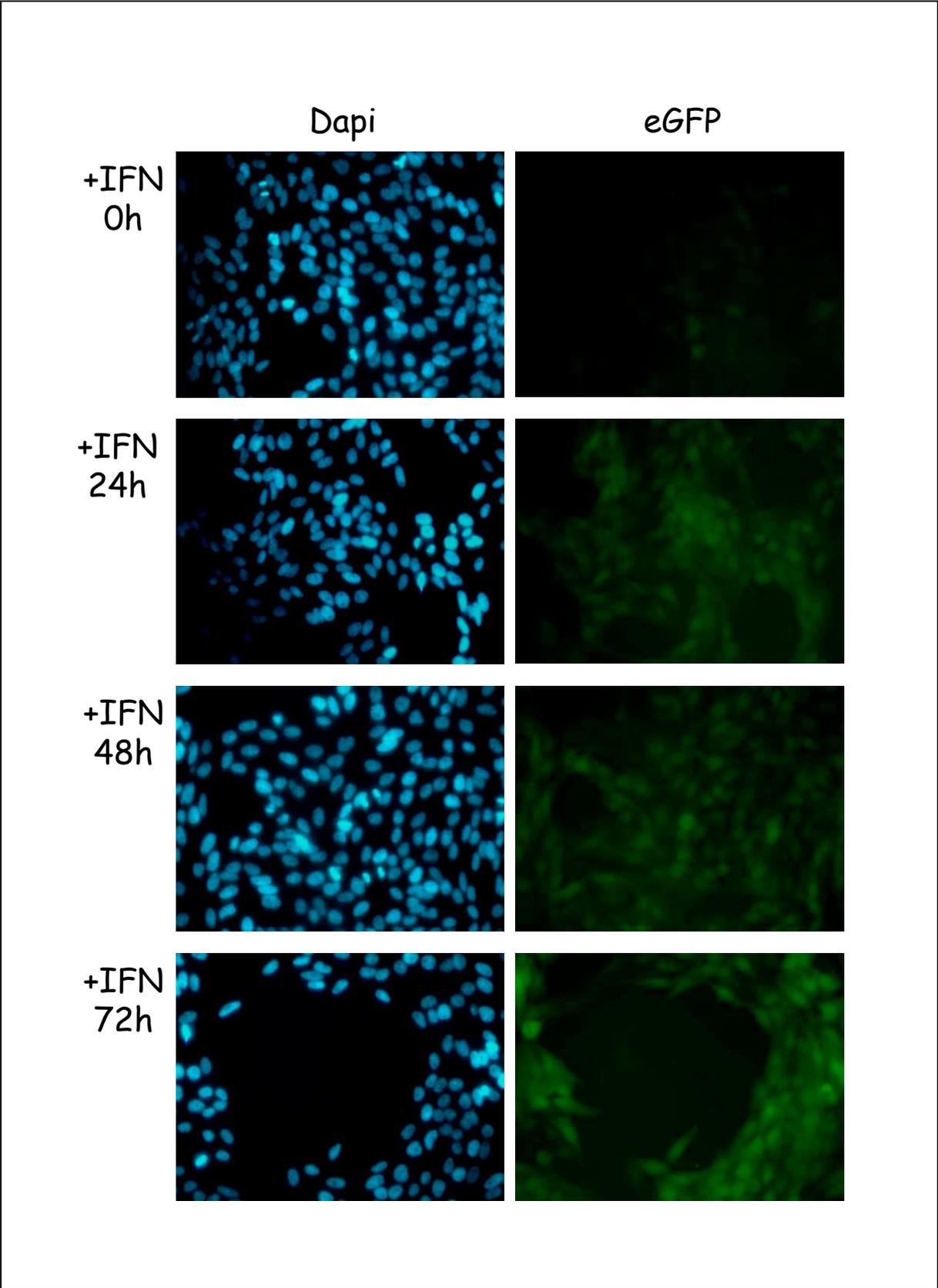


Figure 3.17 A 10-fold increase in eGFP expression was observed when Mx GIPSE cells were treated with IFN for 48h, when compared to untreated cells.

Expression of eGFP was measured in Mx GIPSE cells, either in the presence of 10^3 or 10^4 units of IFN/ml, or in the absence of IFN, using a fluorometer. As observed, eGFP expression was induced in Mx GIPSE cells in the presence of exogenous IFN. Looking at the 5 to 48h interval, the longer the Mx GIPSE cells were subjected to IFN, the stronger the eGFP expression was. The increase in the concentration of IFN from 10^3 to 10^4 units of IFN/ml also resulted in a increase of eGFP expression.

Figure 3.17

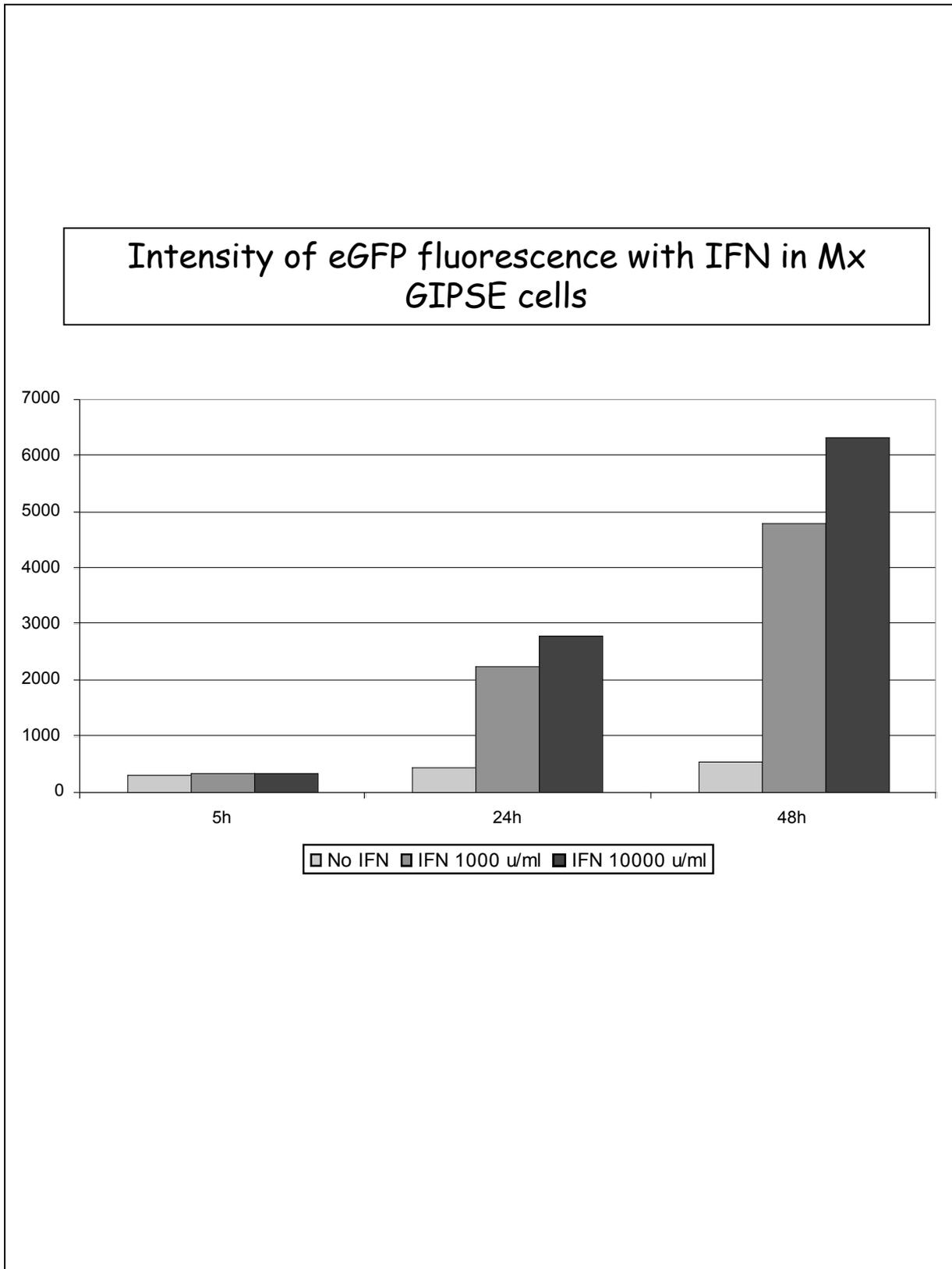
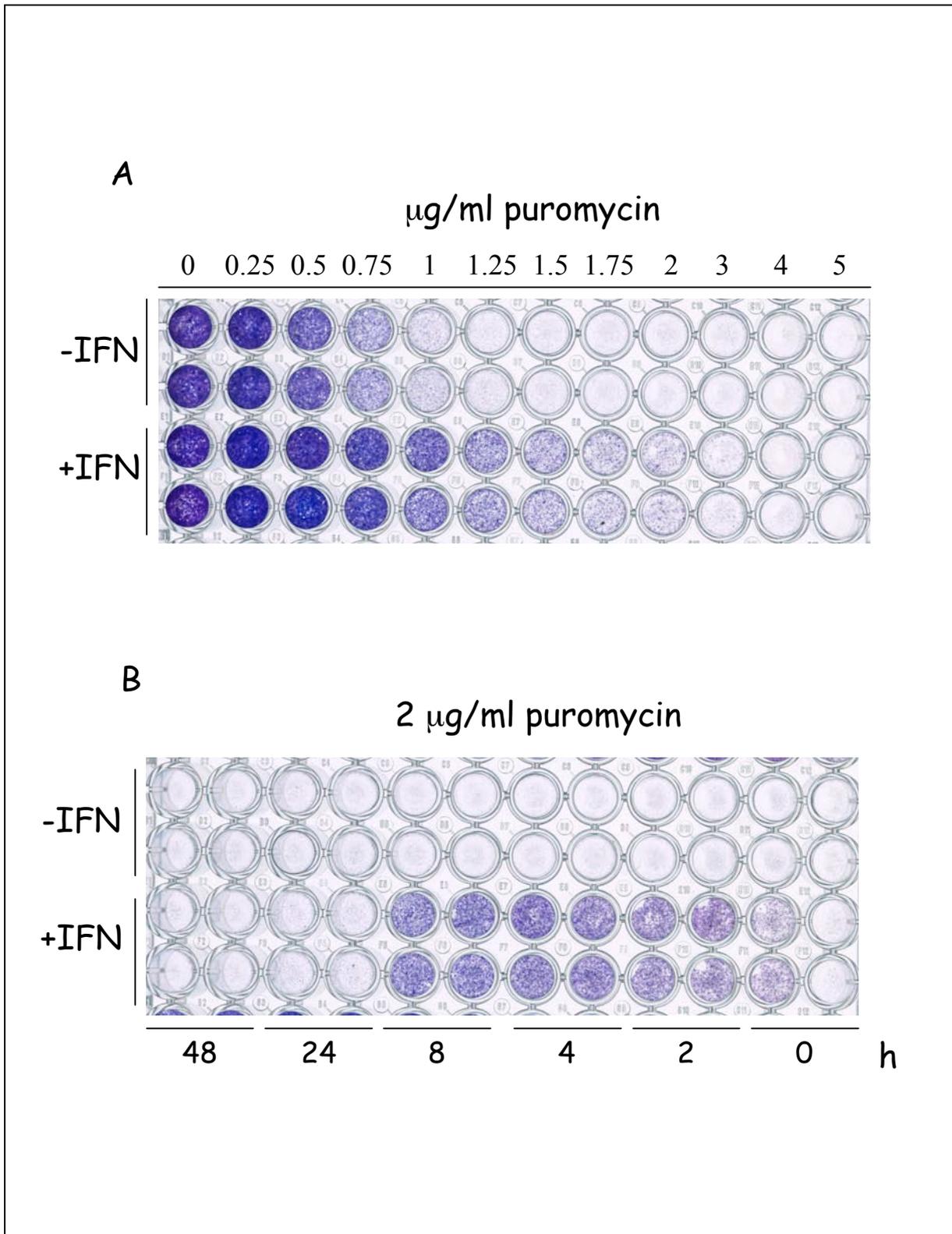


Figure 3.18 Response of Mx GIPSE cells to different concentrations of puromycin and different time-points of addition of puromycin after IFN treatment.

Mx GIPSE cells were setup in 96-well microtitre plates. In panel A, different concentrations of puromycin were added to the culture medium 4h after IFN treatment. In panel B, cells were treated with exogenous IFN at different time points prior to the addition of 2 µg/ml of puromycin. The numbers in panel B refer to the number of hours IFN was present in the culture medium before treatment with puromycin. As observed, Mx GIPSE cells that had been pre-treated with IFN survived the puromycin toxicity when its concentration ranged between 1.25 and 2 µg/ml. It was also observed that puromycin should be added to Mx GIPSE cells 2 to 8h after IFN treatment to allow IFN treated cells to survive the selection process.

Figure 3.18



could be used to attain cell survival when 10^3 units of IFN/ml were added to the culture medium 4h prior to the addition of puromycin. The results in panel B clearly demonstrated that, to allow cells to survive, puromycin ($2 \mu\text{g/ml}$) should be added between 2 and 8h after treatment with IFN- α . As observed, if puromycin was added 24h or later after treatment with IFN, cells did not survive in the presence of puromycin. A possible explanation for this is that the Mx1 promoter is no longer being induced at 24h after the initial treatment with IFN, due to the action of IFN-induced proteins that act in a negative feedback loop, such as the SOCS and PIAS proteins (Kubo *et al.*, 2003, Shuai & Liu, 2005). These proteins serve as negative regulators and inhibitors of the JAK-STAT pathway, and thus might repress the Mx1 promoter, inhibiting the expression of the puromycin resistance gene, leading to cell death when cells are exposed to puromycin. On account of these results, it was decided to use a working concentration of $2 \mu\text{g}$ of puromycin/ml of culture medium to be added 4h after stimulation with exogenous IFN- α in subsequent experiments.

Surprisingly, although eGFP expression seemed to increase gradually with time after IFN treatment in Mx GIPSE cells, the resistance to puromycin was lost if puromycin was added 24h (or later) after IFN treatment. This discrepancy might be related to the half lives of the eGFP and puromycin resistance proteins: the pac protein may have a shorter half life than the eGFP protein.

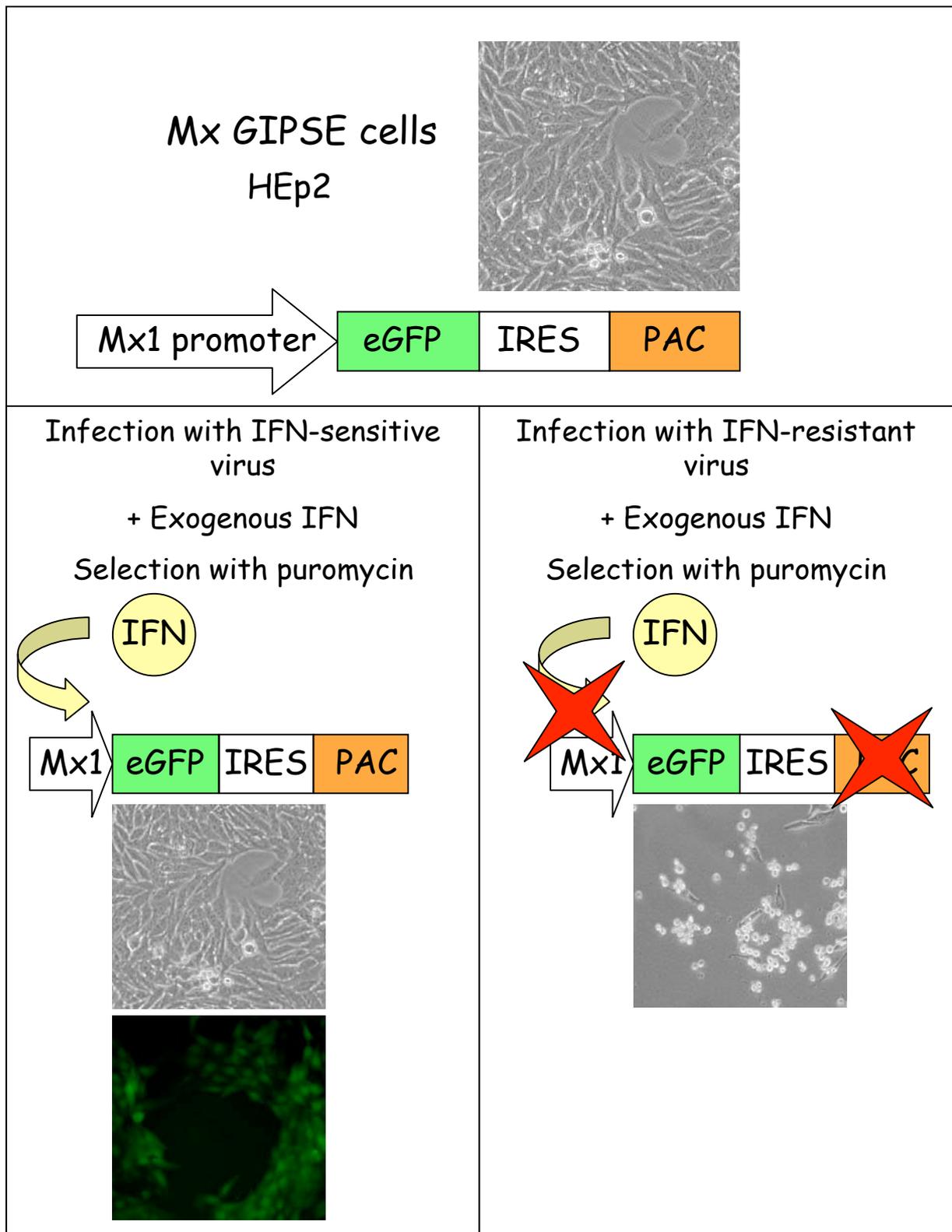
3.2.3.4 Investigation of the use of Mx GIPSE cells to select IFN-sensitive viruses

Figure 3.19 is a representation of how the selection of IFN-sensitive viruses should occur using the Mx GIPSE cell line, exogenous IFN and puromycin. Cells infected with IFN-resistant viruses should not survive the selection process for the viral IFN antagonist should block the induction of the Mx1 promoter. On the other hand, Mx GIPSE cells infected with IFN-sensitive viruses should be capable of surviving the selection with puromycin.

Figure 3.19 Representation of the selection of IFN-sensitive viruses using the Mx GIPSE cell line, exogenous IFN and puromycin.

Mx GIPSE cells should die when infected with an IFN-resistant virus and treated with IFN and puromycin. The action of the viral IFN antagonist(s) protein(s) should block the induction of the Mx1 promoter and consequently also block the production of the puromycin resistance protein. When infected with IFN-sensitive virus and treated with IFN and puromycin, Mx GIPSE cells should survive the selection process, and should express eGFP in fluorescence analysis.

Figure 3.19



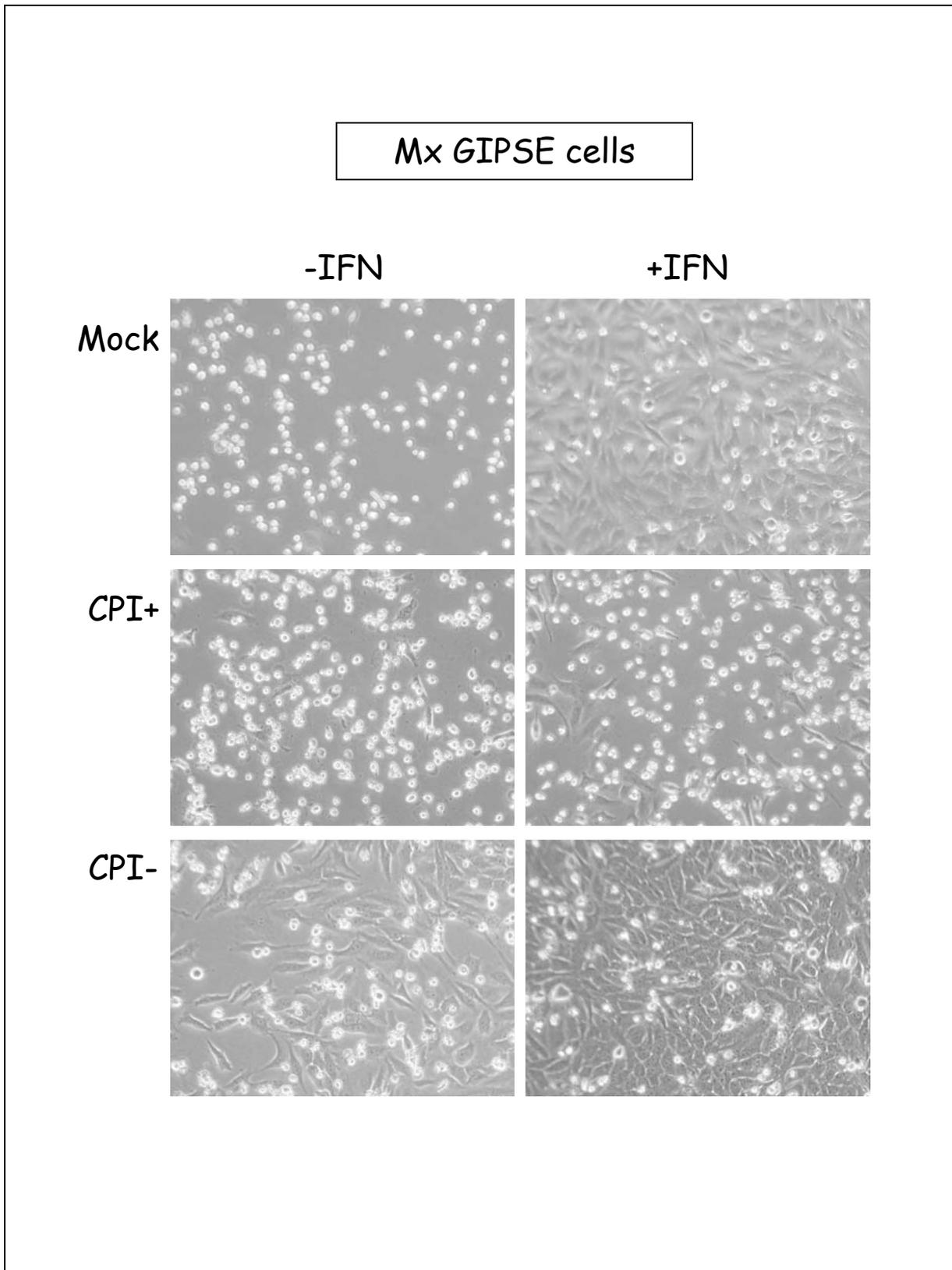
Initially, to determine whether the Mx GIPSE cells could potentially be used to select for IFN-sensitive viruses, cells were infected with either CPI+ or CPI- (or mock) at a m.o.i. of 5 pfu/cell in duplicate wells. Exogenous IFN- α (or mock) was supplemented to cells at 5h pi, puromycin was added to all wells at 9h pi, and surviving cells were visualised at 48h pi (figure 3.20). The results clearly showed that, if infected with IFN-resistant CPI+ virus, the Mx GIPSE cells die in the presence of puromycin, regardless of the prior treatment with exogenous IFN. However, when infected with IFN-sensitive CPI- virus, many cells are capable of surviving the selection process even in the absence of exogenous IFN. The reason why CPI- infected cells survived in the absence of exogenous IFN is because, when infected, HEp2 cells naturally produce IFNs. Since CPI- cannot block IFN signalling, the cellular IFNs produced due to infection are free to trigger the IFN signalling pathway, leading to expression of the puromycin resistance gene. The results from this analysis demonstrated that the developed model system allows for survival of cells that are infected with IFN-sensitive viruses as opposed to cells infected with IFN-resistant viruses.

In order to determine whether IFN-sensitive viruses could be selected and rescued from a mixed virus population using the developed methodology, CPI- and PIV5 W3 viruses were combined in different proportions, including W3 alone, CPI- alone and the following ratios of CPI- to W3: 1:1 , 1:10, 1:100, 1:1000, 1:10000 and 1:100000. Mx GIPSE cell monolayers contained in 25cm² flasks were infected with the W3/CPI- mixtures in duplicate at a m.o.i. of 4 pfu/cell. Exogenous IFN- α was added to the culture medium at 5h pi to one set of flasks (cells remained untreated in the other set of flasks), while puromycin was added to both sets at 9h pi. The response of cells to IFN is a quick process and therefore, cells would have begun producing the IFN-induced antiviral proteins (including the Mx proteins) before the addition of puromycin at 9h pi. After cells had been in the presence of puromycin for approximately 48h, surviving cells were thoroughly washed with PBS to remove all cell debris and any free virus present in the medium. At this stage of the selection, surviving cells should either correspond to uninfected cells or cells infected with CPI- virus.

Figure 3.20 Mx GIPSE cells did not survive IFN and puromycin treatment when infected with an IFN-resistant virus.

Mx GIPSE cells were infected with CPI-, CPI+ or mock at an approximate m.o.i. of 5 pfu per cell. Cells were treated with exogenous IFN at 5h pi (or untreated) and puromycin at 9h pi and photos were taken at 48h pi. As observed, CPI- infection allowed cells to survive the IFN/puromycin selection, while infection with CPI+ did not.

Figure 3.20



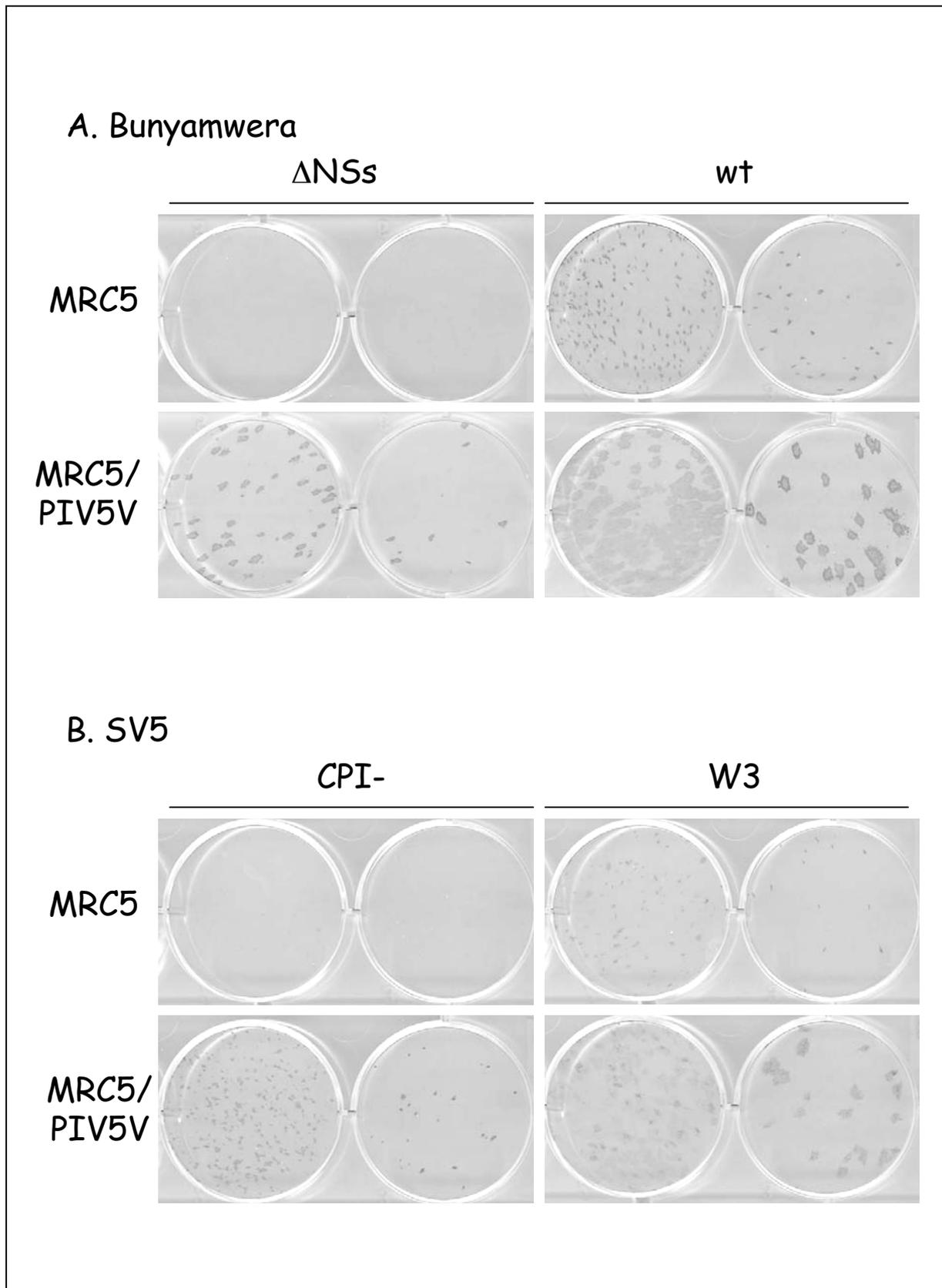
It was necessary to rescue and amplify whatever virus was existent in the surviving cells and therefore, HEp2/BVDVNpro/PIV5V cells (see section 3.1) were added to the surviving Mx GIPSE cells. Because the V protein of PIV5 and the Npro protein of BVDV are IFN antagonists and, in combination, completely block IFN production and signalling in cells, any IFN-sensitive viruses should be able to readily replicate and spread in cells expressing these viral proteins. Viruses were allowed to grow in the permissive cells for 5 days, after which time the supernatant was harvested and frozen at -70°C.

At this stage, it was necessary to ascertain whether the virus population had been enriched in IFN-sensitive viruses. Plaque assays using MRC5 and MRC5/PIV5V cells were setup for this purpose. MRC5 cells are human diploid cells suitable for the production of attenuated viruses for use in humans. Despite having a limited passage number, MRC5 cells are more suitable for plaque assays than HEp2 cells, because the latter overgrow when they reach confluency making it very difficult to identify virus infectious centres. MRC5/PIV5V cells are MRC5 cells that have been engineered to express the V protein of PIV5 and thus to no longer respond to IFN (Young *et al.*, 2003). Previous work has shown that, in comparison to wild-type viruses, IFN-sensitive viruses plaque very inefficiently, if at all, on naïve MRC5 cells but are capable of forming plaques on MRC5/PIV5V cells (Young *et al.*, 2003). Some IFN-sensitive viruses such as BUNΔNSs (Bunyamwera virus from which the NSs gene has been deleted; Bridgen *et al.*, 2001), CPI- and a recombinant PIV5 virus (PIV5VΔC; generated so as to make a truncated version of the V protein and thus to be sensitive to IFN; He *et al.*, 2002) completely fail to form plaques on MRC5 cells but readily form plaques on MRC5/PIV5V cells, while the respective wild-type viruses form plaques on both cell lines (figure 3.21). Therefore, to investigate whether CPI- had been preferentially selected from the mixed virus populations, plaque assays of the selected virus stocks and the original W3/CPI- mixtures were setup on MRC5 and MRC5/PIV5V cells. Plates were fixed at 9 days pi and were immunostained using primary antibodies against the viral proteins, secondary alkaline phosphatase-conjugated antibodies and a third step of incubation with an alkaline phosphatase substrate that detects alkaline phosphatase

Figure 3.21 IFN-sensitive viruses such as Bun Δ NSs and CPI- failed to form plaques on naïve MRC5 cells but readily formed plaques on MRC5 cells constitutively expressing the V protein of PIV5 (MRC5/PIV5V cells).

As observed, the wild-type IFN-resistant viruses Bunyamwera and PIV5 W3 were capable of forming plaques both on MRC5 and on MRC5/PIV5V cells, despite an obvious difference in plaque size between the two cell lines. However, IFN-sensitive viruses Bun Δ NSs and PIV5 CPI- failed to form plaques on naïve MRC5 cells.

Figure 3.21



activity (figures 3.22 and 3.23). As observed, selected CPI- stocks failed to plaque on naïve MRC5 cells, but formed small plaques on MRC5/PIV5V cells (panel A, figures 3.22 and 3.23). Selected PIV5 W3 stocks readily formed plaques on both cell lines in similar numbers, although it was clear that plaques were much bigger on MRC5/PIV5V cells than on naïve MRC5 cells (panel B in figures 3.22 and 3.23). This may be explained if one considers that the ability of PIV5 to circumvent the IFN response is not absolute, as PIV5 does not completely block IFN production. Therefore, in naïve MRC5 cells, infection would result in the production of some IFN molecules which would trigger IFN signalling pathways in neighbouring uninfected cells and the production of antiviral proteins, which would limit viral spread. In MRC5/PIV5V cells, the V protein is expressed constitutively in all cells, thereby blocking IFN signalling and inhibiting the expression of antiviral ISGs, which would result in an enhanced virus spread from cell to cell.

Considering the W3/CPI- virus mixtures, the results clearly showed that there was no preferential selection of CPI- over W3, even when the starting virus preparation corresponded to a ratio of 1 pfu CPI- to 1 pfu of W3 (panel C in figures 3.22 and 3.23). Small plaques corresponding to W3 virus could be observed on naïve MRC5 cells for all the virus mixtures (CPI- fails to plaque on MRC5 cells), indicating that W3 was capable of surviving the selection process, even when exogenous IFN was included in the selection. Furthermore, after counting the number of plaques obtained after puromycin selection and titration of W3 and CPI- independently on MRC5/PIV5V cells (table 3.1), the titres of W3 and CPI- obtained in the absence of exogenous IFN are very similar, indicating that both W3 and CPI- viruses were capable of surviving the selection process. In addition, when the selection was performed in the absence of added exogenous IFN, a mix of small and big plaques was observed for the W3/CPI- mix (ratio of 1:1) on MRC5/PIV5V cells, indicating again that both CPI- (small plaques) and W3 (bigger plaques) viruses survived the selection process. However, for the same virus mix (ratio 1:1), when exogenous IFN was included in the selection process, no small plaques could be observed on MRC5/PIV5V cells, indicating that the addition of IFN at 5h pi depletes the virus population in CPI- virus. Furthermore, the titre of CPI- drops almost 60 fold

Figure 3.22 After selection of a W3/CPI- virus mix (1:1 proportion) on Mx GIPSE cells using puromycin, there is no indication of enrichment of the resulting virus population in CPI- virus.

Viruses (CPI- in panel A, W3 in panel B or a ratio of CPI- to W3 of 1:1 in panel C) were selected using Mx GIPSE cells and puromycin treatment. After amplification of the viruses that survived the selection, resulting virus stocks were titrated on MRC5 and MRC5/PIV5V cells. The selection procedure was not effective enough to allow preferential selection of CPI- over W3 (panel C).

Figure 3.22

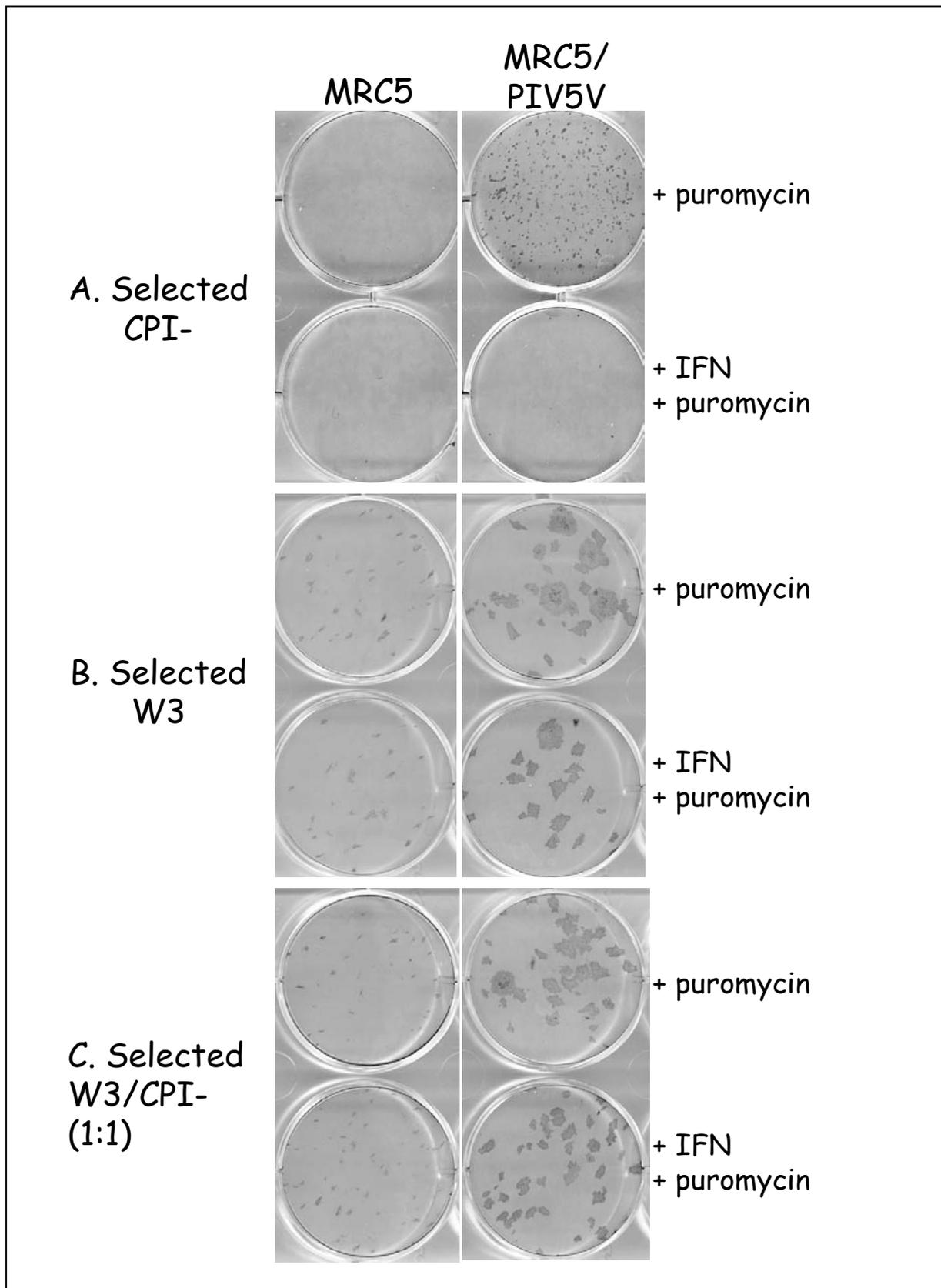
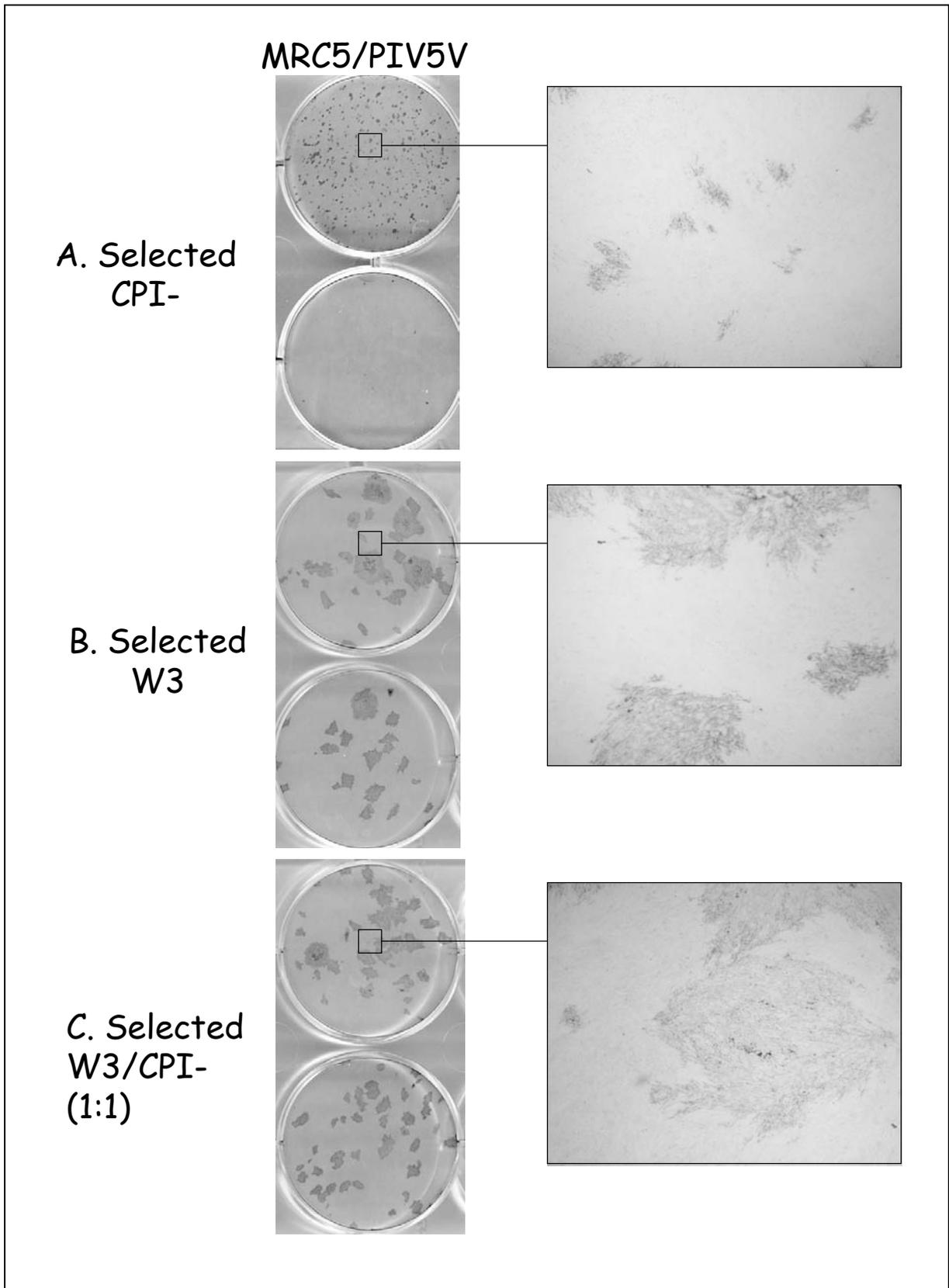


Figure 3.23 Representation of plaque sizes observed on plaque assays of W3 and CPI- on MRC5/PIV5V cells.

Panel A represents the plaque size of CPI- on MRC5/PIV5V cells, while panel B represents the plaque size of W3 virus on MRC5/PIV5V cells. In panel C, a mix of different plaque sizes was observed, indicating a mixed virus population: CPI- (small plaque on the left) and W3 (bigger plaques).

Figure 3.23



(table 3.1) when IFN is included in the selection process when compared to the selection in the absence of IFN. These factors indicate that, if exogenous IFN is to be included in the selection process, it should be added at a later time-point than 5h pi, to give CPI- a chance of surviving the antiviral effects of IFN.

Table 3.1 Number of plaques obtained after selection and titration of W3 and CPI- viruses independently on MRC5/PIV5V cells.

Virus	Type of selection	Number of plaques
W3	Puromycin	4.2×10^5
W3	IFN + Puromycin	2.9×10^5
CPI-	Puromycin	4.0×10^5
CPI-	IFN + Puromycin	7×10^3

At this stage, it seemed that the Mx GIPSE cell line on its own was not adequate for the selection of IFN-sensitive viruses over IFN-resistant viruses. As mentioned before, low levels of eGFP fluorescence were observed in Mx GIPSE cells in the absence of added exogenous IFN, both by immunofluorescence and fluorometer analysis, although these cells would die if treated with puromycin in the absence of IFN. This indicated that the Mx1 promoter might not be tightly regulated, leading to expression of low levels of the puromycin resistance gene in the absence of IFN, which would block the correct functioning of the selection process. To overcome this problem, it should have been possible to select the tightly regulated, inducible Mx GIPSE cells by fluorescence activated cell sorter (FACS) analysis: by sorting the Mx GIPSE cells that did not express eGFP in the absence of exogenous IFN (inducible expression) from the ones that did express eGFP in the absence of exogenous IFN (constitutive expression), although this was not attempted. Another possible solution was to generate an analogous cell line expressing a gene that would allow for a negative selection process to eliminate any cells demonstrating leakiness of the Mx1 promoter. The following section (sections 3.2.3.5) describes the generation of such a cell line.

As a general conclusion, it seems the developed model system was not effective enough to allow preferential selection of CPI- virus (IFN-sensitive virus) over W3 virus (IFN-resistant virus). This type of system requires a tight balance: if IFN is to be included in the selection process, it should be added (together with puromycin that is added 4h after stimulation with IFN) early enough to kill W3-infected cells, but before the IFN-resistant virus has a chance to spread to surrounding cells, and also late enough to allow CPI- virus to survive the IFN-induced antiviral state and the action of the antiviral enzymes. In addition, if there are cells constitutively expressing eGFP and pac in the Mx GIPSE population, these will always survive the selection with puromycin, even if infected with an IFN-resistant virus. These cells would also facilitate the replication and consequent amplification of IFN-resistant viruses.

3.2.3.5 Generation of the pdl Mx TIPSE vector and the Mx TIPSE cell line

Ganciclovir is a guanosine analogue frequently used as a drug to obtain a suicide effect in cells containing the Herpes Simplex virus thymidine kinase (HSV-tk) gene. The tk enzyme phosphorylates ganciclovir to ganciclovir-monophosphate which is further converted to ganciclovir-diphosphate and ganciclovir-triphosphate by host kinases. Ganciclovir-triphosphate is toxic to cells as it causes premature chain termination and apoptosis. It was planned to generate a cell line that would contain both the HSV-tk and the pac genes under the control of the Mx1 promoter. Ganciclovir should pose no threat when added to these cells; however, it should be cytotoxic when used in conjunction with IFN- α , as the tk gene should be expressed, leading to the conversion of ganciclovir to ganciclovir-triphosphate.

The reason for adding the HSV-tk gene to the system in the first instance was to eliminate any cells demonstrating leakiness of the Mx1 promoter. This could be achieved by growing the cell line expressing HSV-tk under the control of the Mx1 promoter in the presence of ganciclovir. Any cells demonstrating leakiness of the Mx1 promoter would have the tk protein being constitutively expressed; consequently, ganciclovir would be converted to ganciclovir-triphosphate in these cells, leading to cell death. Once the cells constitutively expressing tk and pac were eliminated, this cell line would then be tested

to: attempt the selection of IFN-sensitive viruses from an IFN-resistant virus population using the positive selection procedure (figure 3.7 assuming ‘SM1’ is the pac gene and that IFN and puromycin are added exogenously after infection); attempt the selection of IFN-resistant revertant viruses from an IFN-sensitive population using the negative selection procedure (figure 3.8 assuming ‘SM2’ is the HSV-tk gene and that IFN and ganciclovir are added exogenously after infection).

To generate the cell line of interest, the lentivirus vector system previously described was used. Firstly, the HSV-tk gene was amplified by PCR from the LP2AT vector (kindly provided by Dr. Pablo de Felipe, University of St. Andrews) using the fwdtk and revtk primers. The PCR fragment was then directly cloned into the pGEM-T easy vector. Finally, the tk gene was digested with BamHI and NotI restriction enzymes from pGEM-T easy and cloned into the pdl Mx GIPSE vector in the place of eGFP, generating the pdl Mx TIPSE vector (figure 3.24).

To generate the cell line of interest, the pdl Mx TIPSE vector was transfected into 293T cells together with the two helper plasmids to generate a lentivirus stock, and this stock was used to infect HEp2 cells. Exogenous IFN- α (10^3 units/ml) was added to these cells at 48h pi and puromycin (concentration of 2 μ g/ml) was added 4h after IFN. After 48h in the presence of puromycin, the cells were carefully washed and left to grow to confluence. The polyclonal cell line thus generated was subcloned (by a Summer student, Ini Witzel) in the presence of ganciclovir (1 μ g/ml) to eliminate any cells constitutively expressing tk and pac, from which derived the HEp2 Mx TIPSE E5 cell line, referred to as Mx TIPSE cell line from here on for simplicity.

3.2.3.6 Investigation of the use of Mx TIPSE cells to select IFN-sensitive viruses and IFN-resistant viruses

To ascertain whether the selection procedures with both puromycin or ganciclovir alone would work, Mx TIPSE cells were treated with puromycin (2 μ g/ml) or ganciclovir (1 μ g/ml) in the absence or presence of exogenous IFN- α (Figure 3.25). As observed, Mx TIPSE cells survived puromycin toxicity only if in the presence of exogenous IFN, while

Figure 3.24 Representation of the cloning process used to generate the pdl Mx TIPSE lentivirus vector.

The HSV-tk gene was amplified by PCR from the LP2AT vector using taq polymerase (to generate the required 'A' overhangs for cloning into pGEM-T easy) and the following primers: fwdtk (containing a BamHI restriction site) and revtk. The DNA gel represents the generated PCR fragment (last lane of the right). The PCR fragment was cloned into the pGEM-T easy vector to facilitate further cloning procedures. In the next step, the HSV-tk gene was excised from the pGEM-T easy vector using BamHI and NotI restriction enzymes and cloned into the same sites present in the pdl Mx GIPSE vector (which lead to the elimination of eGFP), resulting in the pdl Mx TIPSE vector.

Figure 3.24

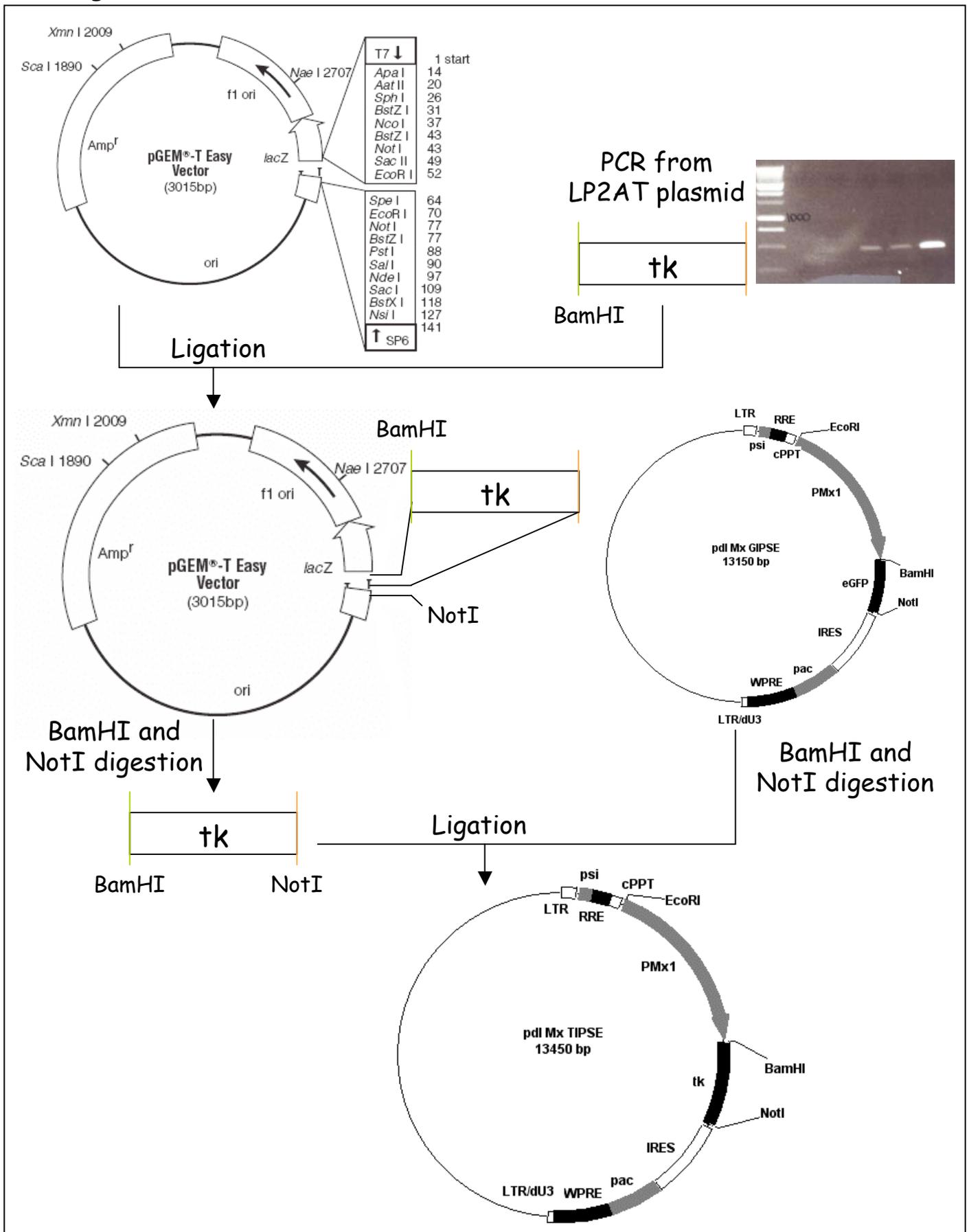


Figure 3.25 Mx TIPSE cells survived puromycin toxicity only in the presence of exogenous IFN and survived ganciclovir toxicity only in the absence of IFN.

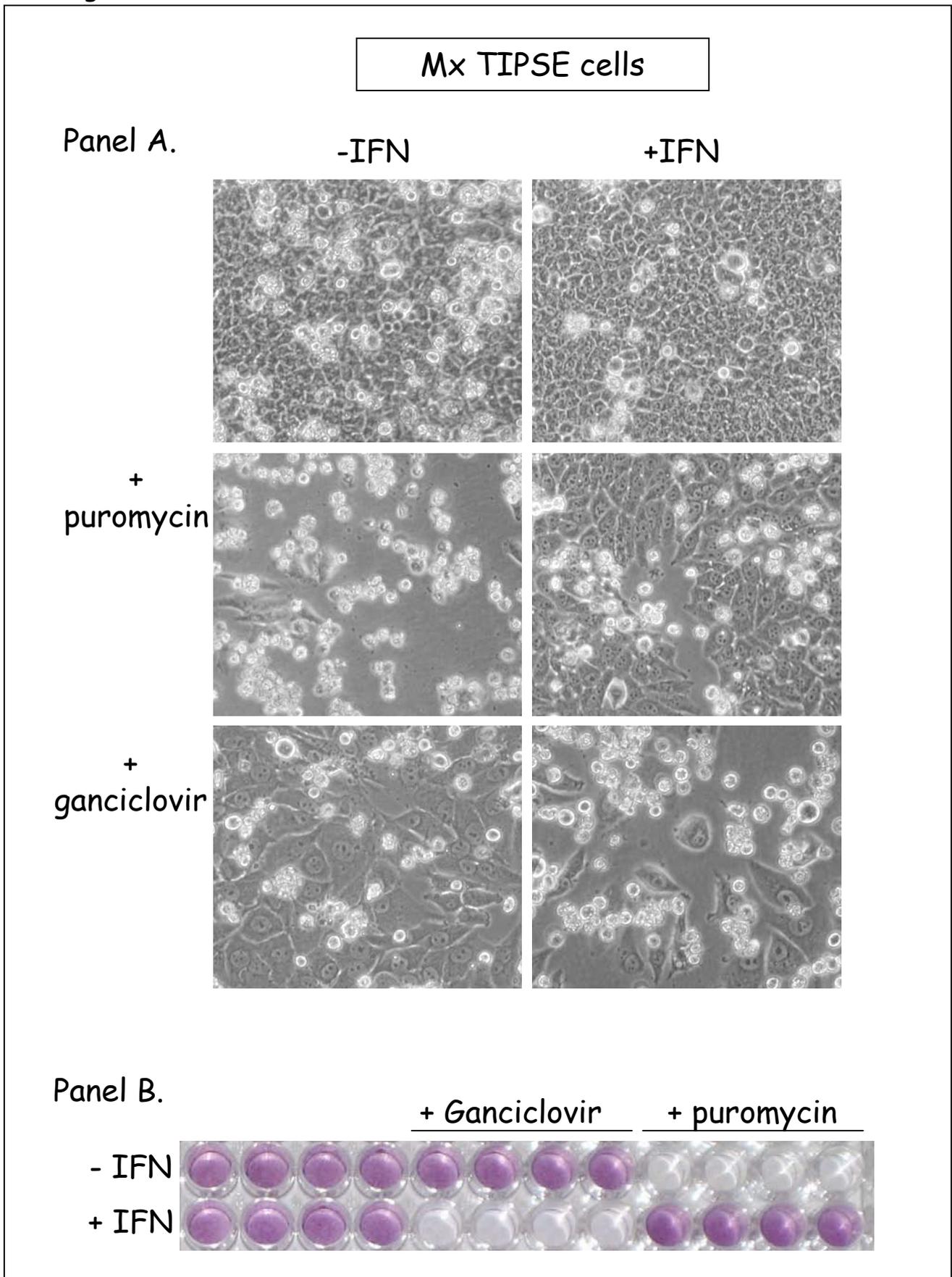
Panel A. Mx TIPSE cells were setup in 6-well plates at a confluency of 20%, 2 days before the stimulation with IFN (or untreated) and subsequent stimulation with puromycin (2 $\mu\text{g/ml}$) or ganciclovir (1 $\mu\text{g/ml}$) at 4h after IFN treatment. Photos were taken 2 to 5 days after treatment with the drugs.

Panel B. Mx TIPSE cell were setup in 96-well plates at a confluency of 40%, 2 days before the stimulation with IFN (or untreated) and subsequent stimulation with puromycin (2 $\mu\text{g/ml}$) or ganciclovir (1 $\mu\text{g/ml}$) at 4h after IFN treatment. Cells were fixed and stained with crystal violet 3 days after treatment with the drugs.

As observed, Mx TIPSE cells survived puromycin toxicity in the presence of added IFN, because IFN induced the expression of the puromycin resistance protein, *pac*, via the IFN-inducible Mx1 promoter. Mx TIPSE cells died in the presence of puromycin and absence of IFN.

Mx TIPSE cells survived ganciclovir toxicity in the absence of added IFN, because the *tk* gene product was not being expressed. However, when Mx TIPSE cells were treated with IFN and ganciclovir, they died after 5 days due to the expression of the *tk* gene product that converted ganciclovir to a toxic product to the cells.

Figure 3.25



they only survived ganciclovir toxicity in the absence of exogenous IFN. This result was an indication that both the selection procedures were working as expected and that the generated cell line should be tested to attempt the selection of IFN-sensitive viruses and IFN-resistant revertant viruses.

However, due to time constraints, it was not possible to attempt the selections of IFN-sensitive viruses or IFN-resistant viruses using the Mx TIPSE cell line. The Mx TIPSE cell line was frozen and stored in liquid nitrogen until further use.

3.3 Other applications for the Mx GIPSE (and potentially Mx TIPSE) cell lines

3.3.1 Use of Mx GIPSE cells in IFN CPE reduction assays

IFN CPE reduction assays are frequently used in our laboratory as a means to titrate the amount of IFN that cells produce under different chosen conditions. Vero cells are generally used in IFN CPE reduction assays; Vero cells are monkey kidney cells that do not produce IFN due to a spontaneous gene deletion (IFN- α/β genes; Desmyter *et al.*, 1968; Mosca & Pitha, 1986), but they do respond to IFN if it is exogenously supplemented to the culture medium. As soon as the different samples requiring testing are harvested, they are subjected to UV irradiation (to kill any virus present in the sample) and are added to confluent monolayers of Vero cells (contained in 96-well microtitre plates) in a series of two-fold dilutions for successive columns. A positive control that is frequently used is exogenous IFN- α , also added at two-fold dilutions to the Vero cell monolayers. At approximately 24h after the samples are added, EMCV is used to infect the Vero cell monolayer at an m.o.i. of approximately 10 pfu/cell. EMCV is an IFN-sensitive virus generally used in IFN CPE reduction assays because, in the absence of IFN, it completely destroys the Vero cell monolayer in only 2 to 3 days, whilst it is unable to destroy the monolayer when IFN is present. Also, the endpoint of the titration is very easy to visualize using EMCV.

An alternative assay to the 'traditional' IFN CPE reduction assay described above was developed using the Mx GIPSE cell line. Mx GIPSE cells are sensitive to puromycin cytotoxicity when IFN is absent from the culture medium; however, in the presence of exogenous IFN- α , Mx GIPSE cells are able to survive in the presence of puromycin. The IFN CPE reduction assay was setup in a 96-well microtitre plate format. Two days after setting up the 96-well plates with confluent monolayers of Mx GIPSE cells, exogenous IFN- α was added to the cells in a series of two-fold dilutions for successive columns, starting at a concentration of 1000 units/ml. Cells were either treated with puromycin (at

a concentration of 2 µg/ml) or EMCV (at a concentration of 2×10^5 pfu/ml) or mock, 4h after the stimulation with exogenous IFN (figure 3.26). As observed, approximately 2 units of IFN/ml were sufficient to induce protection of the Mx GIPSE cells against the EMCV, while 1 unit of IFN/ml (or possibly less) was sufficient to induce protection against puromycin. The results indicated that Mx GIPSE cells can in fact be used in IFN CPE reduction assays and that there is no need to carry out virus infections as puromycin can be used instead. In addition, the assay appears to be more sensitive to IFN when using puromycin, so it should be possible to detect lower amounts of IFN in a given sample; however, the endpoint of the titration is easier to visualise and more defined when using EMCV.

3.3.2 Screening for compounds that inhibit IFN signalling

Compounds that inhibit IFN signalling may be important as anti-inflammatory compounds in certain disease states where there is over-inflammation, such as cystic fibrosis (Hudson, 2004). These compounds may also potentially be used in the laboratory, for example, in assays where it is necessary to mimic a viral IFN antagonist that blocks IFN signalling.

The Mx GIPSE cell line can be used in high throughput screening assays to search for compounds that inhibit or block the IFN signalling pathway. As demonstrated above, Mx GIPSE cells survive puromycin selection if cells are previously treated with exogenous IFN. If however, in high throughput compound screening assays, the Mx GIPSE cells die in the presence of exogenous IFN and puromycin, there are two possible explanations: either the compound is toxic to the cells or it is somehow blocking the induction or action of the puromycin resistance gene, in which case it could be specifically blocking IFN signalling.

To demonstrate the potential of the Mx GIPSE cell line for high throughput screening, cells were plated out in duplicate 96-well microtitre plates at approximately 20% confluency, 24h prior to the addition of a small collection of compounds (compound

Figure 3.26 Mx GIPSE cells and puromycin selection may be used as an alternative to CPE reduction assays.

Mx GIPSE cells were setup in 96-well microtitre plates. Exogenous IFN was added to all wells in a series of two-fold dilutions for successive columns, and puromycin or EMCV (or mock) were added to the wells 4 h after stimulation with IFN. As observed, the assay worked both with EMCV and puromycin; however, there seemed to be an increased sensitivity when using puromycin despite the end-point not being as easy to visualise.

library kindly provided by Dr. N. Westwood, St. Andrews University). The compounds had been stored at a stock concentration of 10 mM in a 96-well plate format, and 100 nl of each stock solution were added per well in duplicate plates. 48h after adding the compounds, exogenous IFN- α (10^3 units/ml) was added to both of the 96-well plates, and puromycin was added to one of the plates at a concentration of 1 μ g/ml 4h after IFN treatment. Plates were fixed and stained with crystal violet 4 days after treatment with IFN and puromycin (figure 3.27). From the control wells in this assay, it can be seen that, as expected, cells survived in the presence of puromycin only if they had been pretreated with IFN. In addition, there are some differences in cell death/survival between the control plate (plate to which no puromycin was added) and the plate to which puromycin was added. In the control plate, wells where cell death was observed indicate that the compounds added to those wells were cytotoxic, at least at the concentration used for this assay. In the plate to which puromycin was added, besides the wells where the compounds were cytotoxic, there were some extra wells where cell death was observed. Compounds added to these wells may possibly be blocking IFN signalling and preventing the induction of the Mx1 promoter. These compounds would have to be investigated further to determine exactly which cellular pathway(s) they interfere with.

3.3.3 Screening for compounds that block the activity of viral IFN antagonists

In addition to screening for compounds that inhibit IFN signalling, the Mx GIPSE cell line can also be modified to screen for compounds that block the activity of viral IFN antagonists. Thus, these compounds might subsequently be developed as novel antiviral drugs.

To test this approach, the Mx GIPSE cell line was engineered to express the V protein of PIV5, generating a new cell line called Mx GIPSE/V. The V protein blocks IFN signalling by targeting STAT1 for proteasome-mediated degradation (Didcock *et al.*, 1999b). Therefore, in a cell line constitutively expressing V, the IFN signalling pathway

Figure 3.27 Mx GIPSE cells were used in a screening assay to search for compounds that inhibit or block IFN signalling.

Mx GIPSE cells were setup in duplicate 96-well microtitre plates at approximately 20% confluency. 24 h later, compounds were added to the wells. Two days after stimulation with the compounds, exogenous IFN was added followed by the addition of puromycin (or untreated in duplicate plate) 4h later. At 4 days after stimulation with puromycin, monolayers were fixed using a formaldehyde solution and later stained with crystal violet stain. Wells from end columns were used as controls as no compounds were added to these wells. These wells guarantee that the Mx GIPSE cells can only survive puromycin toxicity when in the presence of IFN. In panel A, no puromycin was added to wells with compounds and consequently, wells where cell death was observed contained compounds that were toxic to cells. In panel B, puromycin was added to wells, and so cell death may have occurred due to the compound being cytotoxic or due to puromycin toxicity. For example, the compound contained in well B2 might be blocking IFN signalling; it is not cytotoxic because cells survived in panel A, but as observed in panel B, cells died in the presence of the compound, IFN and puromycin.

would be constantly blocked, thus preventing the induction of the Mx1 promoter. Thus, unlike Mx GIPSE cells, Mx GIPSE/V cells should die in the presence of puromycin, even if stimulated with exogenous IFN- α . However, if the Mx GIPSE/V cells survive puromycin selection in high throughput screening assays, then it may be because a particular compound inhibited the action of the V protein.

The Mx GIPSE cell line was engineered to constitutively express the V protein of PIV5, by use of a lentivirus vector called pdl'PIV5V'bla (provided by Dr. Yun-Hsiang Chen, St. Andrews University) that expresses both the V protein and the blasticidin resistance gene under the control of the constitutive SFFV promoter. Lentivirus stocks were made using the pdl'PIV5V'bla plasmid. These stocks were used to infect the Mx GIPSE cell line, and blasticidin was added to the infected cells at 2 days pi at a concentration of 10 μ g/ml. Blasticidin selection was maintained for at least 10 days, after which a polyclonal cell line was generated. The resulting Mx GIPSE/V cell line was characterized by immunofluorescence to check the expression of the V protein. Cells were fixed and stained 48h after stimulation with exogenous IFN. The staining was performed using a PIV5-p-k mAb (figure 3.28). The results indicated that a number of Mx GIPSE/V cells didn't seem to be expressing the V protein. Furthermore, in IFN treated cells, eGFP expression was observed, indicating that the Mx1 promoter was being induced (data not shown). However, when puromycin was added to Mx GIPSE/V cells, 100% of cells death was observed, indicating that indeed expression of the PIV5 V protein was blocking the induction of the Mx1 promoter in these cells. Nevertheless, the Mx GIPSE/V cells were subcloned by a Summer student, Ini Witzel, and tested by immunofluorescence analysis against the V protein, resulting in three positive clones. One of the subcloned cell lines, termed Mx GIPSE/V 5.E2 (figure 3.29), was used in subsequent experiments and will be referred to as the Mx GIPSE/V cell line for simplicity.

To ascertain whether the Mx GIPSE and Mx GIPSE/V cell lines could be used for screening chemicals in compound libraries that block the activity of the V protein, a screening assay was setup in 96-well microtitre plates. The Mx GIPSE cell line was used as a control in this assay, to pinpoint compounds that are either toxic to cells, inhibit the

Figure 3.28 The polyclonal Mx GIPSE/V cells correspond to a mixed cell population, containing cells that express the V protein of PIV5 quite strongly and other cells in which V was not detected by immunofluorescence analysis.

Mx GIPSE and polyclonal Mx GIPSE/V cells were stained with primary antibody against the V protein of PIV5 and an anti-mouse texas red-conjugated secondary antibody. Expression of the V protein was observed in patches in the Mx GIPSE/V cells. The Mx GIPSE cells were used as a negative control.

Figure 3.28

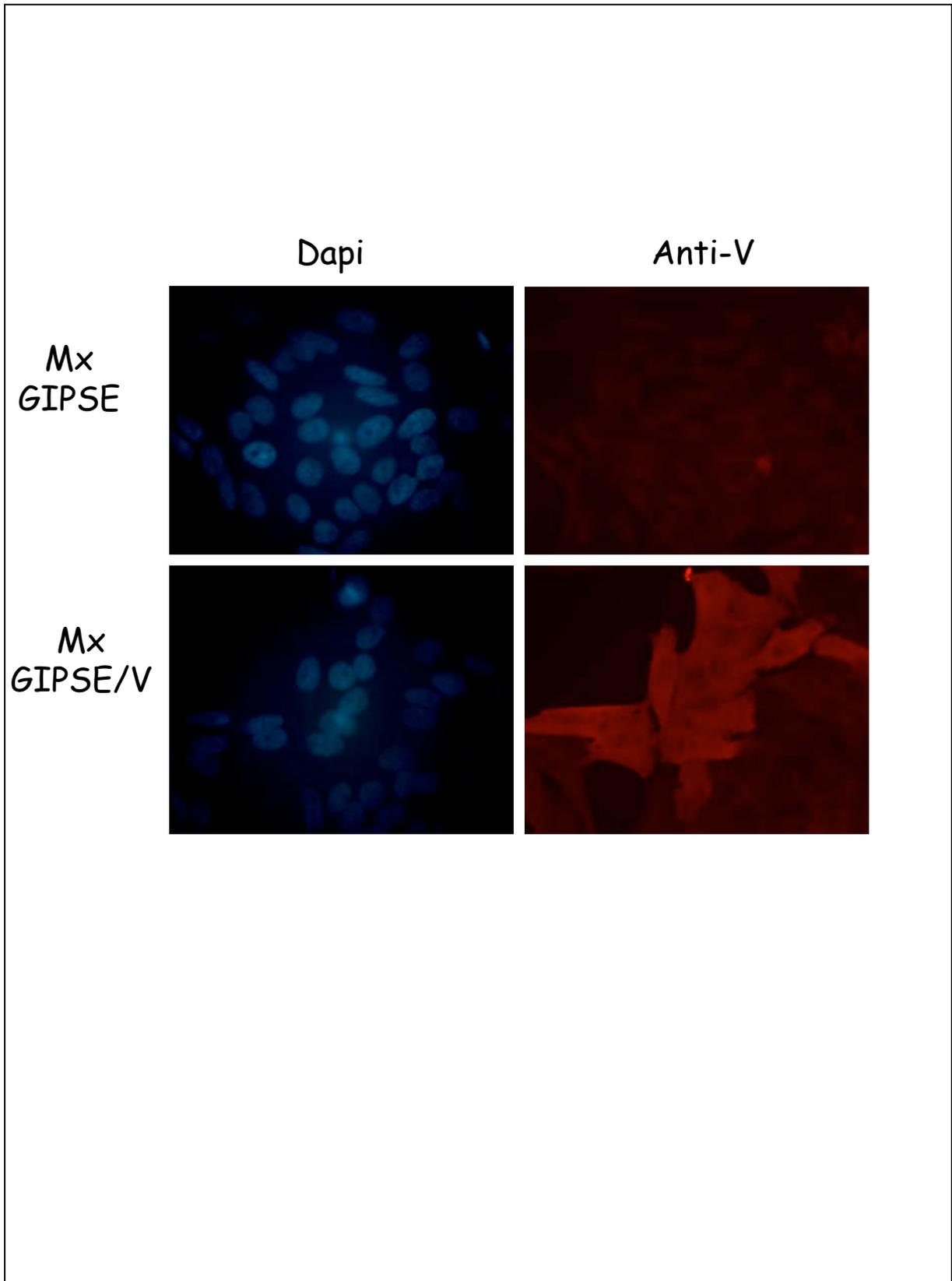
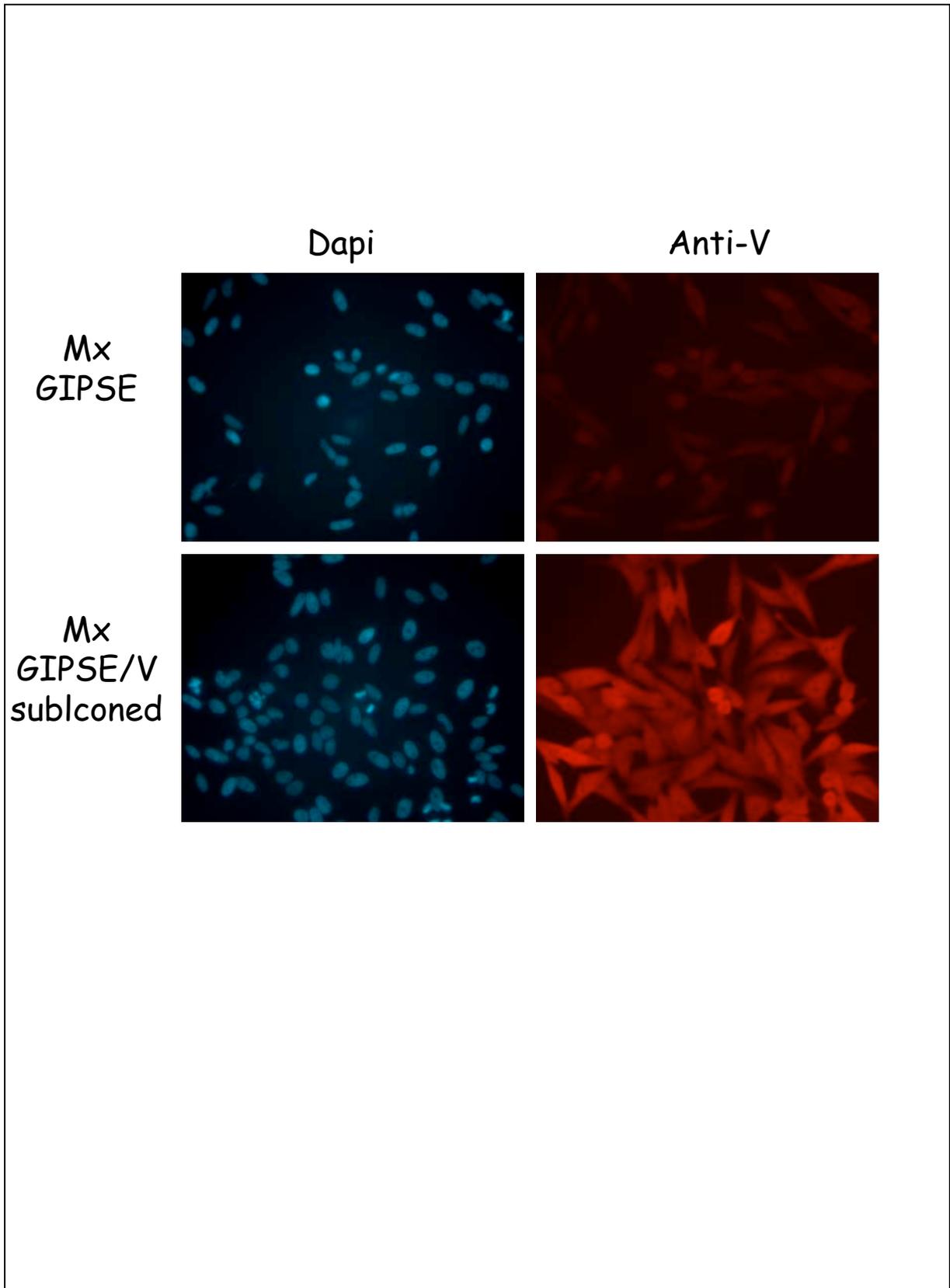


Figure 3.29 The subcloned Mx GIPSE/V cells all expressed the V protein of PIV5 quite strongly, as detected by immunofluorescence analysis.

Mx GIPSE and subcloned Mx GIPSE/V cells were stained with primary antibody against the V protein of PIV5 and an anti-mouse texas red-conjugated secondary antibody. Expression of the V protein was observed in all the Mx GIPSE/V cells while the Mx GIPSE cells were used as a negative control.

Figure 3.29



induction of the Mx1 promoter or block the puromycin resistance gene. Cells were setup at approximately 20% confluency, 24h prior to the addition of the compounds. 48h after addition of the compounds, exogenous IFN- α was added, followed by puromycin (1 $\mu\text{g/ml}$) 4h later. Surviving cells were visualised by staining the plates with crystal violet 4 days after addition of IFN and puromycin (figure 3.30). Although the number of compounds screened in this assay was very low, surviving cells were observed in well A7 panel B, suggesting that this compound may block the action of the PIV5 V protein. These results show that the Mx GIPSE cells can be modified to express a viral IFN antagonist and then used to screen for compounds that block the action of the IFN antagonist.

Figure 3.30 Mx GIPSE/V cells were used in a screening assay to search for compounds that inhibit or block the V protein of PIV5.

Mx GIPSE and Mx GIPSE/V cells were setup in 96-well microtitre plates at approximately 20% confluency. 24 h later, compounds were added to the wells. Two days after stimulation with the compounds, exogenous IFN was added followed by the addition of puromycin 4h later. At 4 days after stimulation with puromycin, monolayers were fixed using a formaldehyde solution and later stained with crystal violet stain. Wells from end columns were used as controls as no compounds were added to these wells. These wells guaranteed that the Mx GIPSE cells could only survive puromycin toxicity when in the presence of IFN, and that Mx GIPSE/V cells could not survive puromycin toxicity even in the presence of added exogenous IFN. In panel A containing Mx GIPSE cells, wells where cell death was observed contained compounds that were toxic to cells or that made cells sensitive to puromycin. In panel B containing Mx GIPSE/V cells, all cells should have died in the presence of puromycin, as the V protein blocks the induction of the Mx1 promoter. However, in well A7, cells survived in the presence of puromycin, indicating that the particular compound added to this well, may be blocking or inhibiting the V protein of PIV5.

4. DISCUSSION

4.1 IFN-sensitive viruses as live attenuated virus vaccines

Vaccines are a potent method of controlling viruses and the diseases they cause in humans and animals. IFN-sensitive viruses (attenuated viruses that cannot block or inhibit the IFN response) could make ideal candidates for the generation of live attenuated virus vaccines, as they should be highly immunogenic but non-pathogenic viruses. IFN-sensitive viruses should contain the attenuating mutation(s) in the protein(s) corresponding to the viral IFN antagonist(s), which are in general, accessory proteins, non-essential for virus replication. This feature would make IFN-sensitive viruses still capable of infecting and replicating in cells, but infection should immediately trigger innate and adaptive defence mechanisms in the infected organism, which would not be blocked or limited by the attenuated virus. This would lead to the elimination of the virus whilst still inducing immunological memory that would protect the host on exposure to the wild-type virus.

IFN-sensitive viruses can potentially be obtained in two ways: through reverse genetics techniques or through the selection of naturally-occurring IFN-sensitive mutant viruses from virus populations.

Reverse genetics confers the ability to engineer specific mutations or deletions into viral genomes; to generate viruses that are sensitive to IFN, one would target the viral IFN antagonist genes. Previous reports have demonstrated the viability of using reverse genetics techniques to generate attenuated viruses that have lost their ability to circumvent the host's IFN response by deletion or truncation of the viral IFN antagonist gene (Ferko *et al.*, 2004, Fernandez-Sesma *et al.*, 2006, Solorzano *et al.*, 2005, Talon *et al.*, 2000b, Valarcher *et al.*, 2003, Weber *et al.*, 2002, Wright *et al.*, 2006). However, deletion of the viral IFN antagonist gene must be handled with precaution as it might bring about an over-attenuation of the virus due to viral IFN antagonists frequently being

multifunctional proteins (e.g. the V protein of PIV5, He *et al.*, 2002, and the NS1 protein of Influenza A, Kochs *et al.*, 2007). Other reports have demonstrated that IFN-sensitive viruses can also be generated using reverse genetics by the infliction of attenuating mutations in the gene that encodes the viral IFN antagonist (Bartlett *et al.*, 2006, Liu *et al.*, 2006, Van Cleve *et al.*, 2006). Attenuated viruses constructed using reverse genetics will probably contain more than one attenuating mutation, which will reduce the possibility of reversion to virulence, as demonstrated in a study to generate hPIV1 vaccine candidates (Newman *et al.*, 2004). In addition, the use of reverse genetics techniques would allow the easy monitoring of all phases of development, manufacture and utilisation of the vaccine in humans because the genetic basis for attenuation would be known and well established. However, reverse genetics techniques are laborious, time-consuming and they have not yet been developed and established for all viruses. In the case of the emergence of a new virus, reverse genetics techniques would prove too slow to generate live attenuated virus vaccine candidates to attempt to control the threat.

The alternative to reverse genetics would be to select and isolate mutant IFN-sensitive viruses from a given virus population. The main attraction of this methodology is the ability to rapidly generate live attenuated virus vaccine candidates against any newly emergent and zoonotic viruses, without the prior need to sequence and analyse the viral genome in search for attenuating point mutations or attenuating gene deletions. However, the success in selecting naturally-occurring IFN-sensitive viruses will always depend on the proportion of IFN-sensitive to IFN-resistant viruses in the starting virus population. If one imagines that possibly one in every 10^6 virus particles in a given wild-type virus stock are IFN-sensitive, what are the chances of selecting and isolating these viruses? An obvious solution is to attempt to increase the proportion of IFN-sensitive viruses in the starting virus population by mutagenesis (e.g. by growing the virus stock in the presence of a chemical mutagen to increase the number of mutant viruses in the population; see below). A second solution is to carry out multiple rounds of selection so that, for every round, the proportion of IFN-sensitive to IFN-resistant viruses increases. Better still would be a combination of both methods: carrying out multiple rounds of selection using a mutagenised virus stock as the starting virus population.

4.1.1 Selection of naturally-occurring IFN-sensitive viruses using Mx GIPSE and Mx TIPSE cell lines

We knew from the start that the development of an effective methodology to select naturally-occurring IFN-sensitive viruses from IFN-resistant virus populations would not be straightforward. However, the potential ability to rapidly generate live attenuated vaccine candidates for any new virus threat was a very attractive possibility. Consequently, a cell-based model system was developed to attempt to select IFN-sensitive viruses that could no longer block or inhibit IFN signalling pathways, from a given virus population, be it a wild-type or a mutagenised virus stock.

The developed methodology relies on an IFN-producing and responsive cell line, termed Mx GIPSE cell line, expressing the eGFP and puromycin resistance genes under the control of the IFN-inducible murine Mx1 promoter. Puromycin is used as the selection drug to kill cells infected with IFN-resistant viruses: these viruses block the induction of the IFN signalling pathways and therefore block the production of the puromycin resistance gene *pac*, leading to cell death in the presence of puromycin (see figure 3.19). The murine Mx1 promoter was chosen as the IFN-inducible promoter in this system as it is a well-characterised, strong promoter (Hug *et al.*, 1988) and is naturally tight, as there is no basal gene expression in the absence of IFN. It was also decided to use the full-length promoter (2.3 kb) as the regulatory elements, such as enhancers and silencers, included in the proximal and distal promoter sequences might prove important in directing the level of transcription of the genes.

The Mx GIPSE cell line was generated and characterised, but it had been observed in immunofluorescence and fluorometer assays that this cell line might not be a homogenous cell line in that the Mx1 promoter might be inducing constitutive expression of eGFP and *pac* in a small percentage of the cells. These cells could potentially aid the replication of IFN-resistant viruses (for they would be resistant to the toxicity of

puromycin) and therefore, reduce the possibilities of selecting IFN-sensitive viruses. These cells might have arisen in the population due to the use of a lentivirus vector system to initially generate the cell line. When lentivirus particles infect cells, the viral genome randomly integrates in the host's DNA. Therefore, the viral genome will be present in different positions in each cell so, although all cells in the population might be expressing the gene(s) of interest, the cell line will never be homogenous unless it is subcloned. In the case of the Mx GIPSE cell line, the lentivirus genome might have integrated in the proximity of a constitutive promoter which could have directed the constitutive expression of eGFP and pac in a small percentage of cells.

A possible solution to this problem would have been to use FACS analysis to separate the constitutive-expressing cells exhibiting eGFP fluorescence in the absence of added exogenous IFN, from the cells that were expressing eGFP in an inducible manner. FACS provides a method for sorting a heterogeneous mixture of cells into two or more containers, one cell at a time, based upon the fluorescent characteristics of each cell. However, this was not attempted as St. Andrews University does not possess a FACS flow cytometer. In addition, it was felt that the second solution of generating an analogous cell line to Mx GIPSE would be advantageous, as the analogous cell line might prove of use in other contexts, such as the selection of IFN-resistant revertant viruses from IFN-sensitive populations (see below).

Consequently, with the aim of eliminating the constitutive-expressing cells, an analogous cell line to Mx GIPSE expressing the HSV-tk protein instead of eGFP, was generated. The presence of the HSV-tk gene and use of ganciclovir as a selection drug allowed for a negative selection procedure to be possible in addition to the positive selection procedure of pac/puromycin: when supplemented to cells, ganciclovir is converted to a toxic product by HSV-tk, whilst it is harmless if the HSV-tk enzyme is absent in the cell. This negative selection process permitted the elimination of cells constitutively expressing HSV-tk and pac and resulted therefore in a homogenous cell line, termed Mx TIPSE cell line. Besides the selection of IFN-sensitive viruses using the positive pac/puromycin selection procedure, the negative HSV-tk/ganciclovir selection process may also allow the selection of IFN-resistant revertant viruses from an IFN-sensitive population. These revertant viruses would be of interest for research purposes and for use in diagnostic

laboratories as they would provide valuable background information about which mutations could lead to reversion to virulence and their location in the viral genome. Unfortunately, due to time constraints, the Mx TIPSE cell line was never tested for its ability to select IFN-sensitive viruses using the positive puromycin/pac selection protocol or IFN-resistant revertant viruses using the negative ganciclovir/HSV-tk selection procedure.

The Mx GIPSE cell line was however, tested for its ability to select IFN-sensitive viruses. In preliminary tests with PIV5 strains (figure 3.20, section 3.2.2.4), the selection procedure using Mx GIPSE cells, exogenous IFN and puromycin allowed the survival of cells infected with CPI- (an IFN-sensitive virus), whilst cells infected with CPI+ (an IFN-resistant virus) were killed under the same conditions by the toxic effects of puromycin. We thus wanted to test the system using the wild-type strain of PIV5 termed W3. However, as mentioned before, there would probably be a very small proportion of IFN-sensitive viruses in the W3 population and consequently, it was decided to mutagenise PIV5 W3 stocks to attempt to increase the proportion of IFN-sensitive viruses. PIV5 W3 stocks were prepared in the presence of varying concentrations (0.01 to 10 mM) of a chemical mutagen, 5-fluorouracil (5-FU) (data not shown). The selection procedure was carried out as previously but, after 48h in the presence of puromycin, 100% cell death was observed in Mx GIPSE cells infected with the mutagenised W3 stocks, indicating that either the 5-FU did not mutagenise the virus enough to allow the generation of sufficient IFN-sensitive viruses or the conditions used in the selection procedure were not adequate.

The next set of experiments with W3 and CPI- mixed virus populations demonstrated that, under the conditions employed, the developed model system using the Mx GIPSE cell line did not allow the preferential selection of CPI- over W3 (figures 3.22, 3.23 and table 3.1).

These experiments demonstrated that the developed methodology has a number of important considerations that were not thought about at the start of the project.

1. The aim of the methodology is to maximise the possibilities of selecting IFN-sensitive viruses. In order to accomplish this, the maximum number of cells should get infected but only with one virus particle each. With the number of IFN-sensitive viruses being so small compared to the number of IFN-resistant viruses (apparently even when the starting virus stock has been mutagenised), we can not take the risk of one cell becoming infected with both an IFN-sensitive and an IFN-resistant virus for this would result in the elimination of the valuable IFN-sensitive virus. It is thus vital to initially infect the cells at the ideal m.o.i. of 1 pfu/cell (and thus to know the exact titre of the starting virus stock), to ensure there will not be a loss of any IFN-sensitive mutant viruses at this initial stage of the protocol.

2. The timing of addition of both IFN and puromycin in the selection procedure is a crucial factor in the developed methodology.

Puromycin needs to be added to the medium after cells infected with IFN-sensitive viruses have had a chance to produce the puromycin resistance protein in response to IFN; however, it is the action of puromycin that will eliminate the IFN-resistant viruses, so it needs to be added early enough, before the IFN-resistant viruses have a chance to spread to neighbouring cells. As demonstrated, puromycin at a concentration of 2 µg/ml should be added to Mx GIPSE cells 2 to 8h after treatment with exogenous IFN (figure 3.18, section 3.2.2.3).

In terms of IFN, how do we know the ideal time-point to add exogenous IFN to the cells in the selection procedure, considering that: enough time has to be allowed before the addition of IFN for IFN-sensitive viruses to establish an infection so that they are not dampened down or eliminated by the antiviral effects of IFN; IFN needs to be added early enough to limit the replication and spread of IFN-resistant viruses to neighbouring uninfected cells? In the current methodology, there are two sources of IFN: the IFN produced by the Mx GIPSE cells in response to the virus infection and the IFN added exogenously during the selection procedure. It would be straightforward to determine the ideal conditions for the addition of exogenous IFN (i.e. the time-point of addition and the concentration of IFN) if the other source of IFN in the system was eliminated. Thus, a possible solution would be to engineer Mx GIPSE cells (or Mx TIPSE cells) to

constitutively express the Npro protein of BVDV, which inhibits IFN production by blocking IRF3 (Hilton *et al.*, 2006); therefore, the presence of IFN in the medium could be controlled and monitored by adding the chosen amount of exogenous IFN at the preferred time-point. In addition to allowing a better control of the IFN levels during the selection process, such a cell line would be useful at the stage of amplification of IFN-sensitive viruses for the expression of the BVDV Npro protein would allow attenuated viruses to replicate more efficiently, as demonstrated previously (see figure 3.6). Other viral IFN antagonists that specifically block the IFN induction pathway could also be used instead of BVDV Npro for this purpose (reviewed in Weber & Haller, 2007, Weber *et al.*, 2004). Another alternative would be to use the pdl'Mx GIPSE lentivirus vector to generate an analogous Mx GIPSE cell line in Vero cells, which are monkey kidney cells which do not produce IFN due to a spontaneous gene deletion (Desmyter *et al.*, 1968, Mosca & Pitha, 1986). However, the transduction of simian cells using lentivirus-based vectors is blocked by the presence of an inhibitory cellular factor in simian cells which restricts lentivirus replication at an early post-entry step (Kootstra *et al.*, 2003). Consequently, it would not have been possible to generate Mx GIPSE Vero cell lines using the lentivirus-based vector system.

3. How long should the Mx GIPSE cells be left under selective pressure before the addition of IFN-permissive cells (HEp2/BVDVNpro/PIV5V, see section 3.1) to the Mx GIPSE surviving cells? The cells need to be in the presence of puromycin long enough to guarantee the death of all cells infected with IFN-resistant viruses. Almost 100% cell death was observed 48h after the infection of Mx GIPSE cells with CPI+ (puromycin had been added at 9h pi, figure 3.20); consequently, for PIV5, cells should be left under selective pressure for at least 39h before washing them and adding the HEp2/BVDVNpro/PIV5V permissive cells. This time-point would have to be optimised for other viruses.

We do not think that the developed system as it stands will work to successfully select IFN-sensitive viruses; however, it might prove very valuable if combined with FACS scanning. Firstly, the constitutive-expressing eGFP cells would have to be eliminated

from the Mx GIPSE cell population by FACS sorting, as previously discussed. Ideally, the next step would be to engineer the homogenous Mx GIPSE cell line to constitutively express the Npro protein of BVDV (or another viral antagonist that blocks IFN induction). The constitutive expression of the Npro protein would block the production of IFN due to virus infection and would therefore allow a much more controlled selection process using exogenous IFN. Once a suitable cell line was generated, cells could be infected with a wild-type or preferably, a mutagenised wild-type virus stock (to increase the number of IFN-sensitive viruses in the starting population), and supplemented with exogenous IFN and puromycin at the optimised time-points. After approximately 24h in the presence of puromycin, surviving cells could be sorted by FACS analysis into cells expressing eGFP (which would correspond to uninfected cells or cells infected with IFN-sensitive viruses) and non-eGFP-expressing cells (which would correspond to cells infected with IFN-resistant viruses). IFN-permissive cells (HEp2/BVDVNpro/PIV5V cell line) could then be added to the eGFP-expressing cells to allow the amplification and growth of IFN-sensitive viruses. Multiple rounds of selection might still need to be carried out to completely eliminate the IFN-resistant viruses and allow appropriate isolation and amplification of the IFN-sensitive viruses.

4.1.2 Panning as an alternative method for the selection of IFN-sensitive viruses

This section describes a different cell-based approach that could be developed to select naturally-occurring IFN-sensitive viruses from given virus populations. As mentioned before, the success in the selection of naturally-occurring IFN-sensitive viruses would depend on their proportion in the starting virus population. If spinner cells were used (see below), the method here described would allow the screening of a large quantity of infected cells, therefore increasing the possibilities of selecting IFN-sensitive viruses, which would be an advantage over the Mx GIPSE/ Mx TIPSE methodology.

The alternative model system would include the use of an immune selection method termed panning. The following description of the panning technique uses *Staphylococcus*

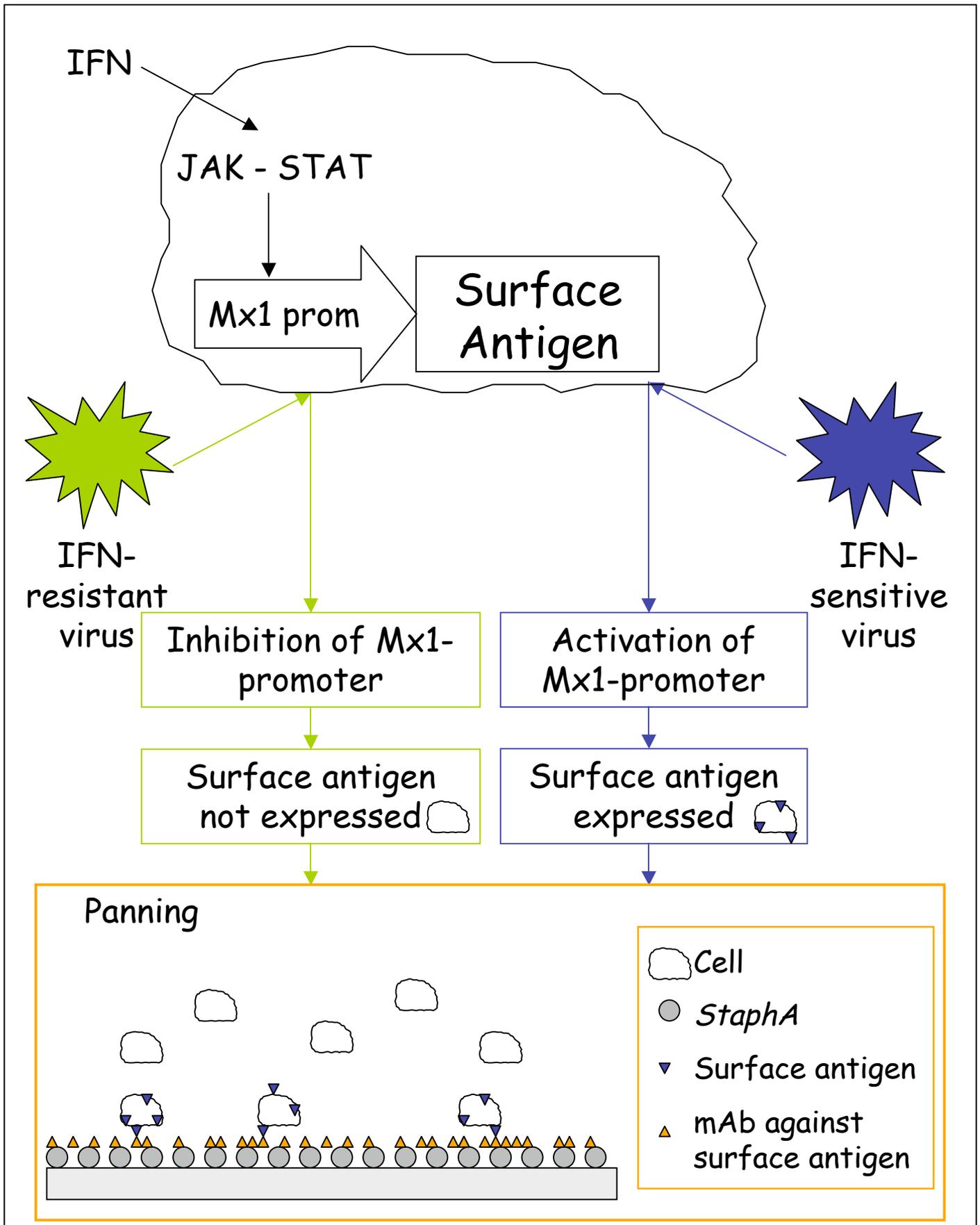
aureus as an example. It has been previously reported that *Staphylococcus aureus* strain A (*StaphA*) form monolayers on plastic tissue culture plates (A plates; Randall, 1983) and that a cell wall component of *StaphA*, termed protein A, has a high affinity for certain classes of IgG immunoglobulins (Goding, 1978). *StaphA* bacteria have been used widely as solid-phase absorbents for the isolation of antigen-antibody complexes because the binding of antibody to protein A does not interfere with the antigen binding capacity of the antibody (Randall, 1983). A plates can therefore be used to separate cells on the basis of their cell surface antigens (for example, cells expressing viral antigens on their surface). A cell line could be specifically engineered to express a surface antigen under the control of an IFN-inducible promoter such as the murine Mx1 promoter (figure 4.1). In this case, if a cell was infected with an IFN-sensitive virus, the Mx1 promoter would be induced leading to the expression of the antigen on the cell surface; however, if a cell was infected with an IFN-resistant virus that blocked IFN signalling, the Mx1 promoter would not be activated and consequently the surface antigen would not be expressed. The panning method would then be utilised (using an antibody against the surface antigen) to separate the cells expressing the surface antigen from the non-expressing cells (figure 4.1), thereby isolating any IFN-sensitive viruses present in the initial virus population. IFN-permissive cells (HEp2/BVDVNpro/PIV5V cell line) could subsequently be added to the selected cells for the *StaphA* monolayer does not prevent the growth of tissue culture cells on the plastic surface (Randall, 1983). The IFN-permissive cells would allow the efficient growth and amplification of IFN-sensitive viruses. We have successfully proved the viability of this method by showing that PIV5 infected cells can be separated from uninfected cells on the basis of the expression of the viral HN surface protein, using a anti-HN mAb (PIV5 HN-4-a) bound to A plates (data not shown).

This IFN-inducible cell line could be generated using adherent, monolayer-forming cells, such as HEp2 cells, or using suspension cells, such as 293 spinner cells. The use of adherent cells might prove difficult during trypsinisation steps in the process: trypsinisation would have to be very efficient to avoid the formation of cell clumps. The use of suspension cells would eliminate the need for trypsinisation steps. In addition, suspension cells are easy to grow and passage in large quantities, so the use of suspension

Figure 4.1 Panning as an alternative method to select IFN-sensitive viruses and IFN-resistant revertant viruses.

Cells lines could be specifically engineered to express a surface antigen under the control of an IFN-inducible promoter, such as the Mx1 promoter. Cells infected with IFN-resistant viruses would not express the surface antigen on their surface for the viral IFN antagonist would block the activation of the Mx1 promoter by blocking IFN signalling. Cells infected with IFN-sensitive viruses would express the surface antigen for the Mx1 promoter would be activated. These cells could then be selected by an immune selection method termed panning. A mAb raised against the surface antigen would be bound to A plates; cells expressing the surface antigen would bind to the mAb, whilst non-expressing cells would remain in the medium. These cells could therefore be separated on the basis of the expression of the surface antigen.

Figure 4.1



cells would facilitate the screening of a large number of cells, resulting in a better chance of selecting IFN-sensitive viruses. Spinner cells would therefore probably be more suitable for this methodology than adherent cells.

In addition, panning could be optimised to select IFN-resistant revertant viruses from an IFN-sensitive population – in this case, the cells of interest would be the cells that did not bind to the antibody bound to the A plates.

4.1.3 Amplification of IFN-sensitive viruses

IFN-sensitive viruses selected through the Mx TIPSE or panning methods would need to be grown to high titres to permit their characterisation and potential use as live attenuated vaccine candidates. IFN-sensitive viruses generally do not grow well in tissue culture cells due to the IFN antiviral response; however, they can be grown to high titres in Vero cells, which are cells that can not produce IFN due to a spontaneous gene deletion (Desmyter *et al.*, 1968, Mosca & Pitha, 1986) but can respond to IFN if it is added exogenously. However, as part of a group effort, it was of interest to generate IFN-permissive cell lines expressing viral IFN antagonists to compare the growth and spread of viruses in these cell lines and in Vero cells. HEp2 cells were the choice for the initial cell lineage as they are easy to grow and maintain, are efficiently transformed by lentivirus vectors and die quickly in puromycin if they are not expressing the vector-contained drug resistance gene. HEp2 cells are not however approved for vaccine development; therefore, in the future, it would be of interest to engineer MRC5 cells to express the viral IFN antagonists, for they are one of the few human cell lines that have been approved for vaccine production. These engineered cell lines expressing viral IFN antagonists would be of interest for the amplification of IFN-sensitive viruses for use as vaccine candidates, for use in diagnostic laboratories and for research purposes regarding the functioning of the IFN system and viral IFN antagonists.

As demonstrated here (figure 3.6, section 3.1) and previously, cell lines expressing viral IFN antagonists can be used to amplify attenuated virus strains (Young *et al.*, 2003). The

best cell line (generated by Dr. Yun-Hsiang Chen), HEp2/BVDVNpro/PIV5V cell line, expresses both the Npro protein of BVDV and the V protein of PIV5. The comparison of the HEp2/BVDVNpro/PIV5V and Vero cell line regarding the amplification of attenuated viruses showed that both cell lines were equally efficient for that purpose (data not shown). However, if IFN-sensitive viruses were selected using the Mx GIPSE or Mx TIPSE cell lines, IFN would still be present in the supernatant after washing the cells. This IFN could lead to the induction of an antiviral state in Vero cells if these were added to the surviving cells, for Vero cells have the ability to respond to IFN. The induced antiviral state would limit the replication of IFN-sensitive viruses. However, if HEp2/BVDVNpro/PIV5V cells were added to the surviving cells, the joint expression of BVDV Npro and PIV5 V proteins would completely block the IFN induction and signalling pathways, in which case the presence of IFN would not induce an antiviral state in these cells. Consequently, HEp2/BVDVNpro/PIV5V cells would be more indicated than Vero cells for the amplification of selected IFN-sensitive viruses using the Mx GIPSE or Mx TIPSE developed methodology.

4.2 Screening for antiviral and anti-inflammatory compounds

After the generation of the Mx GIPSE cell line, its use was considered in assays to screen compound libraries in search of antiviral and anti-inflammatory compounds.

4.2.1 Screening for compounds that block IFN signalling

As demonstrated (figure 3.27, section 3.3.2), the Mx GIPSE cell line can be used in high throughput screening assays to search for compounds that inhibit or block the IFN signalling pathways. Such compounds would be of interest as anti-inflammatory drugs in certain disease states where there is over-inflammation, such as cystic fibrosis (Hudson, 2004), and for research purposes to study and characterise the mechanism that allows the potential block of IFN signalling. The developed screening assay is currently being used to screen a 16000 chemical compound library (kindly provided by Dr. N. Westwood, University of St. Andrews). Any hits resulting from this screening will have to be retested and further investigation will be required to determine if and how these compounds are blocking IFN signalling. The Mx TIPSE cell line could also be used for this purpose instead of the Mx GIPSE cell line.

The pdl Mx GIPSE lentivirus vector was deliberately designed to be a module vector so that the Mx1 promoter and eGFP reporter genes could be easily replaced by other promoters or other reporter genes respectively.

By replacing the Mx1 promoter in the lentivirus vector pdl Mx GIPSE, and generating analogous cell lines to the Mx GIPSE cells, it is possible that the approach described to screen for compounds that block IFN signalling, may be used to screen for compounds that inhibit other cellular activation pathways. For example, the NF- κ B promoter could be cloned in the place of the murine Mx1 promoter to generate a cell line; this would allow the high throughput screening for compounds that block the NF- κ B induction pathway.

The IFN- β promoter could also be cloned to replace the Mx1 promoter, which would lead to the potential screening of compounds that block the IFN- β induction pathway.

4.2.2 Screening for compounds that block viral IFN antagonists

The Mx GIPSE cell line can be engineered to constitutively express the V protein of PIV5 by use of a lentiviral vector, thereby generating the Mx GIPSE/V cell line. As demonstrated (figure 3.30, section 3.3.3), the Mx GIPSE and Mx GIPSE/V cell lines can be used in high throughput screening assays to search for compounds that block the action of the V protein of PIV5. Such compounds would be of interest as novel antiviral drugs that block the ability of the virus to inhibit the IFN response, and for research purposes to study and characterise the mechanism by which the compound blocks the activity of the IFN antagonist protein. The developed assay using Mx GIPSE and Mx GIPSE/V cell lines will be used to screen a 16000 chemical compound library (kindly provided by Dr. N. Westwood, University of St. Andrews), in search for compounds that block the activity of V.

Furthermore, with the aim of finding antiviral compounds that block viral IFN antagonists, similar approaches could be used to make Mx GIPSE cell lines constitutively express other viral IFN antagonist proteins. These antiviral compounds would prove very valuable for they would limit the extent of virus replication in the host and they would probably allow the host's IFN response to eliminate the infecting virus. It may also be possible to develop analogous cell lines to Mx GIPSE/V that express viral antagonist proteins that target other cellular activation pathways, e.g. the NF- κ B pathway, to identify other potential antiviral compounds.

4.3 Conclusion

The aim of this project was to develop methodologies to select IFN-sensitive mutant viruses from IFN-resistant virus populations. In the future, combinations of the developed method using Mx GIPSE or Mx TIPSE cells with FACS analysis and panning techniques could provide rapid and valuable methodologies for the selection of IFN-sensitive viruses to be used as live attenuated vaccine candidates. The potential for these methodologies is huge: they could be used to respond to a new emergent virus threat, by rapidly generating live attenuated vaccine candidates to control the virus. The Mx GIPSE /Mx TIPSE and panning methods could also potentially be developed to select IFN-resistant revertant viruses from IFN-sensitive populations, which would provide valuable information about the IFN system and viral pathogenesis.

The generated Mx GIPSE and Mx TIPSE cell lines are also currently being used to screen for compounds with antiviral and anti-inflammatory potential.

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6. APPENDIX: PRIMER SEQUENCES

Cloning

Primer Name	Sequence (5'→3')	RE site
fwdMx1Pseq	GGTGCCAGAACATTTCTCTATCG	*
revMx1Pseq	GTTTTTGGCGTCTTCCATGGGGC	BamHI
fwdtk	GCGGATCCACCATGGCTTCGTACCCCTGCCAT	BamHI
revtk	GGAATTCATATGTCAGTTAGCCTCCCCCATCTC	
E3L-HAfw	GTCGAGTCATATGTATCCTTATGATGTTCTG	NdeI
E3L-FLAGfw	GTCGAGTCATATGGACTACAAGGACGACGATGAC	NdeI
E3L-HA/FLAGrev	CTCCGATGGCGCCGCTGGATCCTCAGAATCTAATG	NotI

* There was no need to include an EcoRI site in the design of the primer for the Mx1 promoter contained a natural EcoRI site at the start of its sequence.

Sequencing

Primer Name	Sequence	Description
lentiPMx1seqfwd	GAATTCGATAATTCAAGCAAATGAAG	To sequence Mx1 promoter from pdl Mx GIPSE vector
lentiPMx1seqrev	GGATCCCCCTGCCAGGGC	
PMx1fwd	GGTCTGAGCCCTGGCAGGGGGATC	To sequence eGFP from pdl Mx GIPSE vector

The T7 and SP6 promoter primers were also used to sequence any genes cloned into the pGEM-T easy vector (Promega Ltd, United Kingdom).