A comparative study of Interferon evasion by Paramyxovirus V proteins

By

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ABSTRACT

The main conclusion of this thesis is that although all seven of the V proteins of 'emergent' paramyxoviruses studied suppress the activation of the IFNβ promoter via their conserved carboxy termini, only three block IFN signalling in the human and simian cells used. The other viruses, which were all isolated from animal hosts, were unable to block IFN signalling in these cell types, but may be able to block signalling in cells from other animals. Alternatively, these V proteins may have originated from IFN-sensitive viruses, the biological consequences of infection with which are discussed with reference to the establishment of persistent infections.

The V protein from our initial Nipah isolate (Nipah V Geelong) was unable to block IFN signalling and, on comparison with an isolate reported in the literature to antagonise IFN signalling, was found to have three amino acid differences in the V protein. When these amino acid differences were introduced into Nipah V Geelong it was found that a change in only one residue, at position 125, was required to restore function. In contrast to the non-functional Nipah V proteins, the functional proteins created in this study were found to block the IFN-stimulated translocation of STAT1 and STAT2 to the nucleus and to interact with STAT2.

Mapuera V was found to block both IFNα/β and IFNγ signalling in human and simian, but not murine cells, similar to the closely related SV5 V. However, in contrast to SV5 V, Mapuera V does not degrade STAT1 or interact with DDB1, a cellular protein shown to be essential for the targeted degradation of STAT1 by SV5 V. Mapuera V was found to interact with STAT2, as has been observed for SV5 V and other paramyxovirus V proteins, but it is not clear how this interaction relates to the function of Mapuera V. Furthermore, neither STAT2, STAT3 nor p48/IRF9 are targeted for degradation by Mapuera V and currently the mechanism by which it antagonises IFN signalling is unclear.

The regions of the various paramyxovirus V proteins involved in IFN antagonism were also examined using truncated V proteins. It is clear that all V proteins studied can suppress the activation of the IFNβ promoter by expression of the V-unique carboxy terminal alone and that deletion of this region abrogates this function.

However, it was demonstrated that for SV5 V, Mumps V and Mapuera V, neither the amino nor carboxy terminal domains alone are capable of blocking IFN signalling, whereas the amino terminal region of Nipah V is functional.

Evasion of the IFN response, although key to the replication of most viruses, can be restricted by host cell type and single point mutations in the V proteins of paramyxoviruses can switch these proteins from an IFN-sensitive to an IFN-resistant phenotype. Paramyxoviruses have a variety of mechanisms by which they antagonise IFN signalling, but apparently a common mechanism to block the activation of the IFNβ promoter, the details of which are discussed.

DECLARATIONS

i) I, Nicola Stock, hereby certify that this thesis, which is approximately 38,500 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 29/4/04 Signature of candidate

ii) I was admitted as a research student in October 2000 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 Faculty of Sciences at the University of St Andrews between 2000 and 2004.

Date 29/4/04 Signature of candidate .

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

Date 29/4/04 Signature of supervisor

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ABBREVIATIONS

General

U	Unit
IU	International unit
RT	Room temperature
o/n	Overnight
s/n	Supernatant
x*g*	Acceleration of gravity
min	Minute
h	Hour
hpi	Hours post infection
moi	Multiplicity of infection
wt	Wild-type

Reagents

PBS	Phosphate buffered saline
DMEM	Dulbecco's modified Eagle's medium
FCS	Foetal Calf Serum
NBCS	New Born Calf Serum
LB	Luria-Bertani medium
DMSO	Dimethyl sulphoxide
ONPG	*o*-Nitrophenyl-β-D-galactopyranoside
RNase	Ribonuclease A
CIAP	Calf Intestinal Alkaline Phosphatase

Molecular Biology

ORF	Open Reading Frame
IRES	Internal ribosome entry site
IFN	Interferon
STAT	Signal transducer and activator of transcription
ISRE	Interferon stimulated response element
GAS	Gamma activated sequence
DDB1	UV-damaged DNA binding protein subunit 1
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction

Nucleic Acids

DNA	Deoxyribosenucleic acid
RNA	Ribonucleic acid
cDNA	complementary DNA
dsRNA	double-stranded RNA
A	Adenosine
C	Cytidine
G	Guanosine
T	Thymidine

Amino Acids

Gly (G)	Glycine
Ala (A)	Alanine
Ser (S)	Serine
Thr (T)	Threonine
Cys (C)	Cysteine
Val (V)	Valine
Leu (L)	Leucine
Ile (I)	Isoleucine
Met (M)	Methionine
Pro (P)	Proline
Phe (F)	Phenylalanine
Tyr (Y)	Tyrosine
Trp (W)	Tryptophan
Asp (D)	Aspartic Acid
Glu (E)	Glutamic Acid
Asn (N)	Asparagine
Gln (Q)	Glutamine
His (H)	Histidine
Lys (K)	Lysine
Arg (R)	Arginine

Viruses

hPIV2	Human Parainfluenza virus 2
HeV	Hendra virus
MapV	Mapuera virus
MeV	Measles virus
MenV	Menangle virus
MuV	Mumps virus
NiV	Nipah virus
PoRV	Porcine rubulavirus
RSV	Respiratory syncytial virus
RV	Rabies virus
SalV	Salem virus
SV5	Simian virus 5
TiV	Tioman virus
VSV	Vesicular Stomatitis virus

CONTENTS

INTRODUCTION

I. THE PARAMYXOVIRUSES

The *Paramyxoviridae* family (reviewed in Lamb and Kolakofsky 2001) is part of the virus order *Mononegavirales*, which includes all viruses with non-segmented negative strand RNA genomes and contains two other families, the *Filoviridae* and the *Rhabdoviridae*. The *Paramyxoviridae* is divided into two sub-families, *Paramyxovirinae* and *Pneumovirinae,* which are then further sub-divided into genera according to characteristics such as genome organisation, virus morphology, protein characteristics and relatedness of protein sequence (see Table 1).

The *Paramyxoviridae* includes a number of important diseases of both humans and animals including Measles virus, regarded as one of the most infectious viruses known. Viruses such as Mumps virus, the human parainfluenza viruses and Human Respiratory Syncytial virus are common human pathogens and pose significant public health risks, especially to children in developing countries. The *Paramyxoviridae* also includes various animal viruses of economic significance including Newcastle Disease virus, Bovine Respiratory Syncytial virus and Turkey Rhinotracheitis virus. A wide variety of animal species are hosts for paramyxoviruses infections, but the individual viruses are often very host-specific and cross-species spread is usually limited. Paramyxoviruses also have zoonotic potential (reviewed in Wang and Eaton 2001) as had been observed with the newly emergent Hendra and Nipah viruses, which appear to have a natural reservoir in fruit bats but have caused infectious outbreaks in farm animals, domestic animals and humans.

Virion structure

The paramyxovirus virion is typically a spherical enveloped particle, although pleiomorphic and filamentous forms have also been observed, which varies in size from 150 to 350nm in diameter. The virion consists of a helical nucleocapsid core, surrounded by a lipid bilayer of host cell origin, studded with glycoprotein spikes that project from the surface by about 8-12nm (see Fig. 1, Panel A). All paramyxoviruses have two major envelope glycoproteins for membrane fusion (F) and attachment (HN, H or G), which allow the viruses to enter and exit host cells. The fusion proteins are

Family *Paramyxoviridae*

Subfamily *Paramyxovirinae*

Genus *Respirovirus*

Sendai virus
Human Parainfluenzavirus 1 & 3

Genus *Rubulavirus*

Mumps virus
Human Parainfluenza virus 2
Simian virus 5

Genus *Morbillivirus*

Measles virus
Canine distemper virus
Rinderpest virus

Genus *Henipavirus*

Hendra virus
Nipah virus

Genus *Avulavirus*

Newcastle disease virus
Avian parainfluenzavirus 2, 3, 4 & 5

Genus *"TPMV-like Viruses"*

Tupaia virus

Subfamily *Pneumovirinae*

Genus *Pneumovirus*

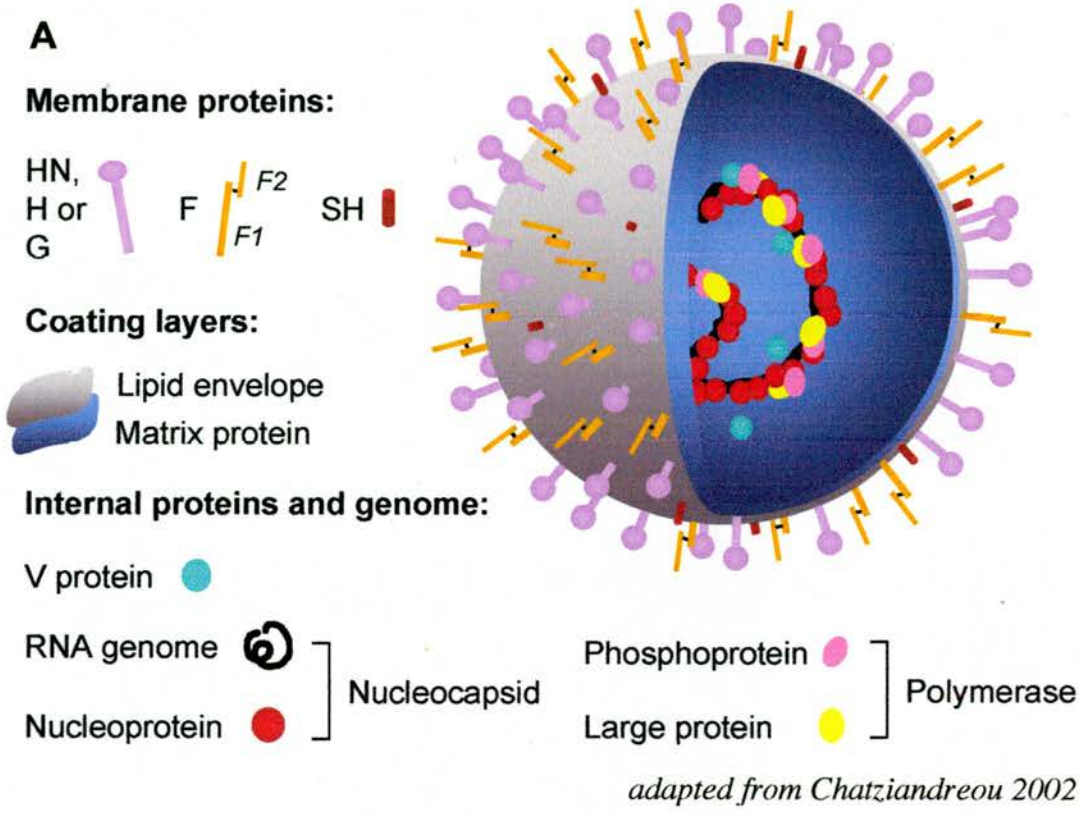
Human respiratory syncytial virus
Bovine respiratory syncytial virus

Genus *Metapneumovirus*

Turkey rhinotracheitis virus

Table 1. Paramyxovirus classification

Current ICTV classification of the *Paramyxoviridae* with examples of viruses from each genus, type viruses in bold.



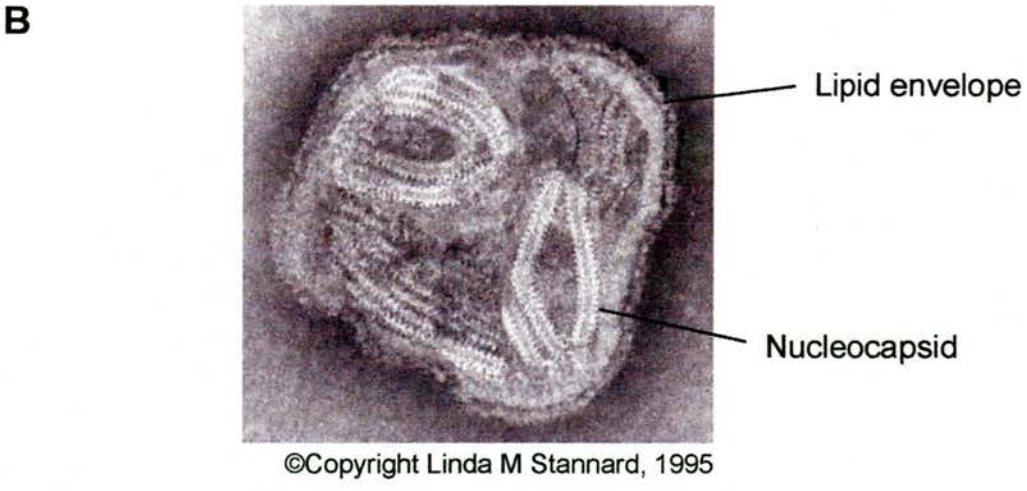


Figure 1: Schematic representation and electron micrograph of a paramyxovirus

Panel A shows a schematic representation of a typical paramyxovirus (not drawn to scale) and lists the components of a typical virion.

Panel B is an electron micrograph of a paramyxovirus particle, showing typical pleiomorphic shape and herringbone nucleocapsid structures. Micrograph by Linda Stannard, taken from Virus Ultrastructure web site: http://web.uct.ac.za/depts/mmi/stannard/paramyx.html

fairly similar throughout the family, but the characteristics of the attachment glycoprotein vary widely between genera, with those of the *Respirovirus* and *Rubulavirus* genera having both haemagglutinin and neuraminidase activity, those of the *Morbillivirus* genus having haemagglutinin activity only and those of the *Pneumovirus* sub-family having neither activity. Members of the *Rubulavirus, Pneumovirus* and *Metapneumovirus* genera also encode an additional small hydrophobic membrane protein (SH), the function of which has yet to be completely elucidated. An additional viral protein, matrix (M), is the most abundant protein in the virion and although is not an intrinsic membrane protein it has been shown to associate with the viral envelope. M protein has also been shown to interact with both viral nucleocapsids and glycoproteins and when expressed alone can self-assemble into sheets and tubes. Electron microscopy studies have shown that the M protein forms a layer directly beneath the lipid bilayer and is thought to be involved in the formation and budding of virus particles.

The paramyxovirus nucleocapsid consists of the single-stranded, negative sense RNA genome encapsidated by the viral N protein, which forms helices visible in infected cells as 'herringbone' structures (see Fig. 1, Panel B). The nucleocapsid is associated with the viral RNA-dependent RNA polymerase (L) and homotrimers of the viral phosphoprotein (P), which together form the minimal unit of infectivity and can transcribe mRNAs *in vitro*. Paramyxovirus nucleocapsids do not disassemble in the course of a viral infection, but the helical structures do exist in a number of different forms of varying pitch. It is thought that the viral polymerase can access the RNA bases only when the nucleocapsid is in the most relaxed form and it is possible that the polymerase is able to uncoil and recoil the nucleocapsid as it travels along the genome, allowing transcription and replication to occur. A number of accessory genes are also encoded by paramyxoviruses, some of which are present in the virion, including the various products of the P gene and in Pneumoviruses the M2 and NS proteins. In many paramyxoviruses these accessory genes are involved in the antagonism of the host immune response, which will be discussed in section IV.

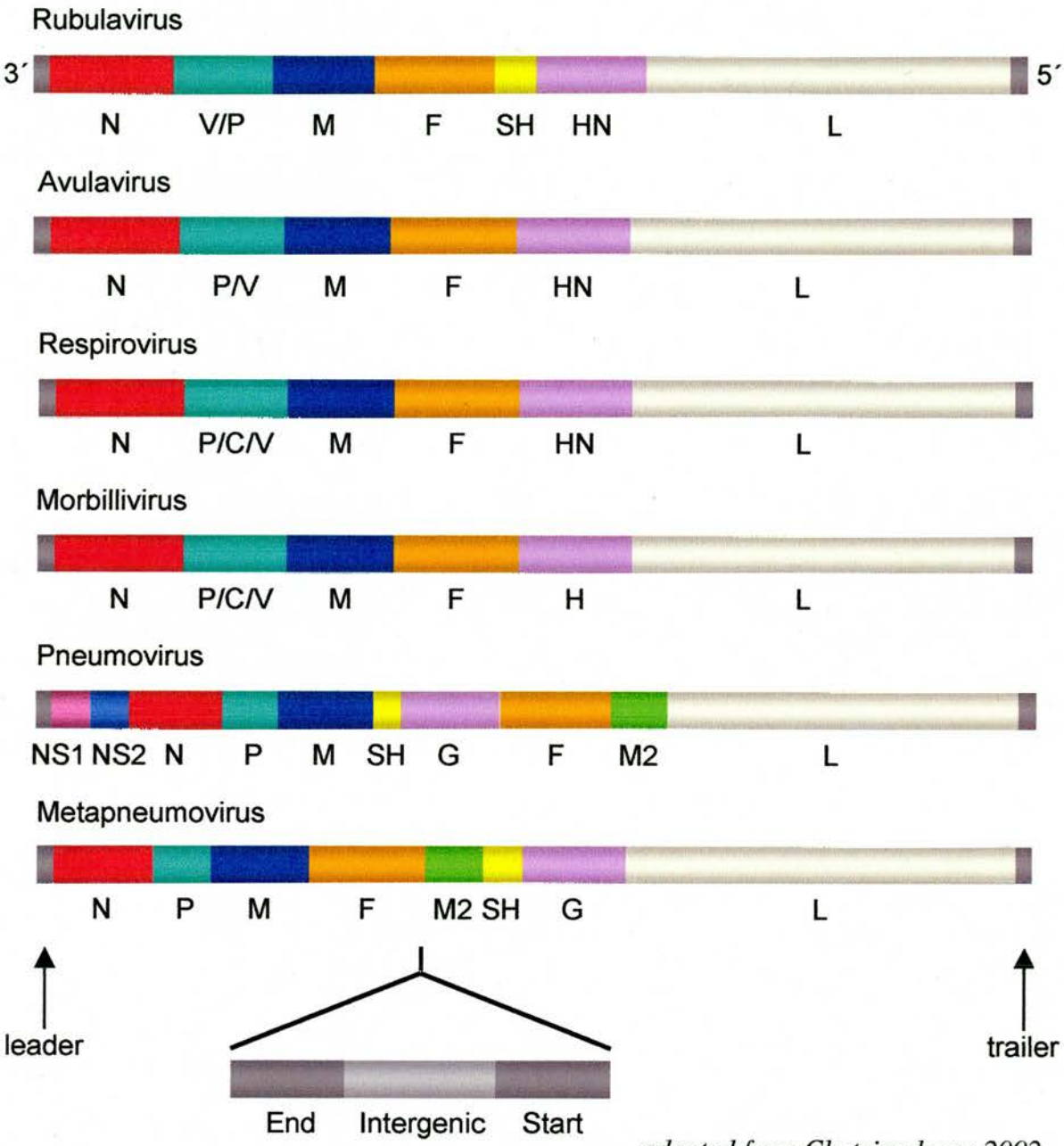
Virus genome and replication strategy

The paramyxovirus genome ranges in size from 15 to 19Kb and consists of 6 to 10 genes, depending on the virus genus, as illustrated in Fig. 2. Extra-cistronic regions are present at both ends of the genome with a 'leader' sequence of 50bp and a 'trailer' sequence of 50-161bp providing control signals for transcription and replication, which in paramyxoviruses are competitive processes. Each gene contains transcriptional control sequences at the start and end of the ORF that are copied into the mRNA and between the gene boundaries there are highly conserved intergenic sequences which vary from a fixed 3nt in length (respiroviruses and morbilliviruses) to variable lengths of 1-56nt (rubulaviruses and pneumoviruses).

i) Primary transcription and translation

Fig. 3 shows a summary of a typical paramyxovirus replication cycle. After virus adsorption to and fusion with the host cell membrane, viral nucleocapsids are released into the cytoplasm where all stages of the virus lifecycle occur. The next event is primary transcription of the encapsidated, negative sense (-) genome into positive sense (+) 5' capped and 3' poly-adenylated mRNAs by the viral polymerase complex. Transcription initiates at the 3' leader sequence of the genome and produces a (+) 3' leader RNA of approximately 50bp, after which the polymerase stops and then re-initiates at the beginning of the first gene. This re-initiation is very important as it defines the transcript as a mRNA rather than a copy of the genome. Individual mRNAs are formed by termination and re-initiation of transcription at each gene junction but as some polymerases fail to re-initiate at each gene, there is a gradient of transcription along the genome with 3' genes being transcribed most abundantly. Different paramyxoviruses display different re-initiation frequencies at each gene junction, allowing them to adjust the relative amounts of each protein expressed and there is some indication from the behaviour of Measles virus in neural and somatic cell lines that cellular factors may be able to alter these re-initiation frequencies, allowing differential expression of viral proteins in different cell types.

In addition to failure of transcription re-initiation, two other methods of transcriptional attenuation of gene expression have been observed in



adapted from Chatziandreou 2002

Figure 2: Paramyxovirus genome organisation

A schematic representation of the genome organisation of the paramyxovirus genera, not drawn to scale. The number of genes vary between 6 and 10 and are encoded in a linear, negative sense, single-stranded RNA genome. Non-coding regions termed the leader and trailer flank the coding regions and between each gene there are conserved regulatory sequences with end, intergenic and start sequences. These non-coding regions have roles in the control of transcription and translation.

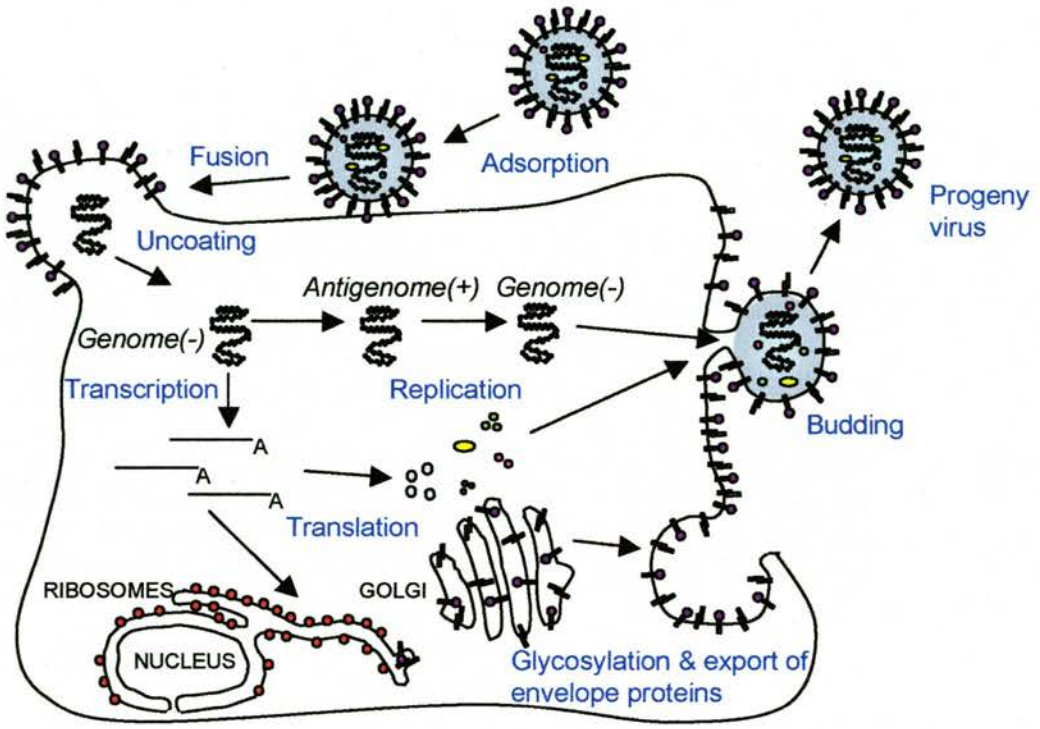


Figure 3: Paramyxovirus Lifecycle

A schematic representation of the different stages of paramyxovirus replication in mammalian cells. Virus particles attach to cell surfaces via their H, HN or G surface glycoproteins and viral and cellular membranes fuse via the activity of the viral fusion protein. The viruses then uncoat and nucleoprotein complexes are released into the cytoplasm where the entire replication cycle occurs. Viral negative-sense genomes are first transcribed by the viral polymerase into capped and poly-adenylated mRNAs which are translated by ribosomes into viral proteins. The glycoprotiens are gylcosylated in the golgi bodies of the cell and exported to the cell membrane where viral matrix protein is involved in the assembly and budding of progeny virions.

Once levels of viral proteins, especially N, have increased to a certain level, the viral polymerase switches from transcription to replication and makes full-length positive sense copies of the genome, the antigenome. From these antigenomes, full-length negative sense copies of the genome are made and packaged into progeny virions with small amounts of P, L and V proteins.

paramyxoviruses. The first involves poor termination of transcription at the end of genes, resulting in a bi-cistronic mRNA, only the first gene of which is translated. The polymerase is still able to terminate and re-initiate downstream of the second gene, so providing a mechanism whereby genes in the middle of the genome can be downregulated. The second method is overlapping ORFs whereby the start sequence of the second gene is upstream of the stop sequence of the first gene. In this case the polymerase will usually terminate at the stop sequence of the first gene, only transcribing the beginning of the second gene and only rarely will the polymerase continue to the end of the second gene. This occurs with the M2 and L genes of HRSV whereby the L mRNA, which is only required in small amounts, is only produced by relatively rare transcriptional read-through events.

ii) Genome replication

Replication of the paramyxovirus genome occurs via a full-length (+) copy called the antigenome, which like the (-) genome is only found in helical complexes with the N protein. Antigenomes are abundant in virally infected cells and can account for up to 40 per cent of all genome-sized RNA molecules and although they are often packaged into virions they have no ORFs and no translational products. Antigenome synthesis occurs once the processes of primary transcription and translation have accumulated sufficient viral proteins in the cell, in particular sufficient free N protein, which begins to encapsidate the nascent (+) RNA chains produced by the viral polymerase. This encapsidation signals to the polymerase to ignore the intergenic regulatory sequences and produce a full-length exact copy of the (-) genome, the antigenome, from which more (-) genomes can be replicated.

iii) Assembly and budding

After replication and assembly of the genome, P_3/L polymerase complexes attach to the helical nucleocapsids containing (-) genomes, which relocate to the cell membrane. Viral envelope glycoproteins, which have been synthesised and glycosylated in the endoplasmic reticulum and golgi, are transported to and incorporated into the cell membrane where virus assembly and budding occurs. The processes of viral assembly and budding at the plasma membrane have not been fully elucidated, but the M protein is thought to play a major role, including interactions

with both nucleocapsids and the viral membrane proteins as cellular proteins are usually excluded from the viral envelope.

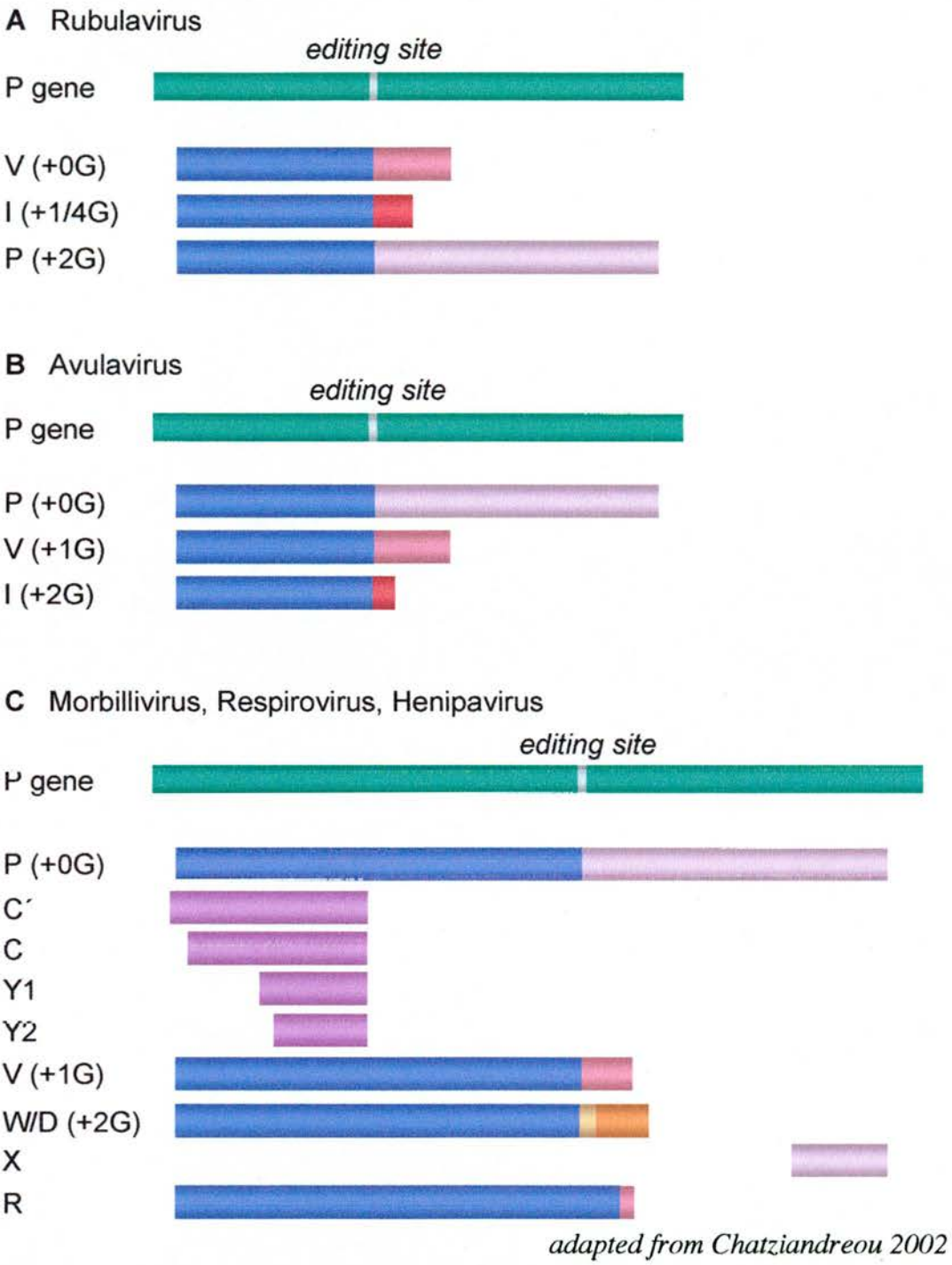
The P gene: multiple proteins encoded by a single gene

An additional unusual feature of paramyxoviruses is their ability to encode multiple proteins within the same section of the genome, illustrated by the various transcription and translation strategies of the P gene (summarised in Fig. 4). In members of the *Pneumovirinae* the P gene encodes a single protein, the phosphoprotein (P), but in all members of the *Paramyxovirinae* it encodes multiple gene products that are accessed by a variety of mechanisms, both transcriptional and translational.

i) RNA Editing

The P genes of all the *Paramyxovirinae* are subject to co-transcriptional RNA editing, first discovered in the laboratory of Prof. R.A. Lamb (Thomas et al. 1988), whereby the viral polymerase pauses at a conserved 'editing sequence' in the gene during transcription and on resuming 'stutters' in a certain number of cases and slips backwards, copying a number of residues for a second time. The RNA editing site consists of a poly-pyrimidine run, poly(U), followed by poly(C) and when the viral polymerase reaches the beginning of the poly(C) stretch it pauses. A small number of the paused polymerases slip back along the template one or two positions, taking the nascent RNA molecule with them and when transcription restarts these one or two residues are copied into the nascent mRNA a second time, adding one or two extra guanosine (G) residues. The addition of these non-templated residues into the mRNA results in a change in the reading frame downstream of the editing site, allowing a second ORF to be transcribed from this point. In this way members of the *Paramyxovirinae* produce proteins that are amino co-terminal up to the editing site and thereafter have unique carboxy termini.

Although all members of the *Paramyxovirinae* use RNA editing to produce additional proteins from the P gene, the details vary between genera. For SeV, the prototype *Respirovirus*, RNA editing of the P gene results in the formation of V mRNA by the addition of a single, non-templated G residue in 25-30% of transcription events (Vidal et al. 1990b; Vidal et al. 1990a; Kato et al. 1997a). The resulting V protein is amino



adapted from Chatziandreou 2002

Figure 4: P gene coding strategies of *Paramyxovirinae*

Viruses in the *Paramyxovirinae* sub-family use a variety of strategies to produce multiple proteins from the P gene. In all genera, RNA editing generates alternative mRNAs encoding V, W, I and D proteins with the number of additional G residues shown in parentheses. In respiroviruses, morbilliviruses and henipaviruses, alternative ribosome start sites for the C protein allow the P mRNA to encode multiple proteins. In addition, Measles virus utilises ribosomal frameshifting to produce the R protein and Sendai virus uses internal ribosome entry to encode the X protein.

co-terminal with P but has a unique C-terminus, containing seven highly conserved cysteine residues. The addition of two G residues by RNA editing also occurs in around 5% of transcripts, giving rise to W mRNA, the product of which is in effect a truncated P protein, as after the –1 frameshift caused by RNA editing there are only two further codons before a stop is reached. Another member of the genus, hPIV3 also uses RNA editing and as for SeV, the addition of two non-templated G residues by the viral polymerase results in a mRNA encoding a protein that is amino co-terminal with P, but has a unique C-terminus. However, this protein is not equivalent to the cysteine-rich V protein of SeV but is rather a different protein, the D protein, which appears to be unique to bovine and human PIV3 (Pelet et al. 1991; Galinski et al. 1992). An ORF for a cysteine-rich V protein is present in the P gene of both bovine and human PIV3 in a third reading frame and these viruses have been shown to add an indiscriminate number of additional residues at the editing site, so producing mRNAs for all three reading frames. However, there are two stop codons after the editing site in the V ORF of hPIV3, suggesting that RNA editing in this virus cannot produce a functional V mRNA. A third member of the *Respirovirus* genus, hPIV1, does not appear to carry out RNA editing at all, despite evidence of a V ORF in the viral P gene. This V ORF contains nine stop codons and so presumably cannot successfully produce a V protein and sequence analysis of a number of more recent isolates of HPIV1 has suggested that the loss of V was not a recent event (Rochat et al. 1992).

Members of the *Morbillivirus*, *Avulavirus* and *Henipavirus* genera have a similar strategy to SeV, as illustrated by MV, NDV and HeV in which the addition of a single non-templated G residue by RNA editing creates a mRNA encoding a cysteine-rich V protein (Cattaneo et al. 1989; Steward et al. 1993; Wang et al. 1998). Additionally in NDV, HeV and NiV the addition of two non-templated G residues by RNA editing creates a third mRNA encoding the W protein, similar to SeV W in that it is, in effect, a truncated P protein (Harcourt et al. 2000). All members of the *Rubulavirus* genus share the same P gene expression strategy as illustrated by SV5, which expresses P and V proteins by RNA editing but in contrast with SeV and MV the faithful transcript of the P gene encodes the V protein and the addition of two non-templated G residues by RNA editing produces P mRNA (Paterson et al. 1984; Thomas et al.

1988). In a small percentage of transcription events, one or four G residues are added at the editing site and the resulting mRNA encodes the I or NS2 protein which terminates soon after the editing site and is roughly equivalent to the amino-terminal common domain of P and V, similar to the W proteins of SeV and NDV.

ii) Ribosomal choice and alternative initiation

In addition to the production of additional mRNAs by RNA editing, some paramyxovirus P genes encode a C protein, the ORF of which is in the P mRNA but has an alternative start site to the P ORF and is accessed by ribosomal choice. Viruses in the *Morbillivirus*, *Respirovirus* and *Henipavirus* genera such as MV, hPIV1, hPIV3, HeV and NiV encode C proteins in a reading frame distinct from that of P and V, in an ORF accessed by alternative ribosomal initiation (Bellini et al. 1985; Spriggs and Collins 1986; Matsuoka et al. 1991; Wang et al. 1998; Harcourt et al. 2000). HeV also has a second potential ORF in the +2 C reading frame, which could be accessed by alternative ribosome initiation and encodes a small basic (SB) protein. This protein has no equivalent in other paramyxovirus but similar proteins are present in other members of the *Mononegavirales* such as Vesicular Stomatitis virus (VSV) and Marburg virus. SeV has a more complex C ORF containing four start sites that encodes a carboxy-terminal nested set of C proteins; C', C, Y1 and Y2 (Curran and Kolakofsky 1988; Curran and Kolakofsky 1989). All four proteins are produced during viral infection, but the 204aa C protein is the most abundant species. Paramyxovirus C proteins are easily detectable in infected cells but are only found in small amounts in virions, leading to some debate over their classification as structural or non-structural proteins.

iii) Ribosomal frameshifting

MV, the prototype Morbillivirus encodes not only P, V and C proteins from its P gene but also has an ORF for the R protein. The R protein is produced via ribosomal frameshifting whereby in a small proportion of P mRNA translation events the ribosome slips into the –1 V reading frame at a conserved sequence 24nt upstream of the V ORF stop codon (Liston and Briedis 1995). The resulting R protein is therefore amino co-terminal with P but has the last eight amino acids and stop codon of the V

protein. A protein of the predicted size for R has been observed in MV-infected cells, but its function is currently unassigned.

iv) Internal ribosomal entry

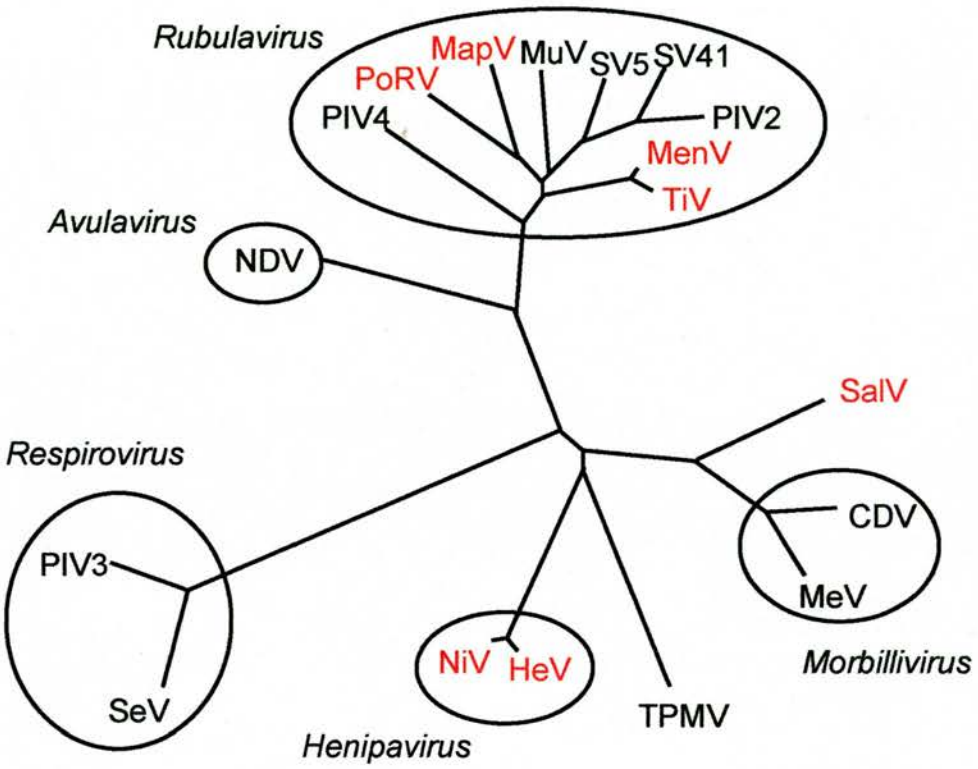
In addition to the P, V, W and C ORFs, the P gene of SeV also contains an ORF for the X protein, which is equivalent to the final 95aa of the P protein. The X start codon is over 1,500nt from the start of the P mRNA and is presumably accessed by internal ribosomal entry (Curran and Kolakofsky 1987).

II. 'EMERGING' PARAMYXOVIRUSES

This thesis investigates the V proteins of a variety of paramyxoviruses, all of which have animal hosts and some of which have caused zoonotic outbreaks in man. It is becoming clear that some wild animal species such as bats are reservoirs for a number of paramyxoviruses (reviewed in Wang and Eaton 2001), some of which have already posed significant threats to public health and that other viruses of unknown pathogenicity to humans and other species are likely to be present and as yet undiscovered in such reservoirs. Due to the unknown origins and characteristics of many of these viruses, they will be referred to as 'emerging' paramyxoviruses and the current information on them is detailed in the following section. The phylogenetic relationships of these 'emerging' paramyxoviruses with other members of the *Paramyxovirinae* are shown in Fig. 5.

Hendra virus

Hendra (HeV), initially known as equine morbillivirus, was the causative agent of two outbreaks of viral disease in horses in Queensland, Australia in 1994 (Murray et al. 1995). Of the equine infections, 16 were fatal and two incidences of zoonotic transmission of the virus to humans had fatal outcomes. The infected horses showed symptoms of acute respiratory disease as did the first human fatality, a horse trainer, while the second man died from relapsing encephalitis thought to be the result of viral infection during the autopsy of infected horses. HeV infects a wide range of tissue culture cells and causes systemic infections in a number of organisms including horses, man, cats and fruit bats. Field studies have suggested that fruit bats (genus *Pteropus*) are the natural host of HeV with around 25% of Australian fruit bats



adapted from Wang & Eaton, 2001

Figure 5: Phylogenetic relationships of viruses within the *Paramyxovirinae*

A phylogenetic tree based on full-length N protein sequences from a range of viruses in the *Paramyxovirinae* sub-family. The solid elliptical lines represent the current grouping of the viruses into five genera and the two viruses not so enclosed are currently unclassified. Viruses labelled in red are the 'emergent' paramyxoviruses investigated in this study. A guide to the abbreviations used can be found in the Abbreviations section.

sampled carrying antibodies capable of neutralising HeV (Young et al. 1996; Halpin et al. 2000) whilst no other wildlife species sampled carried such antibodies. HeV, although clearly a paramyxovirus and having some similar features to members of the *Morbillivirus* and *Respirovirus* genera has been placed in a new genus, *Henipavirus*, due to its overall differences from other paramyxoviruses.

Nipah virus

Nipah virus (NiV) was isolated in 1998 in peninsular Malaysia after an outbreak of severe febrile encephalitis in piggery workers was linked to respiratory illness in pigs. The outbreak spread and was finally brought under control by the culling of over 1 million pigs, but not before 265 human cases of encephalitis, 105 of them fatal, were identified. Cerebrospinal fluid samples from fatal encephalitis cases were used to inoculate Vero cells and an agent causing syncytia formation in these cells was isolated and examined by electron microscopy (Chua et al. 2000). The virus identified had morphological characteristics typical of a paramyxovirus and this classification was confirmed by sequencing of the viral N, P and M genes, which showed strong similarities with known paramyxoviruses, particularly Hendra virus (HeV). The virus was named Nipah virus (NiV) after the Malaysian village from where the initial samples were obtained and was classified as the second member of the *Henipavirus* genus.

Mapuera virus

Mapuera virus (MapV) was isolated in 1979 from an asymptomatic fruit bat, *Sturnira lilium*, in the Brazilian rainforest. Subsequent morphological studies indicated that the virus was a paramyxovirus and this was later confirmed on the basis of viral ultra-structure, its proteins and the sequence of the NP gene which further classified MapV as a member of the *Rubulavirus* genus (Zeller et al. 1989; Henderson et al. 1995). The host range of MapV is not known and while it is not thought to be pathogenic for humans (B. Rima, personal communication), fatal infections of mice can be achieved by intra-cranial injection of virus (Zeller et al. 1989). The V protein of MapV is produced by translation of the faithful transcript of the viral P/V gene as is the case for SV5 and other rubulaviruses and shows some sequence conservation with the V proteins of these viruses, especially in the C-terminal, V-unique region. However,

MapV V also contains a C-terminal extension of 36aa compared with SV5 V, which may be important for the function of the protein.

Porcine Rubulavirus

Porcine rubulavirus (PoRV, also called La-Piedad-Michoacan-Mexico virus, LPMV), a virus causing endemic disease of pigs in Mexico, was initially isolated in 1986 and subsequently classified as a rubulavirus (Moreno-Lopez et al. 1986). Little is known of its pathogenesis although it does have some similarities with MuV, in terms of its molecular biology, tissue tropism and the lesions caused by infection. The PoRV V protein, like that of other rubulaviruses, is produced from the faithful transcript of the P gene and has some homology with other paramyxovirus V proteins, especially in the cysteine-rich C-terminus (Berg et al. 1992).

Menangle virus

Menangle virus (MenV) was isolated from stillborn piglets in New South Wales, Australia in 1997 and identified as the causative agent of an outbreak of reproductive disease resulting in a higher than normal incidence of still births and deformities (Philbey et al. 1998). Neutralising antibodies against MenV were found in *Pteropus* fruit bats in the immediate vicinity of the piggery but not in other wild or domestic animals, suggesting that fruit bats may be the natural reservoir of the virus. Two humans who developed an influenza-like illness after contact with infected pigs were found to be seropositive for MenV, suggesting zoonotic potential for the virus (Chant et al. 1998). Comparison of MenV gene and protein sequences and also the P/V gene expression strategy showed similarity to members of the *Rubulavirus* genus, in which MenV was subsequently placed (Bowden et al. 2001).

Salem virus

Salem virus (SalV) was isolated in Salem, USA in 1992 from the blood of a horse involved in an outbreak of equine illness and was initially thought to be the causative agent of a number of cases of respiratory disease in horses. However, it now seems that SalV was not responsible for the disease outbreak and that its isolation was coincidental, with around 56% of US horses seropositive for SalV (Glaser et al. 2002). The virion structure and sequence characteristics of SalV indicated that it was

a paramyxovirus and investigation of the P/V gene showed that it encodes P, V and C proteins, similar to members of the *Morbillivirus* genus (Renshaw et al. 2000). However, in the single isolate studied it appeared that the V protein was a product of the faithful mRNA transcript of the P gene and that the P mRNA contained multiple G residues added by RNA editing. This is typical of the P/V encoding strategy of rubulaviruses and not morbilliviruses, suggesting that SalV has similarities to both morbilliviruses and rubulaviruses but may be sufficiently different from both to be placed within a new genus and as such the virus is currently unassigned.

Tioman virus

Tioman virus (TiV) was isolated from the urine of fruit bats on Tioman Island off the coast of peninsular Malaysia during a search for the natural reservoir of Nipah virus. Electron microscopy and serology studies indicated that TiV was a paramyxovirus, most closely related to MenV and sequence analysis of the N and V proteins and investigation of the P/V gene expression strategy suggested that TiV should be placed within the *Rubulavirus* genus (Chua et al. 2001), a classification later confirmed by full length sequencing of the TiV genome (Chua et al. 2002).

III. The Interferon System

Interferons, first discovered in the 1950s by Isaacs and Lindenmann in chick cells infected with Influenza virus (Isaacs and Lindenmann 1957), are a family of secreted proteins that are pivotal players in the early immune response, forming part of the innate, non-specific arm of immunity and are the first line of defence against viral infection (reviewed in Goodbourn et al. 2000; Samuel 2001; Kalvakolanu 2003). Interferon (IFN) slows the replication and spread of incoming virus, giving the specific immune response time to marshal its forces and eliminate the infection and evidence from mice lacking IFN receptors shows that IFN is important for protection against viral infection as such mice have little or no resistance to most viruses (Knipe et al. 2001). IFN acts by inducing an anti-viral state inside infected cells via enzymes that limit viral replication and/or interfere with cellular processes such as protein synthesis. They can slow the growth of infected cells by inducing the production of cell-cycle regulatory proteins and they can also induce the apoptosis of infected cells.

Additionally, Interferons have a wide range of immunomodulatory effects and can activate the cells of the adaptive immune response.

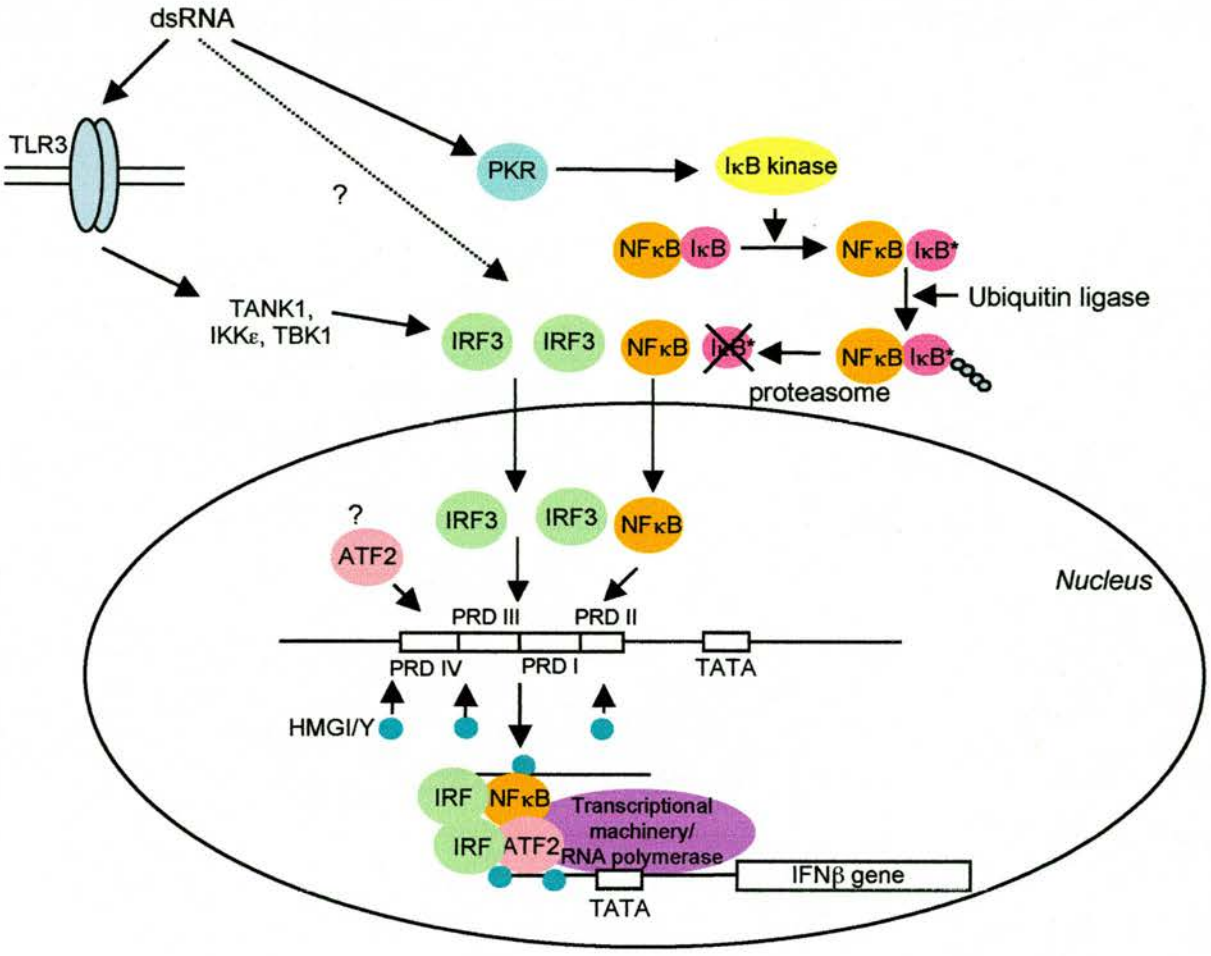
There are two main classes of Interferons, IFNα/β and IFNγ, also referred to as type I or viral IFN and type II or immune IFN. In humans, IFNα is the product of a large multigene family while IFNβ and IFNγ are the products of single genes. The main distinction between the two types of IFN is that IFNα/β is produced by virally infected cells such as fibroblasts and leukocytes, whereas IFNγ is synthesised by immune effector cells such as T lymphocytes and NK cells which have come into contact with and have been activated by infected cells. The two types of IFN molecules do not have any apparent structural homology but do display functional parallels by means of overlapping gene induction.

Induction of IFN genes

IFNβ induction in fibroblasts is the most studied system of IFN induction and much is known about the transcriptional activation of its promoter. The IFNβ promoter is induced by intracellular double-stranded RNA (dsRNA), a unique product of viral infection either as an intermediate stage in viral replication or the viral genome itself and the events that follow stimulation by dsRNA are summarised in Fig. 6.

i) IFNβ promoter activation by PKR

The major transcriptional activator of the IFNβ promoter is NFκB, a ubiquitous transcription factor in animal cells, which in unstimulated cells is bound to an inhibitory protein, IκB and is found predominantly in the cytoplasm. However, in the presence of dsRNA, inactive forms of protein kinase R (PKR) in the cytoplasm are converted into their active form enabling them to phosphorylate and activate a cellular kinase, IκB kinase. The activated IκB kinase phosphorylates IκB, which is then poly-ubiquitinated by an E3 ubiquitin ligase and degraded by the proteosome. The degradation of IκB releases NFκB and uncovers a nuclear localisation signal, resulting in relocalisation of NFκB to the nucleus (reviewed in Israel 2000). Another constitutively expressed transcription factor, IRF3, is also activated by PKR phosphorylation and translocates to the nucleus. In the nucleus, NFκB and IRF3 form part of the 'enhanceasome' complex binding to the positive regulatory domains



adapted from Goodbourn et al, 2000

Figure 6: Activation of the IFNβ promoter by dsRNA.

A schematic representation of the transcriptional activation of the IFNβ promoter by dsRNA, a product of viral replication. dsRNA activates PKR, Toll-like receptor 3 and its associated kinases and possibly other cellular kinases leading to activation and nuclear localisation of transcription factors. PKR activates the IκB kinase which targets IκB for degradation, so releasing NFκB which translocates to the nucleus and binds to the IFNβ promoter with other transcription factors such as IRF3 and the accessory factor HMGI/Y, forming the 'enhanceosome' complex. This complex also associates with the basal transcription machinery and recruits RNA polymerase II to the promoter, resulting in the transcription of the IFNβ gene.

(PRDs) of the IFNβ promoter (reviewed in Thanos 1996), which also includes the transcriptional activators ATF2/Jun and the accessory factor HMG-I/Y. Stimulation of the PRDs by this complex results in the recruitment of the transcriptional machinery and RNA polymerase II to the promoter, transcription of the IFNβ gene and subsequently the production of IFNβ by activated cells.

ii) Toll-like receptors and IFN promoter activation

Toll-like receptors (TLRs) are a family of type I trans-membrane receptors that recognise conserved, pathogen-associated molecular patterns (reviewed in Medzhitov 2001). One member of this family, TLR3, functions as a cell surface receptor for dsRNA and activation of TLR3 by dsRNA leads to the activation of NFκB and the production of IFNα/β (Alexopoulou et al. 2001). Stimulation of TLR3 by dsRNA or viral infection is thought to activate a multi-component protein complex capable of activating both IRF3 and NFκB (reviewed in Williams and Sen 2003) and some components of this complex have been identified. One of these components, TRIF, a TLR adaptor protein, associates with TLR3 and IRF3 and its over-expression activates the IFNβ promoter (Yamamoto et al. 2002). TRIF has since been shown to interact with and activate two non-canonical IκB kinase (IKK) homologs, IKKε and TBK1, which then activate both IRF3 and NFκB (Fitzgerald et al. 2003). It has been suggested that IKKε and TBK1 form part of the 'virus activated protein kinase' (VAK), responsible for activating IRF3 and IRF7 in response to viral infection (Sharma et al. 2003), a complex that may also contain IKKα, IKKβ and TANK1 (Williams and Sen 2003).

In addition, two further TLRs, mouse TLR7 and human TLR8, have been demonstrated to induce IFNα production in response to viral RNA, but in contrast to TLR3 these receptors recognise GU-rich viral ssRNA (Diebold et al. 2004; Heil et al. 2004), suggesting that single-stranded viral RNA may also play a role in the stimulation of IFN production during virus infection in addition to the well characterised involvement of dsRNA.

iii) Activation of IFNα promoters

IFNα promoters do not contain binding sites for NFκB but they do have sites similar to PRD1 and also ATF2 binding elements. The induction of IFNα appears to differ in fibroblasts and leukocytes. In fibroblasts, IFNβ stimulated gene expression appears to be required for IFNα production, as the fibroblasts of mice lacking both copies of the IFNβ gene are unable to produce IFNα (Erlandsson et al. 1998). The protein required for IFNα stimulation is thought to be IRF7, an IFNβ-stimulated gene product. In leukocytes however, IFNβ is not required so there must be a different pathway for IFNα gene expression in these cells.

iv) Activation of the IFNγ promoter

The IFNγ promoter has been studied in $CD4^+$ and $CD8^+$ T cells and has been shown to differ between these two types of cells. In $CD4^+$ cells the promoter consists of a proximal element, activated by cJun and ATF2 and a distal element activated by GATA3 and ATF1. In $CD8^+$ cells the promoter contains only the distal element, which explains why IFNγ expression in these cells is much lower than in $CD4^+$. Cytokines are also important in the expression of IFNγ, particularly IL12 and IL18. These cytokines enhance the production of IFNγ in activated T cells, but together they are also able to stimulate T cells to produce IFNγ in the absence of antigen. NK cells can also produce IFNγ and this production is enhanced by IL18 and stimulated by IL12 produced by antigen presenting cells.

IFN Signal Transduction

IFNα/β and IFNγ have separate receptors present at low abundance in the cell membranes of all major cell types. These receptors are heterodimeric glycoproteins and binding of IFN to them triggers an intra-cellular signal transduction cascade involving Janus family kinases and STATs (signal transducers and activators of transcription), shown in Fig. 7. The result is the transcriptional activation of multiple IFN-stimulated genes (ISGs) via the binding of protein complexes to cis-acting sequences in their promoters.

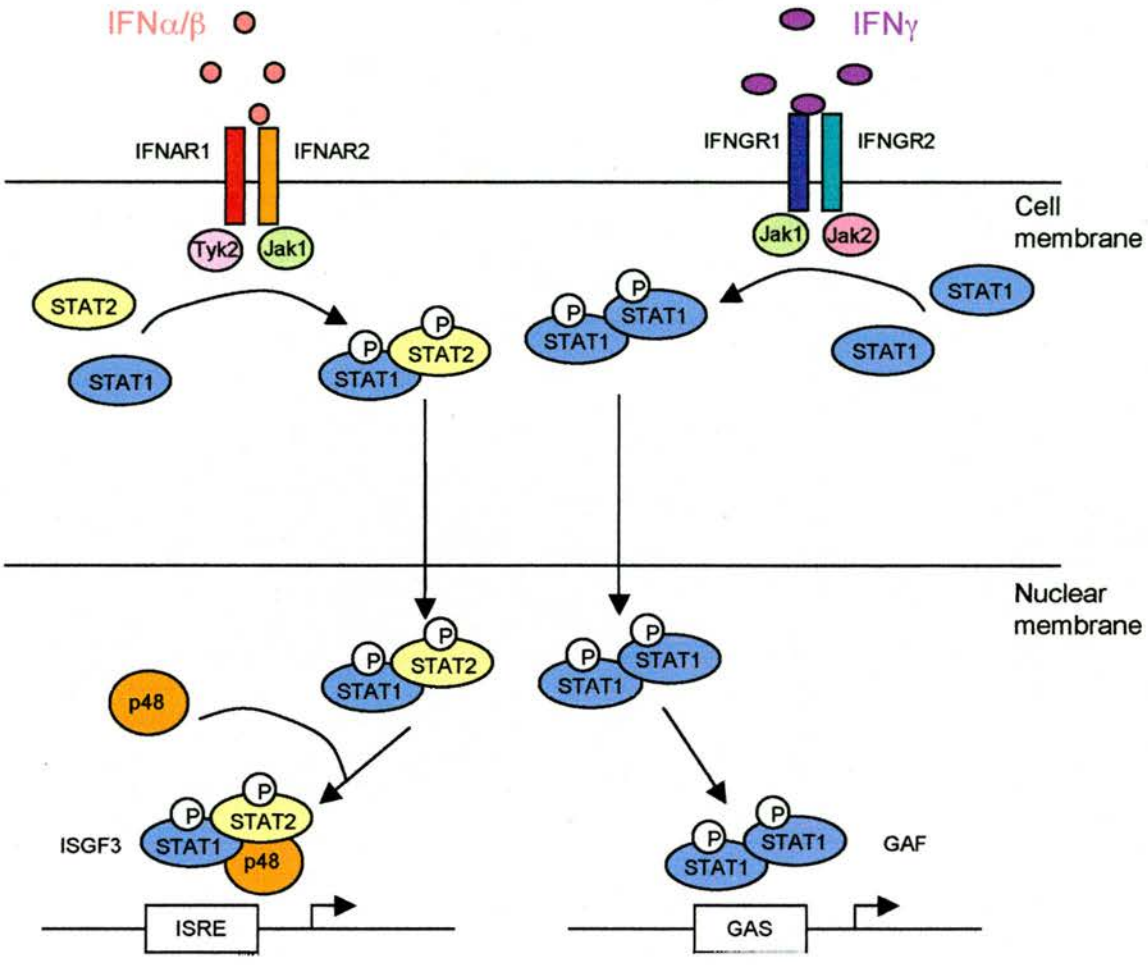


Figure 7: IFNα/β and IFNγ signalling pathways

A schematic representation of IFN signalling pathways in mammalian cells whereby extra-cellular IFN results in the transcriptional activation of IFN-stimulated genes. IFN molecules bind to their cognate receptors on the cell surface causing dimerisation, which brings cellular Janus kinases associated with the receptor subunits into contact. These kinases are activated and subsequently phosphorylate STAT proteins which form heterodimers of STAT1 and STAT2 in IFNα/β signalling and homodimers of STAT1 in IFNγ signalling. These STAT dimers translocate to the nucleus where the STAT1/STAT2 heterodimer in association with p48/IRF9 forms the ISGF3 complex, which binds to IFN-stimulated response elements (ISRE) in the promoters of IFNα/β-stimulated genes, activating transcription. STAT1 homodimers constitute GAF complexes and stimulate transcription via binding to GAS elements in the promoters of IFNγ-stimulated genes.

i) IFNα/β signalling

The IFNα/β receptor consists of a heterodimer of IFNAR1 and IFNAR2 subunits that exist as monomers in unstimulated cells and via their cytoplasmic domains are associated with the Janus tyrosine kinases Jak1 and Tyk2, and also STAT2 in the case of IFNAR2. When IFNα or IFNβ binds to the receptor subunits it stimulates heterodimerisation and the subsequent trans-phosphorylation and activation of Jak1 and Tyk2. Activated Tyk2 phosphorylates a tyrosine residue on IFNAR1 allowing STAT2 to bind via its SH domain and also phosphorylates STAT2 allowing it to recruit STAT1 to the receptor complex. STAT1 is then tyrosine phosphorylated by Tyk2 and a STAT1/STAT2 heterodimer forms and dissociates from the receptor. The STAT heterodimer then associates with a third protein, IRF9 (also known as p48), to form the heterotrimeric ISGF3 complex. IRF9 is the only part of the ISGF3 complex to contain a recognised nuclear localisation sequence and is found in the cytoplasm and nucleus of both resting and IFN-stimulated cells. It is not clear whether the ISGF3 complex forms in the cytoplasm or the nucleus but it has been suggested that IRF9 and STAT2 pre-associate in the cytoplasm, which may allow more rapid formation of ISGF3 after IFNα/β stimulation. Once in the nucleus, ISGF3 binds to the IFN-stimulated response element (ISRE) in the promoters of IFNα/β stimulated genes and activates their transcription.

ii) IFNγ signalling

The IFNγ receptor is also a heterodimeric glycoprotein, consisting of IFNGR1 and IFNGR2 subunits. As for the IFNα/β receptor, in unstimulated cells these subunits are not strongly associated with one another but they are associated with the cellular Janus family kinases Jak1 and Jak2. When IFNγ binds to the receptor subunits they heterodimerise, bringing the two Jak kinases into close contact, which activates Jak2 which then trans-phosphorylates Jak1. Jak1 and Jak2 then phosphorylate a region in the C-terminus of IFNGR1, which forms a pair of binding sites for STAT1. Two STAT1 molecules subsequently bind to IFNGR1, are phosphorylated at Tyr^{701} and dissociate from the receptor, forming a STAT1 homodimer. This homodimer, referred to as gamma-activated factor (GAF), translocates to the nucleus where it binds to gamma-activated sequence (GAS) elements in IFNγ inducible gene promoters and stimulates transcription.

iii) STAT1 transactivation

Common to both pathways, a further phosphorylation event is required before STAT1 can bring about transcriptional activation. Ser^{727} phosphorylation, probably by a cellular MAP kinase, is required for the transactivation function of STAT1 and enables it to interact with the basal transcription machinery, including Creb binding protein (CBP) and p300. STAT2, which lacks a MAP kinase consensus sequence, seems to be able to interact with CBP/p300 without any need for serine phosphorylation.

iv) Attenuation of signalling

The attenuation of IFN signalling is less well understood than its activation. The direct binding of proteins such as IRF2 and IRF8 to ISREs results in the suppression of transcription and this may play a role in preventing ISG expression in the absence of IFN or controlling the response to IFN. Attenuation of signalling may occur via the inhibition of STAT activation by SOCS (suppressors of cytokine signalling), which are a family of proteins induced by IFN and other cytokines that bind to and inhibit activated Janus kinases, preventing STAT phosphorylation. There is also evidence that tyrosine phosphatases such as SHP-1 may dephosphorylate and thus inactivate Janus kinases and STATs, allowing only a transient activation of the IFN signalling pathway. SHP-1 has been shown to associate with a subunit of the IFNα/β receptor after IFNα stimulation (David et al. 1995) and cells from mice lacking functional SHP-1 have higher levels of Jak1 and STAT1 phosphorylation (Haque and Williams 1998). Also involved in the negative regulation of IFN signalling are members of the PIAS (protein inhibitor of activated STAT) family such as PIAS1, which associates with STAT1 homodimers and disrupts their ability to bind DNA (Liao et al. 2000) and PIASy which does not block the binding of STAT1 to DNA but prevents it from activating the transcription of IFN-stimulated genes (Liu et al. 2001).

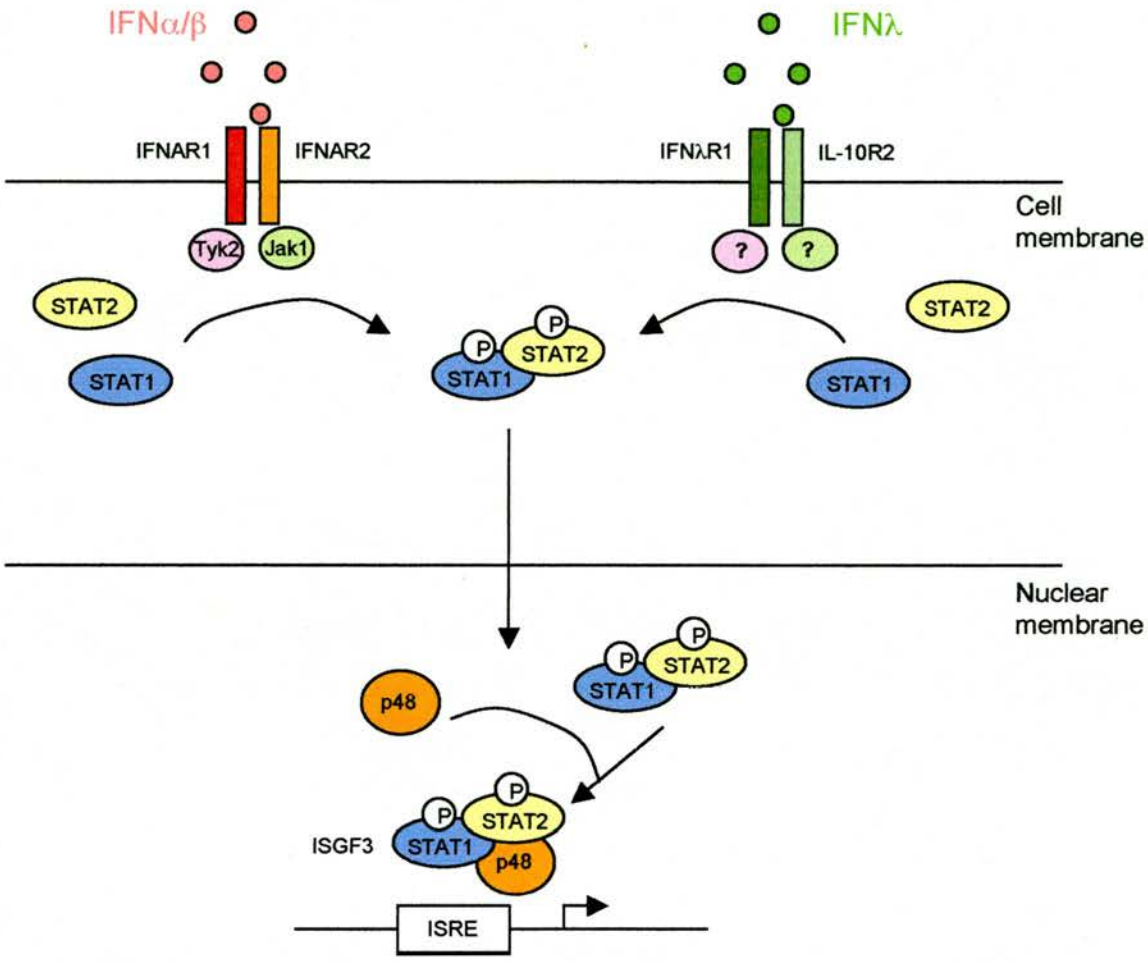
v) Alternative IFN pathways

It should be noted that the Jak-STAT 'classical' pathway is not the only way in which IFN can stimulate the transcription of ISGs. Studies in cells derived from STAT1 null mice have suggested that an 'alternative' pathway exists whereby IFNγ can upregulate certain ISGs and create an effective anti-viral state in the absence of

STAT1, the central component of the classical IFN signalling pathway (Gil et al. 2001; Ramana et al. 2001). Infections of STAT1 null mice with the RNA virus Sindbis virus showed that these mice are only slightly more susceptible to challenge with $1x10^5$ pfu of virus than wt mice, whereas mice lacking IFNα/β and IFNγ receptors exhibit 100% mortality when challenged with as little as 1 pfu (Gil et al. 2001). This study also used microarray analysis to identify a number of ISGs upregulated by IFNγ in STAT1-/- fibroblasts, including SOCS3, an inhibitor of Jak1, suggesting that the classical and alternative pathways may share some regulatory mechanisms. Furthermore, this study demonstrated that the only components of the classical pathway required for the alternative signalling pathway are the IFN receptors and Jak1 and that the lack of STAT1 is not compensated by the upregulation of another STAT family member such as STAT3, suggesting that the IFN receptors and Jak1 participate in an alternative signalling pathway. Currently the importance of this alternative IFN signalling pathway in infected cells remains to be demonstrated but it has been suggested that it may provide a link between IFN and other cytokines and that this, in addition to the cross-talk already observed between IFNα/β and IFNγ signalling pathways may allow "revving up" (Takaoka et al. 2000) of the innate and adaptive immune systems in response to pathogens.

vi) IFNλ: a new class of IFN

Recent work has revealed a novel family of Interferons, IFNλ, which consists of IFNλ1, IFNλ2 and IFλ3, also termed IL-28A, IL-28B and IL-29 (Kotenko et al. 2003; Sheppard et al. 2003; Vilcek 2003). IFNλ is structurally related to IFNα/β and the IL-10 family, is stimulated by dsRNA or viral infection and upregulates the transcription of anti-viral genes such as 2-5 OAS, MxA and MHC class I (see following section for details of these genes). IFNλ signalling is similar to IFNα/β and IFNγ signalling in that IFNλ binds to a heterodimeric surface receptor, one subunit of which is the IFNλ-specific IFNλR1 and the other is the second subunit of the IL-10 receptor, IL-10R2. Binding to the receptor activates cellular kinases, as yet unidentified, to phosphorylate STAT1 and STAT2, which heterodimerise and translocate to the nucleus in combination with p48/IRF9 and activate the transcription of anti-viral genes. This signalling pathway is almost indistinguishable from the IFNα/β signalling pathway, as shown in Fig. 8, and it is likely that the same Janus kinases, Jak1 and Tyk2 are



adapted from Vilček 2003

Figure 8: IFNλ signalling pathway

A schematic representation of the IFNλ signalling pathway in mammalian cells, showing its similarity to the IFNα/β signalling pathway. IFNλ molecules bind to their cognate receptors on the cell surface causing dimerisation, bringing currently unknown cellular kinases associated with the receptor subunits into contact. These kinases are activated and subsequently phosphorylate STAT proteins, which form heterodimers of STAT1 and STAT2, translocate to the nucleus and in association with p48/IRF9 form the ISGF3 complex, as seen in IFNα/β signalling. The ISGF3 complex binds to IFN-stimulated response elements (ISRE) in the promoters of IFNα/β-stimulated genes and activates transcription.

involved in IFNλ signalling. IFNλ also activates STAT3 and STAT5, usually a characteristic of IL-10 signalling. Studies of IFNλ are still in the preliminary stages and its importance in viral infections remains to be evaluated.

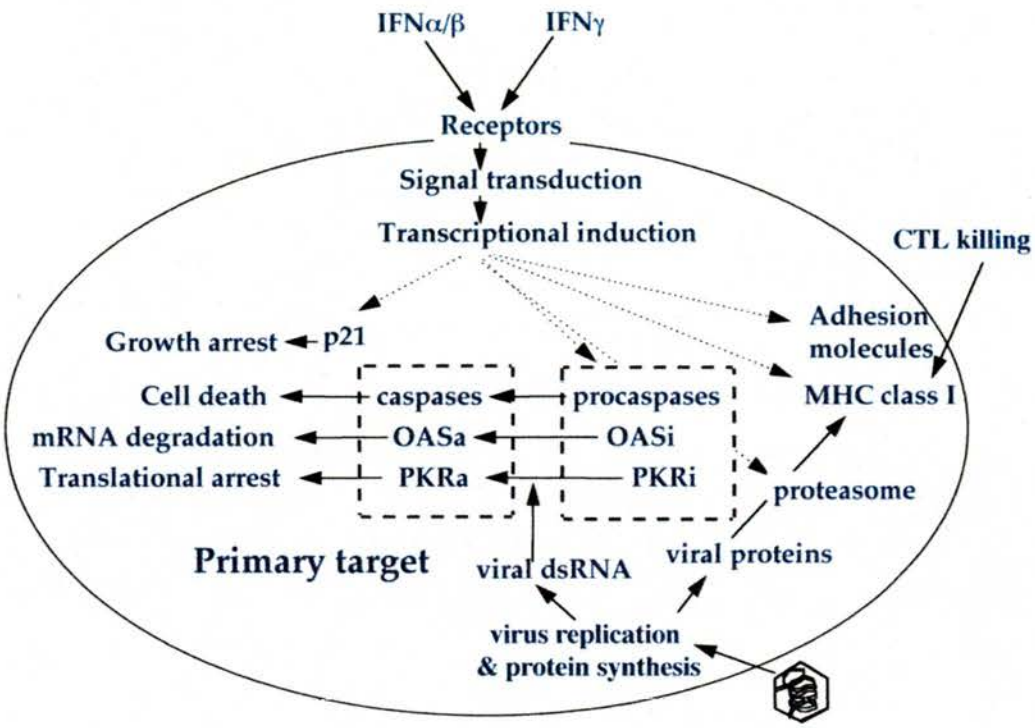
Interferon Stimulated Genes

In general, genes upregulated by IFN stimulation are involved in the establishment of an anti-viral state in the infected cell and also in signalling to neighbouring cells and cells of the adaptive immune system. Several ISG products have been the subject of extensive study and are described below and summarised in Fig. 9.

i) 2'-5' Oligoadenylate Synthetase and RNaseL

A major group of proteins produced as a result of IFN stimulation are those of the 2'-5' oligoadenylate synthetase system (2-5 OAS). These are IFN-induced enzymes which, when activated by dsRNA, catalyse the formation of three to five unit oligomers of adenosine from ATP. These oligoadenylates bind to latent monomers of endoribonuclease L (RNaseL) forming active dimers that can degrade single-stranded RNA such as mRNA and also 28S ribosomal RNA and so inhibit protein synthesis (reviewed in Silverman 1997). As the turnover of oligoadenylates is fairly rapid, active RNaseL is only found in the close vicinity of activated 2-5 OAS, itself only activated by the presence of dsRNA and thus during a viral infection that generates dsRNA, viral mRNAs are thought to be preferentially destroyed compared to cellular mRNAs. RNaseL is constitutively expressed in most cell types where it is present in an inactive form, but its expression is upregulated by IFNα, enhancing its activity in stimulated cells.

The activator RNA for 2-5 OAS during viral infection has not been clearly defined but it is thought that some single-stranded viral RNA which has a significant amount of double-stranded structure could act as 'dsRNA' and activate 2-5 OAS. Other more obvious viral sources of dsRNA are replicative intermediates and the dsRNA genomes of viruses such as reoviruses. The dsRNA binding region of 2-5 OAS has no structural homology with the binding domains of other proteins which bind dsRNA such as PKR and ADAR but, similar to these proteins, the RNA-binding and catalytically active regions of 2-5 OAS are located in separate parts of the protein



from Goodbourn et al, 2000

Figure 9: The biological properties of IFNα/β and IFNγ

After the binding of IFN molecules to their specific surface receptors the transcription of a number of genes is upregulated, the products of which establish an anti-viral state in the cell. Many of these products such as PKR and 2-5 OAS are made in an inactive form which is then activated by viral dsRNA. PKR and 2-5 OAS shut down translation and other anti-viral products are involved in apoptosis, cell-cycle arrest and the upregulation of innate immune responses.

(Sarkar and Sen 1998; Rebouillat and Hovanessian 1999). Inhibitors of RNaseL include a cellular protein, RLI (Bisbal et al. 1995) and in certain cases derivatives of 2-5 OAS, which accumulate in cells infected with certain viruses (Cayley et al. 1984).

In addition to the degradation of RNA, RNaseL has been shown to induce the apoptosis of virally infected cells (Diaz-Guerra et al. 1997). This study showed that RNaseL causes chromosomal degradation and changes in cell morphology characteristic of apoptosis approximately 48 hours after infection with recombinant vaccinia viruses and co-expression of 2-5 OAS was shown to enhance this apoptotic activity, although 2-5 OAS alone has no apoptotic effects. In addition, mice lacking RNaseL have been shown to have defects in apoptosis (Zhou et al. 1997). Currently the mechanism whereby RNaseL triggers apoptosis is unknown, but the cellular oncogene *bcl2* has been shown to block the apoptotic effects of RNaseL and the 2-5 OAS pathway (Diaz-Guerra et al. 1997). It has also been demonstrated that apoptosis via the activation of RNaseL is independent of PKR, as the apoptotic effect of RNaseL is still observed in cell lines lacking PKR.

ii) dsRNA-dependent protein kinase R

The dsRNA-dependent protein kinase R (PKR) is an IFN-inducible serine-threonine kinase with multiple properties related to the control of transcription and translation, present in the cell in an inactive form that, like 2-5 OAS, is auto-phosphorylated and activated on contact with dsRNA (Katze et al. 1991). The active form of PKR is thought to be a dimer, with two molecules of PKR binding one molecule of dsRNA and activation can be achieved by dsRNA viral genomes, artificial dsRNA such as poly(I):poly(C) and highly structured ssRNA species such as HIV TAR RNA and has no apparent sequence specificity (reviewed in Robertson and Mathews 1996). High concentrations of dsRNA and viral RNA can act as inhibitors of PKR phosphorylation (Mathews and Shenk 1991; Clemens and Elia 1997). PKR interferes with translation via its interaction with eIF2α, part of the eIF2 complex which recruits Met-tRNA to the 40S ribosome. Activated PKR phosphorylates eIF2α, which forms a complex with the other two subunits of the eIF2 complex and GTP. This complex initiates translation via interactions with mRNA and the 80S ribosome and the bound GTP is converted to GDP. Normally the complex is recycled by the exchange of GDP for

GTP by another molecule, eIF2B but in the presence of PKR, eIF2B is sequestered by the phosphorylated eIF2α so no recycling of the eIF2 complex is possible and translation of both cellular and viral mRNAs is halted (reviewed in Clemens and Elia 1997). As mentioned previously, PKR is also involved in the phosphorylation of IκB, the inhibitor of NFκB, resulting in IκB degradation, the release of active NFκB and the transcriptional activation of various promoters, including that of the IFNβ gene (Maran et al. 1994; Yang et al. 1995).

PKR is also involved in the apoptosis of virally infected cells (Jagus et al. 1999) and similar to 2-5 OAS-mediated apoptosis this can be blocked by the cellular oncogene *bcl2,* suggesting that PKR and 2-5 OAS may have a converging pathway of apoptotic induction in infected cells, which is blocked by *bcl2* after this convergence (Lee et al. 1997). This study showed that the kinase domain of PKR is required for the stimulation of apoptosis, but *bcl2* does not block the activity of the kinase domain as it has no effect on PKR-mediated inhibition of protein synthesis, suggesting that *bcl2* acts downstream of PKR to inhibit apoptosis.

iii) Mx proteins

Another family of IFN-induced anti-viral proteins are the Mx proteins, which are large GTPases found in all vertebrates (reviewed in Staeheli et al. 1993; Arnheiter et al. 1996). Studies in mice lacking both PKR and 2-5 OAS demonstrated that a significant anti-viral response was still present and that the Mx proteins were responsible (reviewed in Haller et al. 1998). The GTP binding and hydrolysation properties of Mx proteins are essential for their anti-viral activity and studies of ectopic MxA in mice lacking IFNα/β receptors showed that MxA has intrinsic anti-viral activity that does not depend on other ISGs (Hefti et al. 1999). IFN stimulation of cells infected with orthomyxoviruses such as Influenza A virus results in the binding of human MxA to cytoplasmic viral ribonucleoproteins (vRNPs), preventing their translocation to the nucleus and also binding of MxA to nuclear vRNPs and inhibiting their transcription (Weber et al. 2000). Other viruses such as MeV, hPIV3 and VSV are inhibited by MxA and initially the anti-viral activity of Mx proteins was thought to be limited to negative strand RNA viruses. However, recent studies have

demonstrated MxA activity against the positive strand RNA virus Semliki Forest virus (Hefti et al. 1999).

iv) RNA-specific Adenosine Deaminase (ADAR1)

Both cellular and viral RNAs can be edited by the modification of adenosine residues to inosine by deaminases including the IFN-inducible RNA-specific adenosine deaminase, ADAR1 (Patterson and Samuel 1995; Patterson et al. 1995). As inosine is recognised as guanosine by ribosomes, this editing effectively changes the sequence of the mRNA transcript and is likely to have significant effects on the functionality of the encoded protein. It has also been suggested that an RNase with specificity for RNA containing inosine residues, I-RNase, may have anti-viral activity via the cleavage of edited transcripts (Scadden and Smith 1997). The sequence changes introduced by deaminases seem to be involved in the 'biased hypermutation' of negative strand viruses, first observed in the M protein of persistent MeV isolates (reviewed in Cattaneo 1994) and ADAR-mediated hypermutation has been associated with persistent infections (Murphy et al. 1991).

IFN and the Adaptive Immune Response

IFN has wide-ranging effects on the adaptive immune response, mainly via the actions of IFNγ produced by activated subsets of lymphocytes. IFN acts to enhance cell-mediated immunity and thus the anti-viral response via interactions with various components of the adaptive immune system as described below.

i) Major Histocompatibility Complex (MHC)

MHC expression is profoundly influenced by both IFNα/β and IFNγ, both of which upregulate the surface expression of class I MHC, leading to an enhanced display of internal antigens on the surface of infected cells and therefore an improved $CD8^+$ response (reviewed in Boehm et al. 1997). In contrast, only IFNγ can upregulate class II MHC, enhancing the response of $CD4^+$ cells to the display of external antigens. IFNγ also upregulates the expression of cellular proteins involved in antigen processing including proteasomes, which generate antigenic peptides for presentation on class I MHC (reviewed in York and Rock 1996). The additional proteasome subunits expressed in response to IFNγ have different substrate specificities compared

to the normal subunits and result in different peptides being presented to the immune system. IFNγ also upregulates the transport of processed antigen to the endoplasmic reticulum via the upregulation of TAP1 and TAP2, which are proteins involved in peptide transfer, thus increasing the quantity of peptides loaded onto and displayed by class I MHC (Epperson et al. 1992).

ii) Cytokines

IFN also has effects on the production of cytokines, which themselves have wide-ranging immunomodulatory effects. For example, IFNγ affects the balance of the two types of T-helper (TH) cells, which determine whether the immune response to an antigenic challenge is predominantly cell-mediated or antibody-dependent. IFNγ increases the production of IL-12 by antigen presenting cells and this leads to the development of $CD4^+$ TH0 cells into TH1 rather than TH2 cells (Flesch et al. 1995). IFNγ also inhibits IL-4 production (Gajewski and Fitch 1988; Szabo et al. 1995), which normally stimulates the shift to TH2 dominance by increasing TH2 proliferation and a combination of this IFN-induced IL-12 upregulation and IL-4 inhibition causes an overall shift in the balance of TH1 and TH2, making TH1 the predominant type. This results in an upregulation of TH1-mediated cellular immune responses, which are more effective against virus infections than the TH2-mediated antibody response. IFNα/β also upregulates the production of IL-15 (reviewed in Tough et al. 1999), which stimulates the division of memory T cells and it is thought that IFNα/β itself can aid the survival of activated T cells.

iii) Macrophages and Natural Killer cells

IFN has a number of effects on macrophages and natural killer (NK) cells, subsets of leukocytes that recognise and kill virally infected cells. In murine systems, IFNγ has been shown to upregulate the expression of inducible nitric oxide synthase (iNOS), an enzyme that forms the important reactive nitrogen intermediate nitric oxide (NO) and is also implicated in the function of activated macrophages (MacMicking et al. 1997). IFNγ also stimulates the expression of NADPH oxidase in macrophages, an enzyme that generates reactive oxygen intermediates and makes macrophages more active against infected cells. IFNα/β increases the proliferation of NK cells, probably by increasing the production of IL-15 from macrophages and can also increase the

cytotoxicity of NK cells by increasing their production of perforins (Biron et al. 1999).

IV. Paramyxoviruses and Interferon Evasion

All viruses must have some way of evading the IFN response as without such evasion mechanisms virus infections would be rapidly cleared from the host, preventing viral replication and spread. IFN evasion mechanisms have been discovered in all kinds of viruses including DNA viruses such as Herpes simplex virus, Human Cytomegalovirus, Adenoviruses and Poxviruses and RNA viruses such as Influenza virus, Ebola virus and Bunyamwera virus and the IFN evasion strategies of these viruses vary widely and target all aspects of the IFN response. Paramyxoviruses are no exception to this and in recent years many members of the *Paramyxoviridae* have been shown to have IFN evasion mechanisms, described in detail in the following section.

Rubulavirus

i) Simian virus 5 (SV5)

SV5 naturally infects dogs and there is evidence of infection of humans and monkeys but it is not pathogenic in mice, which quickly clear the virus. Experiments in cultured BF cells, derived from murine BALB/c fibroblasts, showed that after an initial burst of replication, SV5 protein synthesis rapidly declined, eventually leading to virus clearance but that viral replication was improved in cells derived from IFNα/β receptor knockout mice or BF cells treated with anti-IFN antibodies, suggesting that the murine IFN response is a factor limiting the replication of SV5 (Young et al. 1997). Further studies demonstrated that in human, but not murine cells, SV5 infection blocked the activation of an IFNα/β-responsive promoter and inhibited the induction of the IFNα/β-responsive gene 6-16, confirming that SV5 is able to antagonise the human but not murine IFN response (Didcock et al. 1999a). SV5 was also able to overcome a pre-established anti-viral state in human cells as was demonstrated by its ability to replicate in IFN pre-treated cells, albeit with less virus-induced fusion that seen in infections of untreated cells.

Further study of SV5-infected human cells showed that both IFNα/β and IFNγ signalling were blocked, as was the formation of both ISGF3 and GAF complexes in response to IFN and the block of IFN signalling was also seen in cells transiently expressing the SV5 V protein (Didcock et al. 1999b). Western blots of infected lysates revealed a loss of STAT1, whereas STAT2 levels were equivalent to those in mock-infected cells and treatment of infected cells with the proteosome inhibitor MG132 restored STAT1 levels, suggesting that STAT1 is degraded in a proteosome-dependent manner as a consequence of SV5 infection and that this results in a block of both types of IFN signalling. It was also demonstrated that viral replication is not required for STAT1 degradation as most STAT1 was degraded by 4-8hpi, before significant viral protein synthesis had occurred and infection with UV-inactivated SV5 also results in degradation of STAT1, suggesting that sufficient V protein is present in SV5 virions to target STAT1 for degradation.

The V protein of SV5 had no previously assigned function, but it had been shown to interact with both viral NP (Randall and Bermingham 1996) and bind zinc via cysteine residues in the V-unique carboxy terminus (Paterson et al. 1995), a region which was also shown to be involved in an interaction with DDB1, the 127kDa subunit of the cellular damage-specific DNA binding protein (Lin et al. 1998). The interaction of SV5 V with DDB1 was reported to slow the cell cycle of HeLa T4 cells infected with SV5 as expression of additional DDB1 in cells expressing V could partially restore normal cell cycle progression (Lin and Lamb 2000) but this has not been seen in all cell lines infected with SV5. Immune precipitations from human 2fTGH cell lines stably expressing SV5 V (2f/SV5 V) confirmed that SV5 V interacts with DDB1 and also an as yet unidentified 150kDa protein and a subsequent study showed that the interaction with DDB1 is essential for SV5 V to function as an IFN antagonist (Andrejeva et al. 2002a; Andrejeva et al. 2002b). The latter study showed that the ability of SV5 V to degrade STAT1 directly correlated with the ability to bind DDB1 and the use of siRNA to knock down DDB1 expression in HeLa cells expressing V (HeLa/SV5 V) resulted in a recovery of STAT1 levels and restoration of IFN signalling, indicating that without sufficient DDB1, V is unable to target STAT1 for degradation. It was also demonstrated that V can target STAT1 for degradation in cells derived from patients with the disease Xeroderma pigmentosum which lack the

normal interaction between the two DDB subunits, DDB1 and DDB2, indicating that DDB2 is not required in the V-mediated degradation process and recently the binding of SV5 V to DDB1 has been shown to displace DDB2 (Leupin et al. 2003). A second study confirmed the requirement for DDB1 in STAT1 degradation by the use of siRNA and also suggested a role for the cellular protein Cul4a in STAT1 degradation, as siRNA specific for this protein made a 10-20% reduction in STAT1 degradation by SV5 V (Ulane and Horvath 2002).

The 2f/SV5 V cell line studies also showed that both phosphorylated and non-phosphorylated forms of STAT1 are targeted for degradation by V and that MG132 treatment of V-expressing cells can prevent the degradation of newly synthesised STAT1 (Andrejeva et al. 2002b). It was also observed that UV-inactivated SV5, which is able to degrade STAT1 in untreated cells was unable to do so completely in IFN pre-treated cells which have higher levels of STAT1 and that 'live' preparations of SV5 were not able to degrade STAT1 in IFN pre-treated cells in the presence of the translational inhibitor cyclohexamide. This suggests that *de novo* viral protein synthesis by SV5 is required to degrade STAT1 in cells with high levels of STAT1 such as IFNα pre-treated cells, whereas the small amounts of V present in virions are usually sufficient to degrade STAT1 in unstimulated cells, indicating that the ability of V to degrade STAT1 is dependent on the concentrations of both STAT1 and V.

It was subsequently revealed that STAT1 degradation by SV5 V also requires the presence of STAT2, as SV5 cannot block IFNγ signalling in U6A cells, which lack STAT2 (Parisien et al. 2002b) or in cells stably expressing the V protein of hPIV2, which targets STAT2 for degradation (Andrejeva et al. 2002b). The former study also demonstrated that STAT1 degradation is independent of IFN signalling, does not require any components of the IFN signalling pathway other than STAT1 and STAT2 and that cell lines lacking STAT1 or STAT2 can be complemented with the expression of exogenous STATs, with no requirement for the presence of tyrosine phosphorylation residues or SH2 domains. Chimeric STAT1/STAT2 proteins and truncations of STAT2 showed that the N-terminal 578 amino acids of STAT2 are required for SV5 V to degrade STAT1. Not only is STAT2 required for STAT1 degradation, it appears that the two proteins are required in roughly equivalent

amounts as demonstrated by experiments which showed that degradation of exogenous, transfected STAT1 by V was not possible unless exogenous STAT2 was also expressed, indicating that the low levels of endogenous STAT2 in the cell were insufficient to allow degradation of the over-expressed, exogenous STAT1 (Andrejeva et al. 2002a).

In addition to the interaction of SV5 V with DDB1, immune precipitations with anti-FLAG antibodies from cells expressing FLAG-SV5 V showed an interaction between V and STAT2 (STAT1 could not be seen as it is subject to degradation in these cells) and GST-SV5 V fusions were shown to interact with STAT1 and STAT2 from cell lysates (Parisien et al. 2002b). It is possible that such interactions were not seen in earlier studies due to the use of V-specific antibodies in immune precipitations, which may have disrupted interactions of V with other proteins such as STAT1 and STAT2. These protein-protein interactions and the evidence suggesting that STAT1 is degraded by the proteosome in cells infected with SV5 or expressing SV5 V led to the suggestion that V acts as an E3 ligase and poly-ubiquitinates STAT1. This is the usual manner in which proteins are targeted for degradation by the proteosome and involves a group of ligases, which activate and transfer ubiquitin moieties to the substrate, which is then recognised by the cellular degradation machinery. A study that attempted to capture the unstable, ubiquitinated STAT1 intermediate by using transfections that produced low levels of V expression showed that ubiquitinated STAT1 is present in such cells, albeit as a small proportion of total STAT1, an observation also made in immune precipitations from transiently transfected 2fTGH cells (Ulane and Horvath 2002; Garcin et al. 2003). The former paper also detailed an *in vitro* E3 ligase assay, in which proteins acting as an E3 ligase are auto-ubiquitinated due to the lack of a substrate. In this assay GST-SV5 V was shown to be ubiquitinated in the presence of ATP and E1 and E2 ligases, suggesting that it can act as an E3 ligase and the requirement for additional E2 ligase was not absolute, indicating that V may also have some E2 ligase activity. From this data it was suggested that the interaction of V with STAT1, STAT2, DDB1 and possibly Cul4a might form a V-dependent degradation complex (VDC), leading to the poly-ubiquitination of STAT1 and its subsequent degradation.

As already described, studies using SV5 V with mutated cysteine residues have showed that these residues are critically required for the interaction of V with DDB1 and the ability of V to antagonise the IFN response. Studies of a recombinant SV5 with a V protein lacking the cysteine-rich C-terminus (rSV5VΔC) underlined the requirement for this region in IFN antagonism as rSV5VΔC did not block IFNα/β signalling or the formation of ISGF3 in infected cells, or target STAT1 for degradation (He et al. 2002). Infection of Vero cells with rSV5VΔC resulted in replication consistent with that seen for wt SV5, but virus replication and titre was reduced in BHK cells and in many cell types infection with rSV5VΔC led to increased cytopathic effect and the appearance of apoptotic cells, possibly as a consequence of an IFN-stimulated, anti-viral state. After a few passages revertant, pseudo-wild-type viruses began to emerge with mutations that abolished the artificially introduced stop codons and allowed full length V to be expressed, indicating strong selective pressure for the recovery of V expression and as this reversion occurred in Vero cells it seems possible that a function of V other than IFN evasion was causing the selective pressure. Initial use of the SV5 reverse genetics system indicated that V was not required for initial viral replication and recovery (He et al. 1997) and since then efforts had been made to create a V(-) virus, but with no success, suggesting that V cannot be classified as a non-essential protein as has been suggested for other paramyxoviruses such as Sendai (Curran et al. 1991).

In spite of the importance of the C-terminus of V in IFN antagonism, studies of two closely related canine isolates of SV5, CPI+ and CPI-, whose V proteins differ at three amino acid positions in the N-terminus, showed that sequence changes in this region can affect the ability to V to antagonise IFN signalling (Chatziandreou et al. 2002). CPI+ infection of both canine and human cells leads a block of IFN signalling via the degradation of STAT1 whereas CPI- infection does not block IFN signalling and has no effect on STAT1 levels and sequential mutation of CPI- V residues to CPI+ V sequence showed that all three residues are required to fully block IFN signalling. However, despite the sensitivity of CPI- to IFN demonstrated in infections of canine cells in which the addition of IFN results in virus clearance from most cells, a few cells in the population remain infected with CPI- and in these cells almost all of the viral P and NP protein is found in cytoplasmic inclusion bodies. These inclusion

bodies are also seen in canine cells infected with CPI- after long periods of infection and have been suggested to be the basis of SV5 persistence, as cells with inclusion bodies have very low levels of both surface glycoprotein and general protein expression which could enable them to escape destruction by the immune surveillance mechanisms of the adaptive immune system. This study also suggested that IFN-sensitive paramyxoviruses such as CPI- may establish persistent infections by residing in inclusion bodies and sporadically reactivating to an IFN-resistant form by the way of point mutations in the V gene.

This phenomenon of persistence of an IFN-sensitive SV5 strain was previously observed in murine cells infected with SV5 (Fearns et al. 1994) and prolonged passage of IFN-sensitive SV5 in murine cells did indeed lead to the isolation of a IFN-resistant virus capable of targeting murine STAT1 for degradation and therefore blocking IFN signalling in murine cells, as well as retaining the ability to antagonise the IFN response in human cells (Young et al. 2001). When the V protein of this isolate was sequenced it was found to have a single amino acid change relative to wt SV5, N100D, in the amino-terminal common domain of V and P and subsequent expression of cloned SV5 V^{N100D} in murine cells was shown to block IFN signalling. In the light of more recent work on STAT1 degradation by SV5 in murine cells, it seems that the N100D mutation allows V to utilise murine STAT2 in the 'V degradation complex', unlike wt V which requires the addition of human STAT2 to enable the degradation of mouse STAT1 (Parisien et al. 2002a). A recombinant SV5, rSV5 V^{N100D}, which incorporated the V^{N100D} mutation into a wt background exhibited prolonged infection and protein expression and increased viral production in murine cells compared to wt SV5, indicating that the ability of rSV5 V^{N100D} to block IFN signalling in murine cells carries with it a clear selective advantage. However, rSV5 V^{N100D} did not replicate as well in murine cells as wt SV5 does in human cells and was not pathogenic in mice, suggesting that the IFN system is not the only constraint on SV5 replication in murine cells.

Initial papers on SV5 IFN antagonism observed that SV5 was not only capable of blocking IFN signalling but was also a poor inducer of IFN (Didcock et al. 1999a) and subsequent studies of rSV5VΔC noted that ISGF3 complexes were formed in

infected cells without the need for IFNα stimulation, suggesting that deletion of the C-terminus of V results in a change to an IFN-inducing phenotype (He et al. 2002). Measurement of IFNβ released during infection showed very little released from cells infected with wt SV5 but large amounts produced by rSV5VΔC-infected cells and this was reflected in the relative amounts of IFNβ mRNA produced in infected cells, indicating that the C-terminus of V is involved in blocking the production of IFNβ during wt SV5 infections (Poole et al. 2002). This was confirmed by assays showing that full-length SV5 V blocks the activation of the IFNβ promoter by poly(I):poly(C) artificial dsRNA and similar assays showed that deletions of up to 126aa from the N-terminus of V are tolerated, but not deletions from the C-terminus. Point mutations of the C-terminal conserved cysteine residues also abrogate the ability of V to block the activation of the IFNβ promoter, confirming the importance of these residues and the V-unique region in this aspect of function. The V proteins of both CPI+ and CPI- isolates were also shown to suppress the activation of the IFNβ promoter, indicating that residues in the N-terminus are not important for this function of V and also shows that the abilities of V to block IFN signalling and production can be separated.

The mechanism of the block of IFN production was investigated at the level of IFNβ promoter activation using various techniques and experiments using truncations of the IFNβ promoter and reporter constructs specific to different transcription factors established that SV5 V blocks the activation of IRF3 and NFκB by viral infection and poly(I):poly(C), but not the ATF2-c-Jun/HMGI:Y module, and that rSV5VΔC leads to the activation of IRF3 and NFκB (Poole et al. 2002). Immunofluorescence studies revealed that in wt SV5-infected cells, IRF3 remains in the cytoplasm but in rSV5VΔC cells it is found in the nucleus and the same pattern can be seen after poly(I):poly(C) treatment of cells transiently expressing SV5 V compared to those expressing SV5 P (He et al. 2002). It is not currently known how IRF3 and NFκB are activated in response to intra-cellular dsRNA and so it is unclear how SV5 V blocks their activation, but it may be via interactions with other cellular proteins, possibly the as yet unidentified 150kDa protein. Although the small amount of V protein in SV5 virions has been shown to block IFN signalling, it appears that higher concentrations of V are required to effectively block IFN production. IFN is produced as a consequence of co-infection with wt SV5 and rSV5VΔC, suggesting that the small

amount of full-length V present in wt virions cannot overcome the stimulation of IFN by the mutant virus and the same effect is seen in rSV5VΔC infection of 2f/SV5 V stable cell lines which express low levels of V. However, transient expression of full-length SV5 V can block IFN production in the presence of rSV5VΔC infection, suggesting that high levels of V can function in *trans*.

The regions of SV5 V required for its different functions have not been fully elucidated but deletion studies have shown that although a deletion of 20aa from the N-terminus can be tolerated for V to retain its STAT1 degradation function, a deletion of 48aa abrogates this function whereas up to 125aa can be deleted from the N-terminus of V and still allow it to block IFN production, suggesting that these two functions of V require different parts of the protein (Andrejeva et al. 2002a; Poole et al. 2002). The C-terminus of V and the conserved cysteine residues therein have been shown to be absolutely required for the antagonism of both IFN signalling and production and the interaction of V with DDB1 and whilst the interaction of V with STAT1 and STAT2 has been shown to require full-length V rather than N and C-terminal truncations, the precise regions responsible for these interactions are unclear (Ulane and Horvath 2002). What is clear is that, in order for V to participate in the varied activities described above, interactions with a number of different cellular and viral protein partners are required, normally something that would be very difficult to accommodate and it has been suggested that the N-terminus of V may be natively unstructured, already demonstrated for the V protein of Measles virus (Karlin et al. 2002). Extreme difficulties in crystallising SV5 V for structural studies lends credence to this suggestion, but until a 3D structure for V is available, perhaps interacting with one or more of its protein partners, it will remain difficult to discover the precise mechanisms of V function.

ii) Human Parainfluenza virus 2 (hPIV2)

After initial studies showed that SV5 blocked IFNα/β signalling, the ability of another member of the Rubulavirus genus, hPIV2, to antagonise the IFN response was investigated and it was demonstrated that hPIV2 infection of human cells blocks the activation of IFNα/β but not IFNγ-responsive promoters and that GAF complexes but not ISGF3 complexes are formed in response to IFN stimulation (Young et al.

2000). This study also demonstrated that STAT2 is targeted for degradation in hPIV2-infected cells, in contrast to the degradation of STAT1 seen in SV5-infected cells, explaining why IFNα/β but not IFNγ signalling is blocked by hPIV2 infection. Subsequent studies revealed that it is the V protein of hPIV2 that is responsible for IFN antagonism, similar to SV5 and that the decrease in STAT2 levels in cells expressing hPIV2 V is due to post-translational degradation of STAT2 in at least a partly proteosome-dependent manner (Parisien et al. 2001). The dependence of STAT2 degradation by hPIV2 V on the proteosome was also demonstrated by the use of MG132 in 2fTGH cells stably expressing hPIV2 V, which prevented the degradation of newly synthesised STAT2 (Andrejeva et al. 2002b). The presence of hPIV2 V seems to have no effect on the levels of STAT2 mRNA, but a study using an *in vitro* translation assay reported that hPIV2 V has an inhibitory effect on the translation of both STAT2 and to a lesser extent STAT1 mRNAs, but no effect on the translation of a control luciferase mRNA (Nishio et al. 2001). Whether this observation is important for the role of V in IFN antagonism is not currently clear.

Development of a reverse genetics system for hPIV2 allowed the creation of a recombinant virus expressing a truncated form of the V protein (rPIV2VΔC) which replicated in Vero cells, although to lower titres than wt, but was completely unable to grow in IFN-competent CV1 and FL cells (Kawano et al. 2001). The yield of rPIV2VΔC from these cells was improved by the addition of antibodies against IFNβ, although not to wt levels, suggesting that hPIV2 lacking a full length V protein is IFN sensitive and also that V may have other roles in the viral life cycle. Indeed, the rPIV2VΔC particles rescued from Vero cells were observed to have anomalous shapes and sizes compared to wt virions, suggesting a role for V in the proper assembly and maturation of virus particles, as has been proposed for SeV C (Hasan et al. 2000).

Studies in 2fTGH cell derivatives lacking certain components of the IFN signalling pathway demonstrated that the degradation of STAT2 by hPIV2 requires the presence of both STAT1 and STAT2 but does not require intact IFN signalling, as is the case for STAT1 degradation by SV5 V (Parisien et al. 2002b). It was also demonstrated that complementation of absent STAT1 or STAT2 with exogenously expressed

protein was sufficient to restore STAT2 degradation by hPIV2 V and required only the first 578aa of the STAT proteins. Interestingly, complementation of STAT2(-) cells with exogenous STAT2 led not only to the degradation of STAT2 but also to a partial loss of endogenous STAT1, in effect a loss of degradation fidelity. This effect was not seen in complemented STAT1(-) cell lines or with SV5 V in any cell line and suggests that precise levels of STAT2 expression may be necessary to preserve hPIV2 STAT degradation fidelity. A similar loss of hPIV2 degradation fidelity has been observed in hPIV2 infections of cell lines from other species (D.F. Young & R.E. Randall, unpublished data).

Like SV5 V, hPIV2 does not successfully infect murine cells and block IFN signalling, but the expression of either full length or aa1-578 of human STAT2 (hSTAT2) in murine cells expressing hPIV2 V resulted in a block of IFNα/β signalling and the partial degradation of both hSTAT2 and murine STAT1, but not murine STAT2 (Parisien et al. 2002a). This reflects the lack of hPIV2 V STAT degradation fidelity seen in human cells complemented with hSTAT2 and indicates that hSTAT2 is required for STAT degradation in murine cells, but that murine STAT2 is not a suitable target for degradation by hPIV2. Similar to SV5 V, hPIV2 V has been found to interact with STAT1, STAT2 and DDB1 (Lin et al. 1998; Parisien et al. 2002b) and there is evidence of STAT2 ubiquitination in cells expressing hPIV2 V, suggesting that hPIV2 V may act as an E3 ligase, enabling the ubiquitination of STAT2 and its subsequent degradation by the proteosome in a similar manner to SV5 V (Ulane and Horvath 2002).

It has been demonstrated that SV5 V is capable of suppressing the induction of IFNβ by blocking the activation of both IRF3 and NFκB and the same study also showed that hPIV2 V blocks IFN production, presumably via the same mechanism (Poole et al. 2002). This aspect of hPIV2 V function may have been involved in an earlier study which observed that the C-terminus of hPIV2 V but not the N-terminus allowed replication of the IFN-sensitive viruses VSV and Sindbis in cells challenged with IFNα/β and suggested that the C-terminus of V was therefore sufficient to antagonise the IFN response (Nishio et al. 2001). Large deletions can be made from the N-terminus of SV5 V with no loss of its ability to block IFN production (Poole et al.

2002) and the same is likely to be true of hPIV2 V, whereas similar truncation studies of SV5 V showed that most of the protein is required to block IFN signalling (Andrejeva et al. 2002a) so it seems unlikely that the C-terminus of hPIV2 V alone is capable of blocking IFN signalling. Indeed, a recent study using chimeras of hPIV2 V and SV41 V (which targets STAT1 for degradation) has shown that, as for SV5 V, residues in both the N and C-terminal domains of hPIV2 V are required for the degradation of STAT2 and has suggested that, although distant in the hPIV2 V sequence, these residues may lie in close proximity to each other in the 3D structure of hPIV2 V (Kozuka et al. 2003).

iii) Mumps virus (MuV)

Studies of human cell lines persistently infected with MuV revealed poor induction of the IFN-stimulated gene products 2-5 OAS, PKR and MxA and undetectable levels of STAT1 after IFNα treatment, suggesting that MuV antagonises the IFNα/β response in these cells via the degradation of STAT1 (Yokosawa et al. 1998). Further studies in another persistently infected human cell line showed that the expression of IFN-stimulated genes and levels of STAT1 could be restored by treatment of infected cells with ribavirin, which inhibits virus replication (Fujii et al. 1999). Persistent infection with MuV was also shown to block the IFNγ-mediated augmentation of apoptosis induced by the drug HPC, indicating that the IFNγ response is also blocked by MuV via the degradation of STAT1 (Hariya et al. 2000). Further studies using the expression of MuV P gene products to rescue IFN-sensitive VSV showed that expression of MuV V and Vc allowed the replication of VSV in the presence of IFNα or IFNγ, whereas expression of MuV P allowed no VSV replication, indicating that the MuV V protein, in particular the C-terminus, was responsible for the IFN antagonism seen in the previous studies (Kubota et al. 2001). This study also showed that STAT1 was degraded in MuV V-expressing cells and that STAT1 levels could be partially restored by treatment with the proteosome inhibitor MG132, suggesting that similar to SV5 V, MuV V may target STAT1 for proteosome-mediated degradation.

In vitro studies have shown that MuV V interacts with STAT1, STAT2 and STAT3 and that the interactions with STAT1 and STAT2 are independent of the conserved, carboxy terminal cysteine residues as judged by the ability to make pairwise

mutations of cysteine residues without any loss of STAT1 or STAT2 binding (Nishio et al. 2002). However, a chimeric V protein with the N-terminus of MuV V and the C-terminus of SeV V was unable to interact with STATs, as seen for full length SeV V, indicating that some residues in the C-terminus of MuV V are important for STAT binding. Both SeV V and MuV V contain a conserved WCNP motif in their C-terminal regions, but MuV V has two additional W residues upstream of this motif that are lacking in SeV V, but are found in hPIV2 V which also interacts with STATs. Mutation of these two W residues in MuV V to the SeV V sequence abrogated the *in vitro* interaction of MuV V with STATs and the reciprocal mutation of the SeV V residues to W in the MuV/SeV V hybrid enabled the chimera to bind STATs, suggesting that the conserved tryptophan motif in the C-terminus of MuV V rather than the downstream cysteine residues is required for the formation of V-STAT complexes *in vitro*. However, a recent study using MuV infections and expression of MuV V constructs in cultured cells showed that the cysteine residues in the C-terminus of MuV V are required for MuV V to block IFN signalling, poly-ubiquitinate STAT1 and target it for degradation and are also involved in an interaction between STAT1 and MuV V (Yokosawa et al. 2002). These cysteine residues are likely to bind zinc in the same way as SV5 V (Paterson et al. 1995) and have been found to interact with DDB1 (Lin et al. 1998) and it is possible that the interaction of MuV with DDB1 may be required for the degradation of STAT1 as demonstrated for SV5 V (Andrejeva et al. 2002a).

Further interactions of MuV V with cellular proteins were studied using MuV Vc as the bait in a yeast-2-hybrid library screen, which pulled out the cellular protein RACK1, an interaction confirmed by *in vitro* interaction studies and immune precipitations from MuV infected and MuV V-expressing cells which also found that the C-terminal cysteine residues of MuV are not required for the interaction (Kubota et al. 2002). RACK1 is thought to mediate the interaction of the IFNα receptor and STAT1 and to investigate whether the presence of MuV V disrupts this function, *in vitro* binding studies of a GST-fusion of part of the cytoplasmic domain of the IFNα receptor (IFNαRβL) were carried out with infected and uninfected cell lysates. These showed that infection with MuV disrupts the normal association of IFNαRβL, RACK1 and STAT1 but has no effect on STAT2 binding to IFNαRβL and suggested

that the interaction of MuV V and RACK1 may result in the dissociation of STAT1 from the IFNα receptor and contribute to its subsequent poly-ubiquitination and degradation in infected cells.

Transiently expressed MuV V has been shown to block IFNα/β and IFNγ signalling in human cells via the degradation of STAT1 and also to block IL-6 signalling in human and murine cells via the degradation of STAT3 (Ulane et al. 2003). This study also showed that IFNγ signalling remained intact in U6A cells that lack STAT2, despite the presence of MuV V, indicating that STAT2 is required for the degradation of STAT1, as seen for SV5 V (Parisien et al. 2002b). However, unlike SV5, which cannot degrade STAT1 in murine cells due to a lack of compatibility with murine STAT2 (Parisien et al. 2002a), MuV V can degrade STAT1 in murine cells. Affinity purification of FLAG-MuV V from transfected cells revealed a number of cellular proteins interacting with MuV V, including STAT1, STAT2, STAT3, DDB1 and Cul4a and also ubiquitinated forms of STAT1 and STAT3, suggesting that MuV V may assemble degradation complexes for STAT1 and STAT3 similar to that proposed for SV5 V (Ulane and Horvath 2002).

iv) Simian virus 41 (SV41)

The V protein of SV41 was demonstrated to function as an IFN antagonist by the use of VSV and Sindbis rescue assays in HeLa cell lines stably expressing SV41 V (Nishio et al. 2001). Expression of SV41 V allowed the replication of IFN-sensitive VSV and Sindbis viruses in the presence of IFNα, IFNβ and IFNγ and resulted in the degradation of STAT1 but this degradation was not affected by treatment with proteosome inhibitors. Thus SV41 appears to have a similar IFN evasion strategy to SV5, but it is not yet clear if the degradation of STAT1 in cells expressing SV41 V occurs in the same way as in cells expressing SV5 V.

Respirovirus

i) Sendai virus (SeV)

The ability to isolate recombinant SeV lacking V expression, rSeV V(-), demonstrated that SeV V is a non-essential, accessory gene. However in contrast with wt SeV, which is lethal in mice, rSeV V(-)infections of mice were non-pathogenic, indicating

that V may be required for efficient pathogenesis in the animal host (Kato et al. 1997a). The same study also noted increased IFN production in the lungs of mice infected with rSeV V(-), compared to those infected with wt SeV, suggesting that V may have a role in IFN antagonism. Further studies using a virus expressing a V protein lacking the cysteine-rich carboxy terminus, rSeV VΔC, demonstrated that this virus was also non-pathogenic in mice, suggesting a role for the C-terminus of SeV V in pathogenicity (Kato et al. 1997b). Subsequently a similar study found rSeV V(-), but not rSeV VΔC to be avirulent in mice, contradicting the results of Kato *et al*, but it is possible that the different strains of mice used in this later study may have contributed towards the conflict with the previous data.

The C-terminus of SeV V, like that of SV5 V and MuV V, binds zinc and point mutations of the conserved cysteine residues in the carboxy terminus reduce zinc binding by varying degrees (Huang et al. 2000). This study also found that recombinant viruses with mutated cysteine residues and therefore a reduced capacity to bind zinc, were also less pathogenic in mice as seen with rSeV VΔC. This suggested that the mutation of a single cysteine residue in the C-terminus of SeV V could reduce its pathogenicity in mice and that this was related to the ability of the protein to bind zinc. Further studies by the same group showed that the mutation of multiple cysteine residues in SeV V impaired zinc binding and pathogenicity in mice as did the mutation of non-cysteine residues directly downstream of the RNA editing site (Fukuhara et al. 2002). One of these mutations was found to significantly reduce the frequency of RNA editing and therefore V expression, which suggests that changes in this region affect pathogenicity by altering the conformation of V or by reducing levels of V, or possibly a combination of the two.

Recent studies have shown that SeV V is capable of blocking dsRNA signalling to the IFNβ promoter when transiently expressed in Vero cells (Poole et al. 2002). This is also a function of SV5 V where it has been shown to depend on the conserved cysteine residues in the C-terminus and so it seems likely that the cysteine residues in SeV V play a similar role in blocking IFNβ promoter activation. It is possible that it is this property of SeV V that allows the virus to maintain high titres in the lungs of mice and cause lethal pneumonia. If SeV V is able to block the production of IFN in

mice, this would explain the observation made by Kato *et al* that lungs from mice infected with rSeV V(-) virus contain more IFN than those infected with wt SeV.

It is worth noting that the suggestion that SeV blocks the production of IFN is in conflict with the established notion that SeV is an efficient inducer of IFN (Didcock et al. 1999a). However, it seems likely that the induction of IFN by SeV is not a consequence of normal viral replication but an artefact, arising from the method of virus purification. Rather than plaque purification, stocks of SeV are often grown as 'von Magnus' preparations whereby virus is serially passaged in eggs at low dilutions (von Magnus 1951b; von Magnus 1951a). It has been demonstrated that these low dilution stocks of SeV are good inducers of IFN in lymphoblastoid cells, whereas SeV passaged at high dilutions is a poor inducer and that this difference in IFN induction can be attributed to the presence of viral defective interfering (DI) particles in the virus stock passaged at low dilution (Johnston 1981). The same effect is seen in MG63 fibroblast cells and is also likely to occur in the various cell types used to study SeV and IFN evasion (S. Goodbourn, personal communication). Whilst plaque purification ensures that only viable viruses are isolated at each passage, the von Magnus technique allows the carry-over of DI particles produced during the course of virus infection. Numbers of these DI particles increase with each passage, as does the ability of the virus stock to induce IFN, until there are too many DI particles in the stock to allow efficient virus replication, at which point virus titres drop away, as does the ability of the stock to induce IFN. Indeed, if SeV is plaque purified, the resulting virus stock is a very poor inducer of IFN, perhaps reflecting the ability of the V protein to suppress the induction of IFNβ (S. Goodbourn, unpublished data). However, the mechanisms by which SeV V operates to suppress the production of IFNβ and any cellular proteins that may be involved remain to be elucidated.

Studies on a tissue culture adapted strain of SeV, SeV^{MVC}, which has two amino acid changes relative to the parent strain (C^{F170S} and L^{E2050A}) showed that it produces more virus in cell culture, has enhanced viral mRNA synthesis and is avirulent for mice, in contrast to the parent strain, suggesting that either the C or L protein has a role in viral pathogenesis (Itoh et al. 1997). Insertion of the C^{F170S} mutation into a wild-type SeV background to create rSeV C^{F170S} showed that this single amino acid change was

responsible for the phenotype of avirulence and restricted viral replication in mice, suggesting that the C protein, like SeV V is involved in pathogenesis (Garcin et al. 1997). rSeV C^{F170S} replicated normally in the mouse lung for 1 dpi but was then rapidly cleared, suggesting a susceptibility to the innate immune system and indicating a role for the wt C protein in the evasion of innate immunity. Further studies using recombinant SeV showed that growth of rSeV C/C'(-) was impaired in cultured cells and the virus was non-pathogenic for mice, despite enhanced expression of Y1 and Y2, suggesting that the shorter Y proteins cannot complement the function of C and C' in tissue culture or in infections of mice (Kurotani et al. 1998). A rSeV 4C(-) virus was also generated in this study and was shown to be viable in cultured cells but highly impaired and avirulent in mice, indicating that the C proteins of SeV are non-essential gene products but crucial for pathogenesis in mice.

Subsequently, a study of SeV infection of murine cells showed that SeV was not affected by the large amounts of IFN produced during infection and that SeV infection could block the activation of an IFNα/β-responsive promoter and inhibit the transcription of the IFN-stimulated gene 6-16 (Didcock et al. 1999a). This effect was also observed in human cells and suggested that SeV has a specific IFN evasion mechanism, functional in both murine and human cells. These results were confirmed by experiments using rSeV, which also demonstrated that the C proteins are crucially required for IFN antagonism (Garcin et al. 1999; Gotoh et al. 1999). C and C' were shown to be important, but some evasion of IFN was seen in C/C'(-) viruses, suggesting that over-expression of Y1 and Y2 can partially compensate for the lack of the longer C proteins, whereas 4C(-) viruses were shown to have no IFN evasion capabilities (Gotoh et al. 1999). Studies using rSeV C^{F170S} demonstrated that the F170 residue in C is essential for IFN evasion as the recombinant virus was unable to block the induction of an anti-viral state (Garcin et al. 1999). These studies also suggested that the blocking of the IFN response by C is an active process as the wt C phenotype was dominant in co-infections of wt SeV and SeV^{MVC}.

IFN signalling assays using IFNα/β and IFNγ-responsive reporter constructs and either SeV infection or the transient expression of C proteins revealed that both IFN signalling pathways are blocked by SeV (Didcock et al. 1999a; Young et al. 2000)

and specifically SeV C proteins (Garcin et al. 2000). The latter publication showed that the C^{F170S} mutation abolished the ability of C to block IFN signalling and that co-expression of Y1 and Y2 blocked IFN signalling to a similar extent to C. It also found that C' alone was slightly less effective at blocking IFNα/β signalling but that all C proteins could block IFNγ signalling. Further studies of the C proteins using HeLa cell lines stably expressing either C, Y1 or Y2 confirmed that these proteins are all capable of blocking IFN signalling and also that all three proteins allow the replication of IFN-sensitive VSV in HeLa cells (Kato et al. 2001). This suggests that even the shortest C protein, Y2, is capable of counteracting the anti-viral state but other studies using recombinant SeV have suggested that only the two longer C proteins, C and C' have this function (Garcin et al. 2001). It is possible that the contradiction in these results is due to difficulties in comparisons of differing experimental approaches such as stable cells lines, transient transfections and recombinant viruses and also the use of different viruses and cell lines.

The mechanism of the C-mediated antagonism of the IFN response remains to be fully elucidated and various theories have been proposed, most of them centring around STAT1 and its phosphorylation status in infected cells. IFN stimulation of SeV-infected 2fTGH cells was observed to cause tyrosine phosphorylation, but not serine phosphorylation of STAT1, suggesting that SeV may block the IFN response by preventing the formation of pS-STAT1, but not pY-STAT1 (Young et al. 2000). Another study reported a block of pY-STAT1 formation and also a partial block of the phosphorylation of the cellular kinase Tyk2 (Komatsu et al. 2000). These two studies appear contradictory but the difference in STAT1 phosphorylation status may be explained by differences in the length of IFN stimulation before the cells were harvested which were 16h and 30min respectively. Subsequent studies have shown that over time, levels of pY-STAT1 accumulate in SeV-infected cells, suggesting that the block of tyrosine phosphorylation is effective at early times post infection but is either leaky or not effective at later times post infection, bringing into agreement the results of Young *et al* and Komatsu *et al* (Takeuchi et al. 2001; Komatsu et al. 2002; Saito et al. 2002). Studies using SeV^{MVC} have shown that the F170 residue in the C protein is required for the block of tyrosine and serine phosphorylation of STAT1 (Garcin et al. 2001). In addition to the blockade of STAT1 phosphorylation, a block

of pY-STAT1 dephosphorylation in C-expressing HeLa cells has also been observed (Saito et al. 2002) but this effect was not seen in SeV-infected MEF cells (Garcin et al. 2003). SeV infection has also been shown to block the formation of pY-STAT2 at both early and late (48h) times post infection, an activity that requires STAT1 as no block of pY-STAT2 formation occurred in U3A cells which lack STAT1 (Gotoh et al. 2003b).

It has been suggested that SeV C protein directly interacts with both STAT1 and pY-STAT1 and that this interaction results in the formation of high molecular weight complexes (HMWCs) with a mass of over 2MDa, containing STAT1, pY-STAT1 and C (Takeuchi et al. 2001). Studies using a HeLa cell line expressing SeV C also showed that pY-STAT1 accumulates in HMWCs in these cells, but could not demonstrate the presence of C in these complexes (Saito et al. 2002). Studies in both infected cells and *in vitro* have shown that all four C proteins are able to bind STAT1 and that the C^{F170S} mutation abolishes this interaction (Garcin et al. 2002). Recent studies suggest that C also interacts with STAT2, but only via its interaction with STAT1 as no interaction between C and STAT2 was detected in U3A cells lacking STAT1 but this could be restored by the expression of exogenous STAT1 (Gotoh et al. 2003b).

There is also evidence that the formation of pY-STAT1 in SeV-infected cells is not inhibited at later times post-infection, but that despite this there are still no functional GAF complexes (homodimers of pY-STAT1) in infected cells at 20hpi (Takeuchi et al. 2001). It was suggested that the interaction between STAT1 and C might somehow prevent STAT1 from binding to the GAS element in IFNγ-responsive promoters or disrupt the interactions of STAT1 with other transcription factors such as CBP/p300. This was also suggested by studies with rSeV, which demonstrated that pY-STAT1 homodimers translocate to the nucleus in C-expressing cells but are unable to activate IFNγ-responsive promoters (Komatsu et al. 2002). These results were confirmed by experiments in cells expressing a truncation of Y2 able to bind STAT1 and block IFN signalling, which showed that aberrant complexes of pY-STAT1 form, which are unable to bind the GAS elements of IFNγ-responsive promoters (Gotoh et al. 2003a).

Many studies in a variety of cell types have shown that the infection of cells with SeV does not lead to a degradation of STAT1, unlike infections with the rubulaviruses SV5 and MuV (Garcin et al. 1999; Gotoh et al. 1999; Komatsu et al. 2000; Young et al. 2000; Kato et al. 2001; Takeuchi et al. 2001). However, experiments in the murine cell line NIH 3T3 MEF, which expresses high levels of STAT1 in the absence of IFN stimulation and appears to be in a permanent anti-viral state have shown that SeV infection leads to STAT1 instability and that this is a property of the C protein that does not correlate with its ability to block IFN signalling (Garcin et al. 2000; Garcin et al. 2001). Further studies using the MEF cell line have shown that SeV infection results in the mono-ubiquitination of STAT1, suggesting that its instability in infected cells may be due to proteosome-mediated degradation similar to that seen in rubulavirus infections (Garcin et al. 2002). It is still not clear whether the ability of SeV to destabilise STAT1 is peculiar to MEF cells or is more widespread and without further study the importance of these observations remains to be established.

As the four SeV C proteins are a carboxy co-terminal nested set, it seems logical that some functions will be common to all four proteins and others may be unique to the longer forms which have N-terminal extensions compared to Y1 and Y2. Studies using rSeV CΔ10-15 which lacks residues from the N-terminal unique region of C and C' have shown that while this virus can block IFN signalling and the formation of pY-STAT1, it is unable to block the establishment of an anti-viral state in MEF cells or cause STAT1 mono-ubiquitination and instability, suggesting that these functions are unique to C and C' (Garcin et al. 2001; Garcin et al. 2002). Studies using SeV^{MVC} and C^{F170S} showed that F170, common to all four C proteins, is required for STAT1 binding and IFN signalling but not for STAT1 destabilisation in MEF cells (Garcin et al. 2000; Garcin et al. 2002; Garcin et al. 2003). It has also been demonstrated that amino acids 10-15 of C, not found in Y1 and Y2 are required for the interaction with pY-STAT1 whereas the region common to all four C proteins is involved in the interaction with STAT1, which can be disrupted by the C^{F170S} mutation (Garcin et al. 2003). This study concluded that while all four C proteins can block IFN signalling, interact with STAT1 and prevent the initial formation of pY-STAT1 by a mechanism dependent on the F170 residue, only the two longer C proteins, C and C' can induce STAT1 instability and counteract the anti-viral state. These results were partially

confirmed by truncation studies of the smallest C protein, Y2, which showed that deletion of 67 amino acids from the N-terminus of Y2 had no effect on the ability of the protein to block IFN signalling, but that removal of a further 28 residues abrogated all IFN antagonism (Kato et al. 2002). The second truncation of Y2 still contained the F170 residue, but appeared to be less stable than longer forms, suggesting that the deleted residues may be required for overall protein stability rather than function. As mentioned previously there is some conflict in the literature on the ability of SeV C proteins to block the establishment of an anti-viral state and this arises again here as the experiments of Kato *et al* showed that full-length and truncated Y2 were able to block the establishment of an anti-viral state and allow the replication of VSV, in contrast to the results of Garcin *et al.*

A recent study using various mutant viruses and a cDNA array designed to study cellular responses to IFNα/β has shown that SeV C proteins, as well as the V protein and viral leader and trailer sequences may be involved in blocking the expression of various non-IFN stimulated genes, including IL-6 and IL-8 (Strahle et al. 2003). Infection with rSeV C^{F170S} and rSeV CΔ10-15 resulted in the upregulation of genes encoding IL-6 and IL-8 compared to expression levels in wt SeV-infected cells and cells infected with rSeV CΔ10-15 secreted higher levels of IL-8. This suggests a role for the C protein in blocking the activation of these genes, the products of which are involved in the inflammatory, adaptive immune response. The C protein mutants and also a rSeV V(-) virus were also found to upregulate the production of IFNβ in an IRF3-dependent manner, confirming the role of SeV V as an antagonist of IFNβ production already observed in transient transfection studies (Poole et al. 2002) and suggesting an additional role for SeV C proteins in the evasion of IFN.

Thus it appears that SeV C proteins are involved in multiple aspects of IFN antagonism with all four C proteins able to block IFN signalling, interact with STAT1 and block the formation of pY-STAT1 in response to IFN, which require residue F170 in the region common to C', C, Y1 and Y2. However, it seems that only the longer C proteins, C and C', can interact with pY-STAT1 and cause mono-ubiquitination of STAT1 and the STAT1 instability seen in some cell types. It also seems that SeV C can influence the expression of other genes, including those for

inflammatory cytokines and IFNβ. So, unlike SV5, which uses a single protein, V, to antagonise IFN signalling and production, SeV divides these two functions between the C and V proteins.

ii) Human parainfluenza virus type 3

Although evidence of IFN antagonism by PIV3 can be found in a publication from over 40 years ago in which PIV3 infection of calf kidney cells was found to enhance the growth of super-infected NDV (Hermodsson 1963), it was only more recently that PIV3 infection of human cells was shown to block both IFNα/β and IFNγ signalling (Young et al. 2000). This study demonstrated that ISGF3 complexes are not formed in PIV3-infected cells but that one of the two GAF complexes observed in mock-infected cells was formed in response to IFNγ, suggesting that PIV3 blocks IFNγ signalling despite the presence of a GAF complex able to bind DNA. PIV3 infection was also found to inhibit the serine but not the tyrosine phosphorylation of STAT1, similar to that seen in SeV infections. An earlier study of hPIV3 infection of human A549 cells had showed that pre-treatment with IFNα inhibited virus yield and replication by more than 90% but that addition of IFNα at 4hpi had little effect on virus yield (Zhao et al. 1996) and this may suggest that viral products generated early in infection can counteract the IFN response. The same phenomenon of reduced viral yield and replication is seen with infections of SV5 and SeV in IFN pre-treated cells and these viruses are both capable of antagonising the IFN response (Didcock et al. 1999a). A more recent study of hPIV3 infection of human fibrosarcoma cells indicated that the IFNγ-stimulated expression of MHC class II is blocked by PIV3, but that expression of other IFNγ-stimulated genes was not inhibited, suggesting that PIV3 does not block IFNγ signalling (Gao et al. 2001). However, the cells were treated with IFNγ at the same time as viral infection which has been shown to inhibit PIV3 replication and it is possible that IFNγ treatment at 4hpi or later would have revealed a general block of the expression of IFNγ-stimulated genes.

The advent of a reverse genetics system for PIV3 has allowed the creation of knockout viruses and enabled the roles of the C, D and V proteins to be investigated (Durbin et al. 1997; Durbin et al. 1999). rPIV3 C(-) virus was found to have reduced replication both in cultured LLC-MK2 cells and in hamsters and monkeys whilst

viruses lacking D or V expression replicated to wt titres *in vitro* and in hamsters and were only partially attenuated in monkeys. The F170 residue in SeV C had already been shown to be important for function (Garcin et al. 1997) and an equivalent residue in PIV3 C, F164, was mutated to assess its effect on virus replication. rPIV3 C^{F164S} was shown to replicate to wt titres *in vitro* but was attenuated *in vivo*, although not to the same extent as rPIV3 C(-). This data suggests that PIV3 C, like the C proteins of SeV and MV, is not essential for virus replication but is required for full replicative efficiency in both cultured cells and *in vivo*. The C protein of hPIV3 appears to be important for pathogenesis *in vivo* and the F164 residue appears to have similar importance to C function as the corresponding F170 residue in SeV C, despite a sequence homology of only 38% between the two C proteins. The D and V proteins of hPIV3 appear to be unimportant for replication both *in vitro* and *in vivo*, but a double mutant lacking expression of both proteins was partially attenuated *in vivo*, suggesting a role for these proteins in pathogenesis, perhaps as an interacting pair. It is also worth noting that rPIV3 D(-) was not a true D knockout but a truncation and the virus was still able to express a D protein with 61aa of the D-unique region, so it is possible that the truncated D was able to function normally, explaining the lack of a D(-) phenotype.

The functional similarities between the C proteins of PIV3 and SeV suggested by these studies have been emphasised by recent studies showing that the L and C proteins of hPIV3 and SeV can interact and that hPIV3 C is an inhibitor of viral transcription, like SeV C (Malur et al. 2004; Smallwood and Moyer 2004). Whether the similarities between hPIV3 and SeV can be extended to their strategies for IFN evasion remains to be seen but it is tempting to suggest that that hPIV3 C is responsible for IFN antagonism in the same way as SeV C. Currently the roles of hPIV3 D and V proteins are unknown but further study should reveal whether they too have roles in the evasion of the IFN system.

Morbillivirus

i) Measles virus (MeV)

Early studies of the IFN-induced gene 2-5 OAS in cells persistently infected with MV strains showed that in many cases the expression of this gene in response to IFN was

suppressed, suggesting that MV has strategies for IFN evasion, although differences in the expression of 2-5 OAS were observed between the various cell types and viral strains studied (Fujii et al. 1988; Fujii et al. 1990). Later studies of various vaccine and wild MV strains revealed differences in their ability to induce IFN production and their sensitivity to IFN treatment and it was shown that wild MV induced less IFN than vaccine strains but was more sensitive to IFN treatment (Naniche et al. 2000). It was also demonstrated that pre-infection of peripheral blood lymphocytes with wild MV could prevent 75-80% of the IFN induction by subsequent MV Ed infection, but not IFN induction by dsRNA, suggesting that some strains of MV have mechanisms to block the induction of IFN and some are capable of evading the effects of the IFN response. Studies of the effects of MV on gene expression using a cDNA array showed that wild MV infection upregulates the expression the IFNβ gene expression by 2.1-fold, whereas infection with MV Ed had no effect and the same study used RT-PCR to show that both wild MV and MV Ed upregulate the IFNα gene, albeit by small amounts, 1.1 and 2.3-fold respectively (Bolt et al. 2002). Measurement of IFNα in the supernatant of infected cells showed increases of around 20-fold for wild MV infection and 70-fold for MV Ed infection compared to uninfected cells, confirming that different MV strains differ in their ability to induce IFN. However, recent studies have suggested that it is the amount of DI particles in the MV stock, rather than characteristics of the virus itself, which determine the amount of IFN induced by MV infection (Shaffer et al. 2003), a phenomenon that has also been observed for SeV (Johnston 1981).

Whatever the reasons behind the varying induction of IFN by MV strains, it has recently been clearly demonstrated that MV has the ability to evade the IFN response. Studies of wild MV strains showed that infection with MV enables the replication of IFN-sensitive VSV in the presence of IFNα but not IFNγ and that signalling to an IFNα/β-responsive reporter was blocked, but not signalling to an IFNγ-responsive reporter, suggesting that MV can antagonise IFNα/β but not IFNγ signalling (Yokota et al. 2003). The same study found no evidence of STAT1, STAT2 or IRF9 degradation in MV-infected cells, but it did observe a block on the IFNα-stimulated phosphorylation of STAT1 and Jak1 but not on the IFNγ-stimulated phosphorylation of STAT1. Another study using HeLa cell lines stably expressing the V protein from a

wild MV strain found that V-expressing cells blocked the induction of an anti-viral state by IFNα, but not IFNγ, suggesting that the V protein of MV is responsible for IFNα/β evasion (Takeuchi et al. 2003). This study also observed no degradation of STAT1 and STAT2 in MV V-expressing cells and a partial block of both tyrosine and serine of phosphorylation of STAT1 in response to IFNβ. It also showed that STAT2 phosphorylation is completely blocked in cells expressing MV V and suggested that the block of STAT2 phosphorylation is the most important factor in MV evasion of IFNα/β. However, another study which used MV Ed V found that expression of V in 293 cells blocked both IFNα/β signalling and IFNγ signalling and also showed that MV Ed V has an effect on IL-6 and vSrc signalling via STAT3 and suggested that this may allow MV to influence other aspects of host immunity (Palósaari et al. 2003). It also found that expression of MV Ed V did not affect the IFNα-stimulated phosphorylation of STAT1 and STAT2 or the IFNγ-stimulated phosphorylation of STAT1. It is possible that this difference to the results of Yokota *et al* and Takeuchi *et al* may be due to the use of MV Ed V, rather than a wild MV V, or possibly to the use of transient expression of V rather than infection or stable expression.

A study using HeLa cells stably expressing wild MV C did not allow the replication of IFN-sensitive VSV in the presence of either IFNα or IFNγ, suggesting that MV C does not have a role in IFN evasion (Takeuchi et al. 2003). However, another investigation using a rMV Ed C(-) virus showed that C(-) virus is restricted in immune-competent human cells, but not immune-competent simian cells and that the addition of anti-IFNα/β antibodies restored C(-) virus replication in human cells to wild type levels (Shaffer et al. 2003). This suggests that the MV Ed C functions as an IFN antagonist in human cells and reporter assays in the same study demonstrated that transiently expressed MV Ed C blocks IFNα/β signalling in Vero cells and reduces IFNγ signalling in the same cells by 50%.

Two studies have investigated potential interactions of MV and host proteins using immune precipitations from cells either infected with wild MV or transiently expressing the MV Ed V. Immune precipitations of wild MV-infected cell lysates with an antibody against part of the IFNα/β receptor showed that a complex including MV V and C proteins as well as STAT1 and RACK1 cellular proteins is formed with

the IFNα/β receptor in infected cells, whereas an antibody against the IFNγ receptor did not show any interaction with MV V or C (Yokota et al. 2003). It is suggested that the presence of MV V and C proteins disrupts the normal interactions of the IFNα/β receptor, RACK1 and STAT1 and "freezes" the complex, thereby blocking the phosphorylation and activation of STAT1. The lack of interaction with the IFNγ receptor demonstrated by this study may explain why IFNγ signalling is not blocked in cells infected with wild strains of MV or expressing wild MV V protein. The second study used transient transfections of 293 cells with FLAG-MV Ed V and showed that MV Ed V co-precipitates a number of proteins, including STAT1, STAT2, STAT3 and IRF9 (Palosaari et al. 2003). This study also compared the proteins immune precipitated in FLAG-MV V expressing cells with those from FLAG-SV5 V expressing cells and showed that the complexes formed are distinct in that the MV V immune precipitates did not include Cul4a and only trace amounts of DDB1 were seen which is perhaps not surprising as these proteins are thought to be involved in the SV5 V-mediated ubiquitination and degradation of STAT1, which is not seen as a consequence of MV infection.

The current data on MV and IFN is confusing and it is not clear whether both MV V and C proteins are IFN antagonists in all strains of MV and in all cell types, but what is clear is that the MV mechanism of IFN evasion is unlike that described for other paramyxoviruses. There is currently no evidence for the degradation of any components of the IFN signalling pathway such as the STAT degradation characteristic of rubulavirus infections. There is also no consensus on the effects of MV on the phosphorylation of STAT proteins, although the current data suggests that wild MV blocks STAT phosphorylation whereas MV Ed does not. There is also no real agreement on the viral protein responsible for IFN evasion with studies on wild MV suggesting that V alone is responsible for IFN antagonism and work on MV Ed indicating that both V and C proteins are involved. Further studies on both wild and vaccine strains of MV and their V and C proteins are required to resolve these ambiguities.

Avulavirus

i) Newcastle Disease virus (NDV)

Studies using an IFN-sensitive rNDV-GFP virus to infect non-permissive chick embryo fibroblasts expressing various NDV proteins showed that expression of full-length NDV V or the cysteine-rich C-terminus of NDV V (Vc) enabled viral replication, whereas expression of NDV P or the N-terminus of NDV V (Vn) did not, indicating that NDV V is able to antagonise the IFN response in these cells via its cysteine-rich carboxy terminus (Park et al. 2003b). Other studies using reverse genetics demonstrated that the replication of rNDV lacking full-length V expression was impaired in IFN-competent chicken cells, suggesting a role for NDV V in IFN antagonism (Mebatsion et al. 2001). Later studies using an editing-defective rNDV demonstrated the presence of selective pressure for the efficient expression of NDV V and W proteins as after a few passages a compensatory mutation had appeared in most of the viruses in the population, which increased RNA editing and thus levels of V and W mRNA (Mebatsion et al. 2003). Another study showed that expression of NDV Vc enhances the growth of both an editing-defective rNDV virus and a recombinant virus expressing Vn rather than full-length V protein (Huang et al. 2003).

It was also reported by Huang *et al* that NDV infection of 2fTGH cells and the transfection of 2fTGH and Vero cells with a construct expressing NDV Vc leads to the degradation of STAT1. The observation that NDV infection leads to the degradation of STAT1 is not surprising as several rubulaviruses, to which NDV is fairly closely related, also antagonise the IFN response via the degradation of STAT1. However, it is slightly surprising that the C-terminus of NDV V alone is capable of targeting STAT1 for degradation as in rubulaviruses the whole V protein seems to be required. It is possible that NDV V is different to rubulavirus V proteins, but the data presented for the degradation of STAT1 by NDV Vc is not totally convincing. The anti-STAT1 western blot data shown in the publication represent total cell lysates from 2fTGH and Vero cells transfected with NDV Vc and it is widely accepted that transfection efficiencies in these cells are of the order of 0.5-5% and up to 50% respectively and certainly not close to 100%. Even if NDV Vc were targeting STAT1 for degradation in the transfected cells, one would expect normal levels of STAT1 in

untransfected cells to at least partially mask this degradation, unless the transfected cells were selected in some way, which does not appear to have been done. Unless complete transfection efficiency was achieved in these experiments, which seems very unlikely, it is not clear how the lack of STAT1 in the cells transfected with NDV Vc can be explained. Ideally this experiment should be repeated using lysates from cells either infected with a rNDV Vc virus at high multiplicity or stably expressing NDV Vc to ensure that all cells are expressing the viral protein. Alternatively, the ability of NDV Vc to block IFN signalling could be tested in a simple IFN-responsive reporter assay which would clearly demonstrate which regions of NDV V are capable of antagonising IFN signalling.

Another recent publication, again using rNDV viruses lacking full-length V expression or the ability to produce V and W by RNA editing, has suggested that NDV V is functionally interchangeable with Influenza virus NS1 protein and is a determinant of viral host range (Park et al. 2003a). The introduction of full-length NDV V or Influenza NS1 between the HN and L genes of rNDV V(-) viruses restored viral growth in immune-competent cells to wt levels, suggesting that these proteins have similar properties. Influenza NS1 is a known antagonist of IFN production (Garcia-Sastre et al. 1998) as are the C-termini of several paramyxovirus V proteins (Poole et al. 2002) and the sequence conservation between the C-terminus of NDV V and other paramyxovirus V proteins makes it likely that NDV V is also capable of suppressing IFN production by antagonising dsRNA signalling to the IFNβ promoter. In this respect NDV V and Influenza NS1 may be functionally equivalent in their blockade of IFN production, despite their lack of sequence identity. The same study also demonstrates that wt rNDV blocks IFN production in chicken cells but not human cells whereas a recombinant virus expressing Influenza NS1 protein blocked IFN production in both cell types and suggests that the ability of NDV to block IFN production in different cell types may be a factor in viral host range. Recent experiments have showed that expression of NDV V does not appear to block dsRNA signalling to the IFNβ promoter in Vero cells as demonstrated for SV5 and SeV V proteins, suggesting that this function of NDV V may be restricted to avian cells (S. Goodbourn, personal communication).

Henipavirus

i) Nipah Virus (NiV)

Recent studies on the products of the NiV P gene have found that the NiV V protein is capable of blocking IFNα/β and IFNγ signalling when expressed in human cells (Rodriguez et al. 2002). Immunofluorescence revealed that NiV V has a cytoplasmic distribution in transiently transfected cells and that the expression of NiV V alters the subcellular localisation of STAT1 and STAT2 in response to IFN, preventing their nuclear translocation. NiV V was shown to immune precipitate endogenous STAT1 and STAT2 from 293 cells transiently transfected with NiV V and evidence of high molecular weight complexes of NiV V and STATs was obtained by gel filtration chromatography. Contrary to other paramyxovirus IFN evasion mechanisms such as that of SV5, STAT2 is not required to block STAT1 translocation in the presence of NiV V and there was no evidence of STAT1 or STAT2 degradation, although the phosphorylation of STAT1 appeared to be blocked or at least reduced. This evidence has led to the suggestion that NiV V may function by the direct binding and cytoplasmic sequestration of STAT1 and STAT2, so blocking both the IFNα/β and IFNγ signalling pathways.

Experiments with an IFN-sensitive rNDV-GFP virus and transiently expressed NiV proteins demonstrated that both V and W proteins enabled the replication of rNDV-GFP in IFN-competent chicken embryo fibroblast cells, suggesting that both of these proteins have roles in antagonising the IFN response (Park et al. 2003b). Expression of NiV C allowed some replication of rNDV-GFP, but not to the same extent as V or W, suggesting that C may be a weaker antagonist of the IFN response in these cells. The same system showed that a construct expressing the N-terminus of NiV V (NiV Vn) enabled rNDV-GFP replication but that a construct expressing only the cysteine-rich C-terminus (NiV Vc) did not. Assays using a CAT reporter gene under the control of an IFNα/β-responsive promoter in Vero cells showed that NiV V, NiV W and NiV Vn could block IFN signalling but NiV Vc could not, confirming that at least two products of the NiV P gene have roles in antagonising the IFN response. Further to this, sequence analysis of the Nipah V and W proteins has suggested that they may be natively unfolded as demonstrated for the V proteins of Measles virus and Sendai virus and this may have some connection with their ability to evade the IFN response.

ii) Hendra Virus (HeV)

Recent studies have shown that, similar to NiV V, HeV V blocks IFNα/β and IFNγ signalling in human cells (Rodriguez et al. 2003). The mechanism appears to be very similar to that of NiV V in that nuclear translocation of STAT1 and STAT2 is blocked in cells expressing HeV V and there is evidence for interactions between HeV V, STAT1 and STAT2. Cells transfected with HeV V expression constructs show evidence of larger than normal ~550kDa complexes containing STAT1 and STAT2 and it has been suggested that the mechanism of HeV V antagonism of the IFN response is via binding and cytoplasmic sequestration of STAT1 and STAT2.

Pneumovirus

i) Human and Bovine Respiratory Syncytial Virus (HRSV; BRSV)

Studies in human cells have revealed that HRSV replication is relatively unaffected by pre-treatment of cells with IFNα/β, the addition of IFNα/β after infection or production of endogenous IFN stimulated by treatment with poly(I):poly(C), regardless of virus load (Atreya and Kulkarni 1999). However, HRSV infection did not prevent the IFNα/β-stimulated expression of the anti-viral protein MxA or allow the subsequent replication of an IFN-sensitive virus, suggesting that although HRSV is resistant to the effects of IFNα/β, it does not block the establishment of an anti-viral state in cells. The ability of HRSV to replicate in cells stably expressing MxA indicates that HRSV may instead be resistant to the activities of anti-viral proteins.

Further studies in human cells showed that HRSV was resistant to both endogenous and exogenous IFN but did not block either IFNα/β or IFNγ signalling or the formation of ISGF3 and GAF complexes, in contrast to other paramyxoviruses resistant to IFN such as SV5 and SeV (Young et al. 2000). Increased levels of STAT1 expression and phosphorylation were observed in HRSV-infected cells, suggesting that the stimulation of ISGs was intact in these cells which added further weight to the claims of Atreya & Kulkarni that HRSV is resistant to the effects of cellular anti-viral products. A recent publication has suggested that HRSV blocks IFNα/β signalling via the proteosome-mediated degradation of STAT2 (Ramaswamy et al. 2004), in much the same way as the rubulavirus hPIV2, but to date this is the only study to observe

such a phenomenon and it is possible that STAT2 degradation by HRSV is peculiar to the respiratory epithelial cells used in this study.

In the light of work with other paramyxoviruses, it seemed likely that HRSV and BRSV would use products of the viral P gene to evade the IFN response. However, none of the viruses in the *Pneumovirinae* sub-family encode more than a single product, the P protein, from the P gene. However, pneumoviruses encode two unique, non-structural genes, NS1 and NS2, which are found at the 3' end of the genome from where they are abundantly transcribed in infected cells and the use of reverse genetics techniques has created recombinant HRSV and BRSV lacking NS1 and NS2, indicating that they are accessory genes, dispensable for virus replication in cell culture (Buchholz et al. 1999; Teng and Collins 1999; Teng et al. 2000). NS1 has been shown to inhibit viral transcription and replication (Atreya et al. 1998) and interact with the HRSV M protein (Evans et al. 1996) and NS2 is required for optimal replication in cultured cells, to the extent that rHRSV with an artificial stop codon in the NS2 ORF reverted at high frequency to a form that could express NS2 (Teng and Collins 1999). Animal studies have shown that both rHRSV ΔNS1 and rHRSV ΔNS2 are highly attenuated in chimpanzees, suggesting a role for the NS proteins in *in vivo* pathogenesis (Teng and Collins 1999; Whitehead et al. 1999; Teng et al. 2000).

Work on BRSV showed that deletion of the NS1 and/or NS2 accessory genes gives BRSV an attenuated phenotype in cultured cells and that this was especially severe in MDBK bovine cells, which are optimal for the growth of wt BRSV (Schlender et al. 2000). The mutant viruses were less attenuated in Vero cells, but the addition of supernatants from infected MDBK cells impeded the growth of rBRSV ΔNS1/NS2 in Vero cells and this effect could be reversed by treatment of Vero cells with an antibody specific to the IFNα/β receptor, suggesting that rBRSV ΔNS1/NS2 was sensitive to IFN. Addition of IFNα/β to BRSV-infected cells decreased virus yield in a dose-dependent manner and the effect was most severe on the ΔNS mutants which all had equivalent IFN sensitivity, indicating that both NS1 and NS2 are involved in the resistance to IFN. It was also shown that BRSV is more resistant to the effects of IFNα/β in bovine cells than simian (Vero) cells, suggesting differences in IFN resistance in cells from different species. It was also demonstrated that expression of

NS1 and NS2 from recombinant Rabies viruses (RV) could protect RV from IFN, but only when both NS1 and NS2 were expressed together, showing that NS1 and NS2 can protect an unrelated virus from the effects of IFN.

As the NS proteins of BRSV and HRSV are closely related (69% identity for NS1 and 84% identity for NS2), the NS proteins of HRSV were tested in the rRV system for their ability to protect against the effects of IFN (Bossert and Conzelmann 2002). As found for BRSV, NS1 and NS2 of HRSV protected rRV from IFN in MDBK cells, but only when expressed together and the study also found that combinations of bovine and human NS proteins can protect against IFN, although not effectively as wt in all cases. However, when a rBRSV expressing HRSV NS1 and NS2 was engineered, it was found to replicate as well as wt BRSV in Vero and Hep2 cells but was highly attenuated and sensitive to IFN in bovine MDBK and Klu cells, suggesting that HRSV NS proteins can function in a BRSV background in human and simian cells, but not in bovine cells. It has also been observed that HRSV is sensitive to the effects of IFN in murine cells (Hanada et al. 1986), suggesting that the NS proteins of BRSV and HRSV are best adapted to counteract the IFN responses of their natural host and may act as determinants of viral host range.

A second study of BRSV NS mutants investigated the amounts of IFN produced in cells infected with rBRSV and showed that while rBRSV ΔNS1 is the least attenuated in bovine NT cells and infection does not produce significant amounts of IFNα/β, rBRSV ΔNS2 and ΔNS1/NS2 are both severely attenuated and cells infected with these viruses produce large amounts of IFNα/β (Valarcher et al. 2003). The same study showed that infection with rBRSV ΔNS2 stimulates the transcription of IFNβ mRNA whereas wt BRSV does not and suggested a specific role for NS2 in blocking the transcriptional activation of the IFNβ promoter. This appears to be a dominant effect as super-infection of cells infected with rBRSV ΔNS1/NS2 with either wt BRSV or rBRSV ΔNS1 virus resulted in a block of IFN production. A subsequent study confirmed the role of BRSV NS proteins in blocking the production of IFN and showed that infection with wt BRSV blocked the activation of the IFNβ promoter by both viral infection and poly(I):poly(C) whilst rBRSV ΔNS1/NS2 had no effect (Bossert et al. 2003).

A recent study using HRSV NS deletion mutants found that cells infected with these mutants had significantly higher levels of IFNα and IFNβ mRNA and increased secretion of IFNα/β, suggesting that HRSV NS proteins also have a role in blocking the production of IFN (Spann et al. 2004). Although the double ΔNS1/NS2 mutant had the most effect on IFN levels, HRSV ΔNS1 induced more IFN than ΔNS2 and ΔNS1/NS2 super-infection of cells infected with wt HRSV, ΔNS1 or ΔNS2 did not significantly increase IFN levels, suggesting that the NS proteins can block IFN induction either individually or in combination but, in contrast to BRSV, NS1 seems to be more important than NS2. This study also investigated the effects of NS protein deletion on the induction of IFNλ1 and IFNλ2/3, finding that similar to IFNα and IFNβ, IFNλ is induced by ΔNS1/NS2 infection and that the NS proteins may block the induction of IFNλ in wt HRSV infections.

To elucidate the mechanism of the BRSV NS-mediated block of IFN production, reporter assays using plasmids responsive to activated NFκB, AP1 and IRF3 were carried out and showed that wt BRSV but not rBRSV ΔNS1/NS2 blocked the activation of IRF3, but not NFκB or AP1 (Bossert et al. 2003). Studies of infected cell lysates showed that IRF3 was not phosphorylated in cells infected with BRSV, whereas normal IRF3 phosphorylation occurred in rBRSV ΔNS1/NS2 infected cells, indicating that BRSV blocks IFN production via a block of IRF3 phosphorylation and that this requires both NS1 and NS2, although NS2 alone was found to have some ability to block IFN production. This is similar, but distinct to part of the IFN evasion mechanism of the SV5 V protein, which inhibits IFN production by blocking the activation of both IRF3 and NFκB (Poole et al. 2002). It is not currently clear whether HRSV NS1 and NS2 proteins block IRF3 phosphorylation and there is some conflict in the literature as to whether IFN is produced during HRSV infections, with evidence both of IFN production during infections of human cells (Krilov et al. 1987; Garofalo et al. 1996; Spann et al. 2004) and a lack of IFN production in cultured cells and human infections (Hall et al. 1978; McIntosh 1978; Hall et al. 1981; Roberts et al. 1992). However, the evidence that BRSV and HRSV NS proteins are to some extent interchangeable suggests that their IFN evasion mechanisms will be similar.

AIMS

The aims of this project were to examine the abilities of a number of paramyxovirus V proteins to antagonise IFN signalling, in order to further investigate the different IFN evasion strategies used by viruses from this family and perhaps to discover novel IFN evasion mechanisms. It was also hoped that by gaining a better understanding of paramyxovirus IFN evasion strategies, further details of the cellular pathways involved in the anti-viral IFN response might be elucidated.

In addition, during the course of the project work in our laboratory showed that the V proteins of several paramyxoviruses, including SV5 and SeV, could suppress the production of IFNβ by blocking the activation of the IFNβ promoter in response to dsRNA. It was therefore of interest to investigate whether the V proteins of the paramyxoviruses in this study were also able to block IFNβ promoter activation and whether the mechanisms used were as varied as those used to antagonise IFN signalling.

A final aspect of this study was to investigate the role of IFN evasion in viral host range. Previous work in our group had suggested that paramyxoviruses whose V proteins were unable to antagonise IFN signalling in certain animal cells were also unable to successfully infect these cells, suggesting that the ability to evade the IFN response might be a determinant of viral host range. As all of the V proteins in this study were originally isolated from viruses in animal hosts or from humans during zoonotic paramyxovirus outbreaks, it was of interest to determine their ability to antagonise IFN signalling in a variety of cell types.

MATERIALS AND METHODS

I. DNA: CLONING AND ANALYSIS

Polymerase Chain Reaction

DNA was amplified using Taq polymerase (Promega UK), Vent polymerase (New England Biolabs (UK) Ltd.) or PfuI (produced in-house) as per manufacturers instructions, using a GeneAmp PCR System 2400 (Applied Biosystems).

Clean-up of DNA after PCR

DNA products were either cleaned up directly using a PCR purification kit (Qiagen Ltd., UK) as per manufacturers instructions or Sodium Iodide/Silica extraction (see below).

Agarose Gel Electrophoresis

Agarose gels were made by melting powdered agarose (Invitrogen) in TAE buffer (0.04M Tris-acetate, 0.001M EDTA, pH 8.0) to achieve the desired percentage, adding Ethidium Bromide to 0.125μg/ml and casting in a H1-set gel rig (Bioscience Services). Gels were run at a constant voltage of 70V in TAE buffer.

Sodium Iodide/Silica Purification of DNA from Agarose Gels

Silica suspension was prepared by adding 10ml PBS to 1g silica (SiO_2, Sigma-Aldrich Co., UK), which was allowed to settle, 2h, after which the s/n was removed. This was repeated, the silica pelleted by gentle centrifugation (2,000 x*g*, 2min) and all remaining PBS removed. The silica was resuspended in 10ml 3M NaI and stored in the dark.

To purify DNA, a slice containing the DNA band was excised from a TAE-agarose gel, weighed and 3 slice volumes 6M NaI was added. The slice was melted in a 55°C water bath for about 5min, 10μl silica suspension added and vortexed gently. The suspension was pelleted in microfuge (13,000 x*g*, 1min), the s/n removed and the pellet resuspended in 1ml wash buffer (50mM NaCl, 10mM Tris pH 7.5, 2.5mM EDTA, 50% Ethanol). This was pelleted in a microfuge as before, the s/n thoroughly removed and the pellet resuspended in 10-100μl TE to elute the DNA. After a short

incubation the silica was pelleted and the s/n containing DNA transferred to a clean tube

Restriction Enzyme Digests of DNA

i) Cloning

10μg vector DNA was mixed with 10U appropriate restriction enzyme(s) and buffer (Promega UK, New England Biolabs (UK) Ltd.) in a total volume of 50μl and incubated @37°C, 2-4h.

ii) Screening clones

5μl Miniprep DNA was incubated with 2U appropriate restriction enzyme(s) and buffer in a total volume of 20μl and incubated @37°C, 2-4h.

5' Phosphorylation of blunt-ended insert DNA

Purified DNA was added to a 50μl reaction containing 1 unit T4 Polynucleotide Kinase (Promega UK), Kinase buffer and 1mM ATP. Reaction incubated @37°C, 30min and cleaned up prior to ligation.

Dephosphorylation of linearised vector DNA

Typically 50μl digested plasmid DNA was added to a 100μl reaction containing 1U CIAP and CIAP buffer (Promega UK). Incubated @37°C, 30min for sticky ends or @37°C, 15min then @56°C, 15min for blunt ends. DNA cleaned up prior to ligation.

Ligation of DNA

Insert and vector DNA was prepared by digestion with appropriate restriction enzymes and mixed with T4 DNA Ligase and ligase buffer (New England Biolabs (UK) Ltd.) in a total reaction volume of 10μl. Reactions were incubated @16°C o/n. For rapid ligation the volume was increased to 20μl and reactions were incubated @RT, 10min.

Bacterial strains

Escherichia coli strains DH5α [ϕ80dlacZΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K-, m_K+), sup*E*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169] and JM109 [*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 (r_K-, m_K+), *relA*1, *supE*44, Δ(*lac-proAB*), [F′, *traD*36, *proAB*, *lacI*^{q}ZΔM15]]were used for cloning and BL21 [F-, *ompT*, *hsdS*$_B$, (r_B-, m_B-), *dcm*, *gal*, λ(DE3)] was used for expression.

Growth of bacteria

Bacterial cells were grown in liquid Luria-Bertani (LB) medium (10g/L bacto-tryptone, 5g/L yeast extract, 10mM NaCl, pH 7.0) or plated on solid LB medium (LB plus 1.5% (w/v) agar, 10mM $MgSO_4$).

Preparation of chemically competent cells

A single bacterial colony was inoculated from an LB agar plate into 2.5ml LB and incubated o/n @37˚C with shaking. The entire o/n culture was sub-cultured into 250ml LB supplemented with 20mM $MgSO_4$ in a 1L flask. Cells were incubated @37°C until A_{600} reached 0.4-0.6. Cells were pelleted by centrifugation @4,500 x*g*, 5min @4°C, gently resuspended in 100ml ice-cold TFB1 (30mM Potassium Acetate, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM RbCl, 15% Glycerol, pH 5.8 with 1M acetic acid, filter sterilised) and incubated on ice 5min. Cells were again pelleted by centrifugation @4,500 x*g*, 5min @4°C, gently resuspended in 10ml ice-cold TFB2 (10mM MOPS/PIPES, pH 6.5, 75mM $CaCl_2$, 10mM RbCl, 15% Glycerol. pH 6.5 with 1M KOH, filter sterilized) and incubated on ice 15-60min. Cells were aliquotted into pre-chilled microfuge tubes, 200μl per tube and snap frozen in liquid Nitrogen before storage @-70˚C.

Preparation of chemically competent cells – Inoue Method (Inoue et al. 1990)

10 colonies were picked from a freshly streaked LB agar plate into a 2 litre flask containing 250ml SOB and incubated @18°C with vigorous shaking until OD_{600} = 0.6 (up to 2 days). Cells were incubated on ice for 10min, pelleted by centrifugation @2,500 x*g*, 10min @4°C and resuspended in 80ml ice-cold TB (TB Solution 10mM PIPES, 15mM $CaCl_2$, 250mM KCl. pH 6.7 with KOH, added 55mM $MnCl_2$, filter sterilised). Cells were incubated on ice for 10min then pelleted by centrifugation

@2,500 x*g*, 10min @4°C and resuspended in 20ml ice-cold TB. 1.6ml DMSO was added immediately with gentle swirling and the cells were incubated on ice for a further 10min. Cells were aliquotted into pre-chilled microfuge tubes, 500μl per tube and snap frozen in liquid Nitrogen before storage @-70°C.

Transformation of DNA into chemically competent cells

100μl competent cells were incubated on ice until just thawed. DNA (in plasmid or ligated form) was added and the cells mixed gently and incubated on ice, 10-30min. Cells were then heat-shocked in a 42°C water bath, 90s and replaced on ice, 5min. 400μl SOC was added and the cells recovered @37°C, 1h with shaking. Cells were then plated onto L-Agar plates with appropriate antibiotic and incubated @37°C o/n.

DNA Minipreps

Plasmids minipreps were prepared either using QIAprep Spin Miniprep kits (Qiagen Ltd., UK) as per manufacturers instructions or by Alkaline Lysis (see below).

i) Alkaline Lysis Minipreps

Single bacterial colonies were inoculated into 5ml LB with appropriate antibiotic and grown o/n with shaking @37°C. 3ml of each o/n culture was transferred to a microfuge tube and pelleted @13,000 x*g*, 2min. Cell pellets were resuspended in 200µl Solution I (50mM Glucose, 25mM Tris-HCl, pH 8.0, 10mM EDTA, pH 8.0) and cells were lysed by the addition of 200µl Solution II (0.2M NaOH, 1% SDS). Cell debris and chromosomal DNA was precipitated by the addition of 200µl Solution III (3M Potassium Acetate, 115ml/L glacial Acetic Acid) and mixing by inversion. Debris was pelleted by centrifugation @13,000 x*g*, 10min and the cleared s/n was transferred to a fresh microfuge tube. Plasmid DNA was precipitated by the addition of 1ml 100% Ethanol and incubation @-20°C for 30'. DNA was pelleted by centrifugation @13,000 x*g* 10min and the s/n carefully aspirated off. DNA pellets were washed with 200µl 70% Ethanol, allowed to dry completely and resuspended in 50µl TE-RNase (20μg/ml, Sigma-Aldrich Co., UK).

DNA Maxipreps

Plasmid maxipreps were prepared using QIAfilter Plasmid Maxi kits (Qiagen Ltd., UK) as per manufacturers instructions.

DNA Sequencing

DNA was sequenced using appropriate oligonucleotide primers and an ABI Prism® 377 DNA sequencer (Applied Biosystems). Sequence analysis was carried out by Alex Houston at the DNA Sequencing Unit, University of St Andrews.

II. CELL CULTURE

Cell lines

Cultured cells of human, simian and murine origin were used in this study and are described below.

i) Human cells

2fTGH (Pellegrini et al. 1989; McKendry et al. 1991): human fibroblast cells. Provided by Dr. S. Goodbourn, St George's Hospital Medical School, London, UK

HeLaE: human transformed cell line, derived from cervical carcinoma cells. Provided by Dr. S. Goodbourn, St George's Hospital Medical School, London, UK

293: human embryo kidney cells, transformed with sheared human Ad5 DNA. Provided by Prof. R.T. Hay, University of St Andrews, UK

ii) Simian cells

Vero: simian fibroblast-like cells, originally from kidney cells of African Green Monkey. Obtained from ICN Pharmaceuticals Ltd., UK

iii) Murine cells

BF: murine fibroblast cells, cloned from a primary BALB/c mouse embryo cell line. Produce non-functional Mx proteins due to large deletion in Mx gene (Staeheli et al. 1988).

Maintenance of cultured cells

Cultured cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Ltd., UK) supplemented with 10% foetal calf serum (HeLaE, BF) or 2% foetal calf serum + 8% newborn calf serum (2fTGH, 293). Cells were routinely passaged, trypsinised (Trypsin, EDTA; Becton Dickinson UK Ltd.) and diluted according to growth rate of the cell line.

Freezing of cultured cells

Adherent cells were trypsinised, resuspended in a small amount of normal growth medium, pelleted @400 x*g*, 5min and resuspended in the required volume of ice-cold freezing medium (5ml for a confluent 25cm^2 flask). The resulting cell suspension was aliquotted into the required number of pre-labelled, sterile cryovials (1ml per vial) making sure that the lids were firmly screwed on. The vials were placed in a polystyrene box and left at -70°C overnight before being transferred to liquid nitrogen for long-term storage.

Resuscitation of cultured cells

Cryovials were removed from liquid nitrogen and the cells rapidly thawed in a 37°C water bath. The vials were centrifuged @250 x*g*, 5min to pellet the cells, the supernatant was aspirated and the cell pellet resuspended in 1ml normal growth medium. The cells were transferred to a flask or plate containing a generous amount of growth medium and allowed to grow at 37°C. The medium was changed on the following day to remove any traces of DMSO.

Transient transfection of mammalian cells

Adherent mammalian cells were transfected with DNA 24h after trypsinisation and seeding using either Fugene™ 6 (Roche Diagnostics, UK) or Lipofectamine™ (Invitrogen) as per manufacturers instructions.

Preparation of stable cell lines

Mammalian cells in 75cm^2 flasks were transfected with a pEF.IRES.neo DNA construct containing the gene of interest under the control of a constitutively active promoter and the neomycin resistance gene under the control of an IRES. 24h post-

transfection the cells were trypsinised and plated into 60cm dishes at varying dilutions. The medium was then supplemented with the antibiotic G418 (Melford, UK) and the cells incubated, with regular changes of antibiotic-containing medium, for about 14 days until resistant colonies formed. Individual colonies were picked, amplified and screened for the expression of the gene of interest.

III. INTERFERON ASSAYS

Reporter plasmids

All of the reporters described below were provided by Dr S. Goodbourn, St George's Medical School, London, UK.

i) IFNα/β-responsive plasmid

p(9-27)4tkΔ(-39)lucter (King and Goodbourn 1998). Contains a minimal tk promoter and four tandem repeats of the ISRE from the IFN-inducible gene 9-27, fused to –17 of the firefly luciferase gene (de Wet et al. 1987).

ii) IFNγ-responsive plasmid

p(GAS)2tkΔ(-39)lucter (King and Goodbourn 1998). Contains a minimal tk promoter and two tandem repeats of the IRF1 GAS site, fused to –17 of the firefly luciferase gene.

iii) dsRNA-responsive IFNβ promoter plasmid

pIF(-116/+72)lucter (King and Goodbourn 1992). Contains human IFNβ sequence from –116 to +72, fused to –17 of the firefly luciferase gene.

iv) β-gal plasmid

pJATlacZ (Masson et al. 1992). Contains the β-galactosidase gene under the control of the rat β-actin promoter. Used as a transfection standard in IFN assays as known not to be affected by IFN.

Transfection of luciferase reporters

Cells were seeded in 6 well plates and once 50% confluent were transfected with three plasmids; the appropriate luciferase reporter, β-gal control and V expression construct (or empty expression vector). DNA solutions were first made up in serum-free DMEM and 0.7μg each of the luciferase reporter and β-gal plasmid and 0.95μg expression plasmid was mixed to a total volume of 127μl. This DNA mix was added to 127μl 8% Lipofectamine™ in serum-free DMEM, incubated 45min and added dropwise to 0.5ml serum-free DMEM in each of two wells of the 6-well plate (previously washed twice with 4ml serum-free DMEM). After 6h, 4ml 10%-FCS DMEM was added to each well and the cells incubated @37°C.

Stimulation of IFN-responsive reporters with IFN

48h post transfection, medium was removed from transfected cells and replaced with appropriate growth medium containing either human IFNα (1.8×10^4 IU/ml; Roferon®-A, Roche Diagnostics, UK), human IFNγ (1.0×10^4 IU/ml; R&D Systems, UK. Cat. #285-IF) or 'Universal Type I IFN' (1.0×10^4 IU/ml; PBL Biomedical Labs, New Brunswick, USA. Cat. #11200) and incubated @37°C, 4h.

Induction of β-IFN Promoter with dsRNA

dsRNA in the form of poly(I):poly(C) (Amersham Pharmacia Biotech, UK) at 1mg/ml was heated @55°C to denature any secondary structure and then diluted to 20μg/ml in serum-free DMEM, 125μl per well to be stimulated. A solution of 8% Lipofectamine™ in serum-free DMEM was prepared, sufficient for 125μl per well, combined with the poly(I):poly(C) solution and incubated @RT for at least 15min. Meanwhile, transfected cell monolayers in 6-well plates were washed twice with 4ml serum-free DMEM and 1ml fresh serum-free DMEM was added to each well. 250μl poly(I):poly(C)-Lipofectamine™ mix was added to each well to be stimulated and 250μl serum-free DMEM alone was added to unstimulated wells and the cells were incubated @37°C, 9-12h.

Luciferase Assay

Cell monolayers in 6 well plates were gently washed with 5ml PBS and after careful aspiration of this wash, 200µl Luciferase Buffer A (25mM Tris phosphate pH 7.8, 8mM $MgCl_2$, 1mM DTT, 1mM EDTA, 1% Triton X-100) was added to each well, distributed by gentle swirling and incubated @RT, 2mins to lyse cells. 200µl Luciferase Buffer B (30% glycerol, 0.8mM ATP, 2% BSA in Luciferase Buffer A) was added to each well and mixed immediately by gentle swirling. Lysates were transferred to 1.5ml microfuge tubes and centrifuged @13K rpm, 30s to pellet debris. 300µl of each cleared s/n was transferred to a clean luminometer tube and loaded into either a Lumat LB9501 or Sirius luminometer (Berthold Detection Systems GmbH, Germany). 100µl 0.6mM luciferin (D-luciferin, sodium salt, Molecular Probes Inc.) was injected and relative light units measured. After taking the luminometer reading, samples were retained for a β-galactosidase assay.

β-Galactosidase Assay

800µl lacZ buffer (60mM $Na_2HP_2O.7H_2O$, 40mM $NaH_2PO_4.H_2O$, 10mM KCl, 1mM $MgSO_4.7H_2O$) and 200µl ONPG (4 mg/ml in 0.1M Sodium phosphate buffer, pH 7.5) were added to each luciferase reaction including the negative control. The reactions were incubated @37°C until lemon yellow, transferred to cuvettes and A_{420} measured vs. the control using a spectrophotometer. If reactions were very fast (<2h) they were stopped by the addition of 500µl 1M Na_2CO_3 prior to measurement.

IV. PROTEIN PREPARATION AND ANALYSIS

Antibodies

A number of different primary and secondary antibodies were used for Indirect Immunofluorescence, Western blots and Immune Precipitations and are described below.

i) Epitope tag Antibodies

Anti c-myc mouse MAb (produced in-house from the hybridoma cell line 9E10)
Anti-FLAG mouse MAb (Anti-FLAG® M2, Sigma-Aldrich Co. Ltd., UK. Cat #F3165)

ii) STAT Antibodies

Anti-STAT1 mouse N-terminal MAb (BD Transduction Laboratories, Europe. Cat #61015)

Anti-STAT1 rabbit PAb (BD Transduction Laboratories, Europe. Cat #610119)

Anti-STAT2 rabbit C-terminal PAb (anti-STAT2 (C20), Santa Cruz Biotechnology, Inc., USA. Cat #sc-476)

Anti-STAT3 mouse MAb (Transduction Laboratories, Cat #S21320)

iii) Other Primary Antibodies

Anti-IRF9/p48 rabbit PAb (obtained from Dr S.Goodbourn, St George's Hospital Medical School, London, UK).

Anti-SV5 V mouse MAb (anti-SV5-Pk, produced in-house from a hybridoma cell line)

iv) Secondary Antibodies

Anti-mouse IgG HRP linked Ab (Amersham Biosciences UK Ltd. Cat #NA9310)

Anti-rabbit IgG HRP linked Ab (Amersham Biosciences UK Ltd. Cat #NA934)

Anti-mouse Ig Texas Red conjugated Ab (Oxford Biotechnology Ltd., UK. Cat #1010-7)

Anti-mouse Ig FITC conjugated Ab (Oxford Biotechnology Ltd., UK. Cat #1010-02)

Anti-rabbit Ig Texas Red conjugated Ab (Oxford Biotechnology Ltd., UK. Cat #4010-07)

Anti-rabbit Ig FITC conjugated Ab (Oxford Biotechnology Ltd., UK. Cat #4010-02)

Immunofluoresence

Cells were seeded on 10mm coverslips and at a suitable time, harvested and fixed in fixing solution (5% formaldehyde, 2% sucrose in PBS), 10min. Cells were then permeabilised in permeabilisation buffer (0.5% IGEPAL, 10% sucrose in PBS), 5min and washed in 1% calf serum PBS. To detect the proteins of interest, cells were incubated with 20μl appropriate primary antibody, diluted in antibody dilution buffer (1% calf serum, 2% sucrose, 0.1% (w/v) Sodium azide in PBS), at least 1h @RT. Cells were subsequently washed with 1% calf serum PBS and incubated with 20μl appropriate secondary antibody with 0.5μg/ml DAPI (Sigma-Aldrich Co Ltd., UK),

1h @RT. Cells were then washed with 1% calf serum PBS, water and mounted on slides using Citifluor AF-1 mounting solution (Citifluor Ltd., UK). Slides were examined using a Nikon Microphot-FXA microscope.

Immune precipitation from transfected cell lysates

Cells were seeded in 60mm dishes and once 50% confluent were transfected with V expression constructs using Fugene™ 6 (Roche Diagnostics, UK), as per manufacturers instructions. 48h post transfection the cells were washed with 5ml ice-cold PBS and then lysed in 250μl Immune Precipitation Buffer (IPB; 10mM Tris-HCl pH 7.8, 5mM EDTA, 0.5% IGEPAL, 0.65M NaCl) plus protease inhibitors (Complete Mini EDTA-free Protease Inhibitor tablets, Roche Diagnostics Ltd., UK). Lysates were then centrifuged @13K rpm, 10min in a microfuge to pellet cell debris and the cleared s/n (soluble antigen) was transferred into clean microcentrifuge tubes. A suitable antibody was added to the soluble antigen and incubated 1h, on ice, after which Protein A Sepharose (Sigma-Aldrich Co. Ltd., UK) was added and incubated o/n @4°C, with mixing. The samples were then pelleted and the pellets washed with 1ml IPB, seven times. After the last wash, all s/n was removed and the beads resuspended in sample buffer (0.05M Tris-HCl pH 7.0, 0.2% SDS, 5% 2-mercaptoethanol, 5% glycerol), boiled and separated by SDS-PAGE.

Immune precipitation from mixtures of in vitro transcribed and translated proteins

Proteins were *in vitro* transcribed, translated and labelled with ^{35}S-Met using a TNT® T7 Coupled Reticulocyte Lysate System (Promega UK). These proteins were mixed together and incubated on ice, 30min. A suitable antibody was then added to the mixture and incubated on ice, 30min. Protein A Sepharose (Sigma-Aldrich Co. Ltd., UK) in 100μl IPB was then added and the samples incubated 30min @4°C, with mixing. The samples were then pelleted and the beads washed with 1ml IPB, seven times. After the last wash, all s/n was removed and the beads resuspended in sample buffer, boiled and separated by SDS-PAGE.

SDS-PAGE

Protein samples were prepared in sample buffer and boiled, 5min prior to electrophoresis. Proteins were separated using 10% SDS-PAGs in the Bio-Rad mini-protean II electrophoresis system (Bio-Rad, UK) at 150V, until maximum resolution of polypeptide bands was achieved.

Western Blots

Samples separated by SDS-PAGE were transferred to a PVDF membrane (?) using the Bio-Rad Trans-Blot Cell (Bio-Rad, UK) with the unit submerged in transfer buffer (0.025mM Tris, 0.19M Glycine, 20% (v/v) methanol) at either 40mA o/n or 200mA, 2h. Following transfer, the membrane was incubated in blocking buffer (5% (w/v) skimmed milk powder in TN buffer (10mM Tris-HCl, pH 8.0, 150mM NaCl), 1h @RT, to block non-specific protein binding sites. Proteins were then detected using appropriate specific antibodies, diluted in blocking buffer and incubated for at least 1h @RT or o/n @4°C. The membrane was then washed (3x 10min) in TNT buffer (0.1% Tween-20 in TN buffer). The membrane was then incubated in blocking buffer with an appropriate HRP-conjugated secondary antibody, 1h @RT. The membrane was then washed as before and the protein bands visualised by enhanced chemiluminescence (ECL; Amersham Biosciences UK Ltd.) according to the manufacturers instructions.

Expression of GST-fusion proteins

GST-fusion constructs were transformed into an appropriate *E.coli* expression strain (e.g. BL21, B834) and a single colony was inoculated into 10ml LB with an appropriate antibiotic and incubated @37°C o/n. Cells were subcultured at 1:100 into a larger volume (e.g. 10ml for test expression, 2L for large scale prep [500ml in each of 4 flasks]) and incubated @37°C with shaking until $OD_{600} \approx 0.5$. Cells were induced with 1mM IPTG and incubated @30°C with shaking, 3h. Cells were harvested by centrifugation, @4,500 x*g*, 10min @4°C and cell pellets were resuspended in Cell Resuspension Buffer (50mM Tris-HCl, pH 8.0, 200mM NaCl + 1mM DTT) plus protease inhibitors (Complete Mini EDTA-free Protease Inhibitor tablets, Roche Diagnostics Ltd., UK). 500µl was used for a 10ml test culture, 7ml for 1L and 14ml for 2L culture. Cells were broken using either a French press (Cell Disruption

Systems, UK) for large volumes or sonication (10 amplicons, 3x 30s, on ice) for smaller volumes. Lysates were spun briefly @1,600 x*g*, 2min to reduce foaming, then cell debris was pelleted by ultracentrifugation @30K rpm, 30min. The s/n (soluble protein) was carefully removed, filtered through a 0.45μm filter and stored on ice prior to further purification.

Glutathione-Agarose Preparation

To prepare 1ml glutathione-agarose beads, 82.8mg glutathione-agarose (Sigma-Aldrich Co., UK) were added to 16ml sterile dH_2O and allowed to swell for 30min @RT. The beads were washed in 10ml sterile water, pelleted and resuspended in 2ml water to give a 50% suspension. Glutathione-agarose columns were prepared by loading a suitable amount of 50% bead suspension onto a 2ml disposable column (Pierce Biotechnology Inc.) allowing to settle and washing with ten column volumes of an appropriate buffer.

Purification of GST-V

Soluble total protein from bacterial expression (see Expression of GST-Fusion Proteins) was applied to Glutathione-agarose column, the flow through collected and reapplied to the column to maximise binding. The column was washed with ten column volumes CR buffer (50mM Tris-HCl, pH 8.0, 200mM NaCl), until no more protein was detected in the flow through (tested by staining 2µl of flow through with Naphthalene black on nitrocellulose). GST-V was eluted from the column with freshly made 10mM Glutathione in 1ml fractions until no more protein was detected in the eluate. The peak fractions were identified using Naphthalene black staining, pooled and dialysed o/n into 2L CR buffer to remove excess glutathione. The resulting protein solution was quantified on an SDS-PAG vs. standard dilutions of BSA after which 10% glycerol was then added and the protein stored @-70°C.

GST Pulldown of in vitro transcribed/translated proteins

GST fusion proteins (prepared as per method above) were mixed with 10µl 50% suspension of glutathione-agarose for 30min @4°C. The beads were pelleted by centrifugation @6,000 x*g*, 30s and washed three times with 1ml CR buffer (50mM Tris-HCl, pH 8.0, 200mM NaCl). ^{35}S-Met labelled *in vitro* transcribed/translated

proteins were made using a TNT® T7 Coupled Reticulocyte Lysate System (Promega UK) with 0.5µg DNA and 10µCi ^{35}S-Met in a total volume of 25µl. The reactions were incubated @30°C, 90min. For each pulldown, 5-10µl labelled protein was diluted in 1ml CR buffer (50mM Tris-HCl, pH 8.0, 200mM NaCl) + 0.1% Tween-20 and 1% BSA. 10µl of the pre-bound GST-fusion glutathione-agarose beads was added and mixed @4°C, 30min with tumbling. The beads were pelleted by centrifugation @6,000 x*g*, 30s and washed five times with 1ml CR buffer + 0.1% Tween-20. The s/n was removed and the beads resuspended in 30μl 4x Disruption buffer, boiled and loaded onto an SDS-PAG with 1μl labelled protein as a positive control. After separation, the gel was stained, dried and exposed to a phosphorimager plate o/n.

CLONING STRATEGIES

All of the experiments detailed in the Results section depend on the use of cloned V proteins, both full length and truncated, tagged and fused to GST, under the control of various promoters. All of these clones were constructed from scratch for the purposes of this study and their construction was a major part of the work described. The individual cloning strategies are detailed in the following section.

V expression construct cloning

In order to study the ability of the various V proteins to block IFN signalling it was necessary to clone them into a suitable mammalian expression vector and tag them with a suitable epitope tag, as there were no specific V MAbs available. The following section describes the vectors and cloning strategies used to create the N-terminal myc tagged V constructs used to study IFN antagonism.

The basic mammalian expression vector used in the laboratory is pEF.plink2, shown in Fig. 10, which contains the EF-1α promoter driving high-level constitutive expression of proteins cloned into the MCS between the UTRs from the human β-globin gene and can be selected using ampicillin. A derivative of this vector, pEF.myc.plink2, shown in Fig. 11, has a myc epitope tag directly upstream of the MCS in frame with the NcoI site and can be used to express proteins with an N-terminal myc tag. A second derivative of pEF.plink2 is pEF.IRES.neo, shown in Fig. 12, which in addition to the ampicillin resistance gene contains a neomycin resistance gene (Neo^R) under the control of a poliovirus IRES. This IRES is positioned downstream of the multiple cloning site (MCS) and allows translation of both the protein cloned into the MCS and the downstream Neo^R gene from the bi-cistronic mRNA generated by transcription from the EF-1α promoter. Thus in cells expressing the protein cloned into the MCS, the Neo^R gene should also be expressed, making pEF.IRES.neo ideal for the creation of stable cell lines.

The initial strategy for cloning the various V proteins was to clone into pEF.IRES.neo so that both transient and stable expression could be achieved using the same vector. This strategy was used for the initial V proteins that were cloned, but later V proteins were cloned into pEF.myc.plink2 as this was a much simpler strategy.

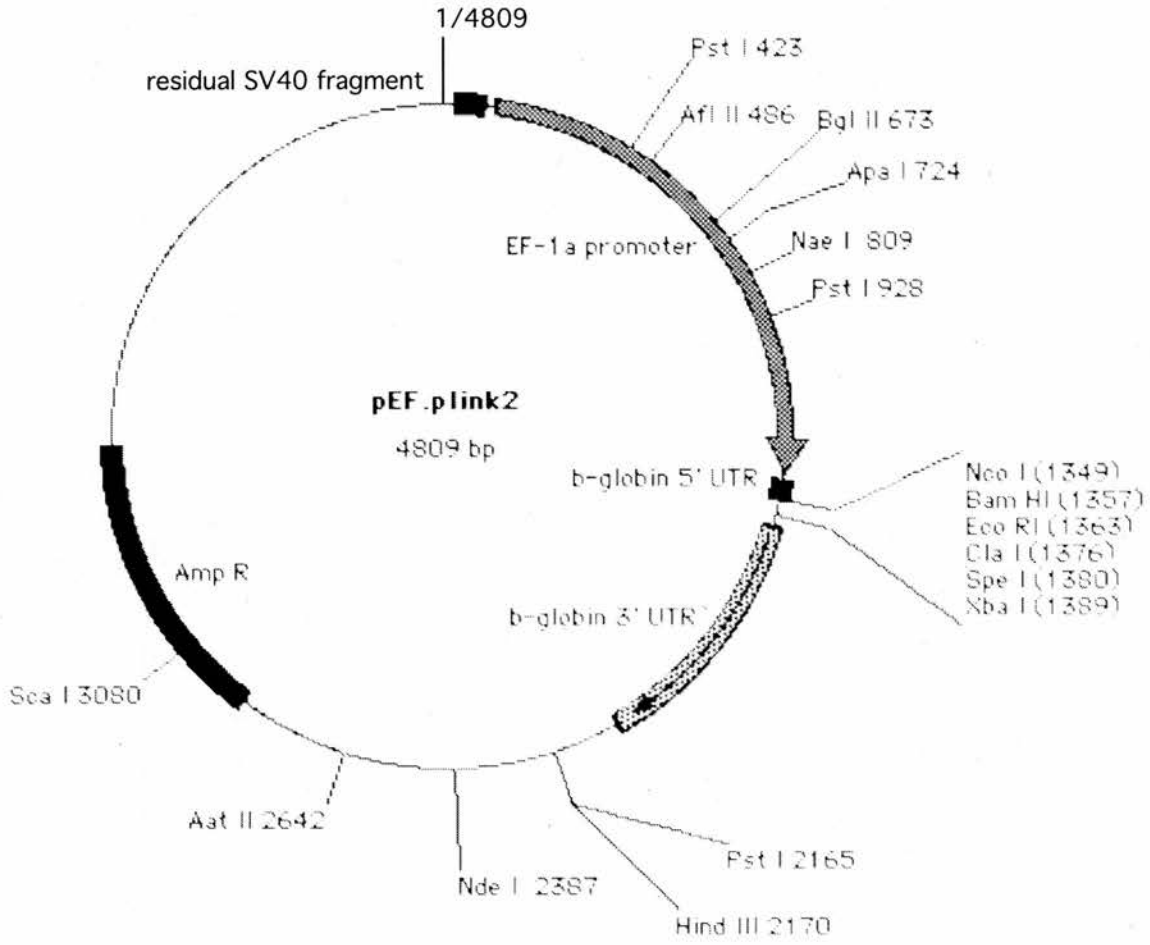


Figure 10: A schematic representation of the pEF.plink2 mammalian expresssion vector

Vector diagram of pEF.plink2 expression vector showing approximate positions of EF-1α promoter, multiple cloning site and ampicillin resistance gene. Unique restriction sites are shown with their nucleotide posistion in the vector relative to the origin.

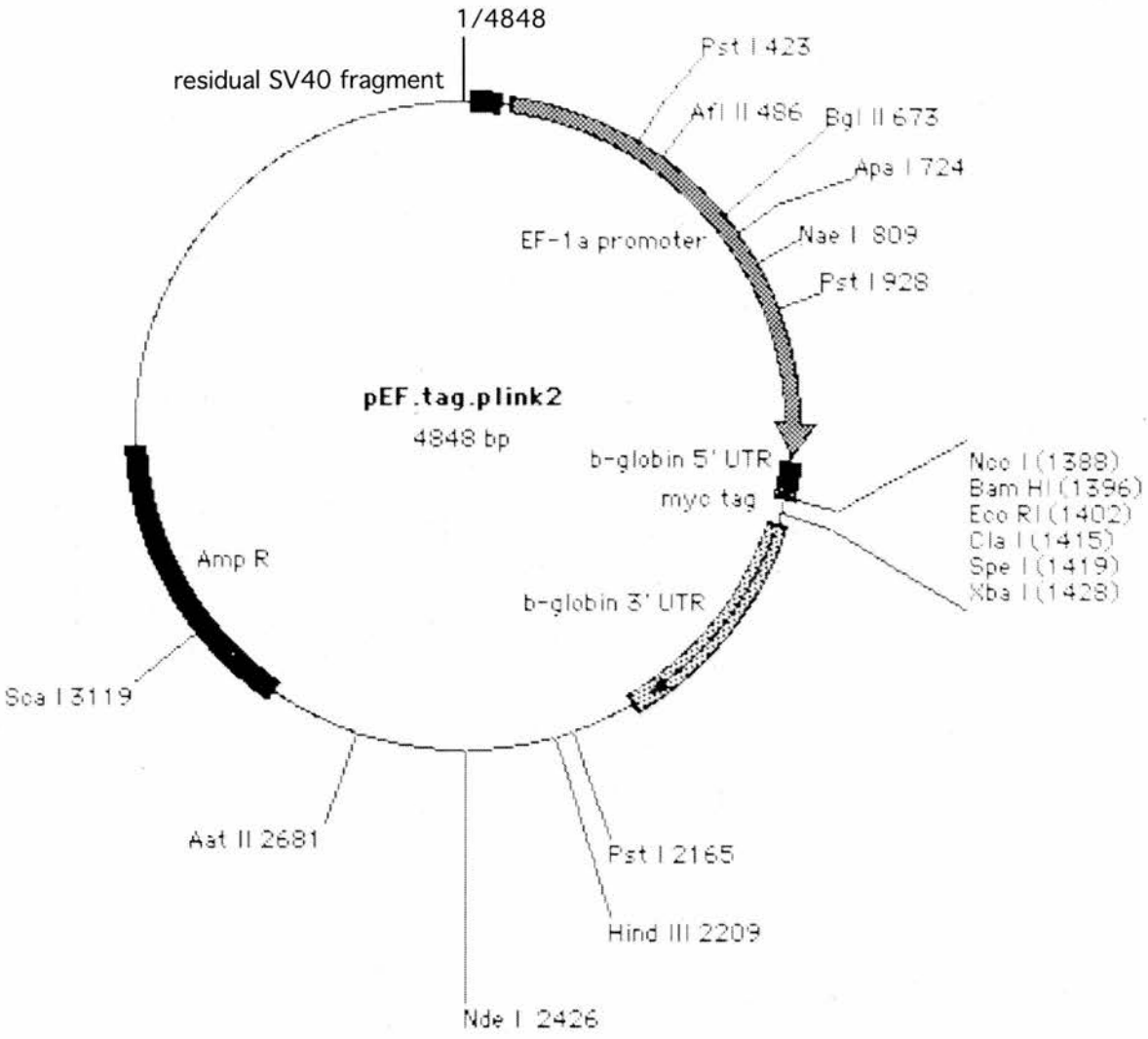


Figure 11: A schematic representation of the pEF.myc.plink2 mammalian expresssion vector

Vector diagram of pEF.myc.plink2 expression vector showing approximate positions of the EF-1α promoter, myc epitope tag, multiple cloning site and ampicillin resistance gene. Unique restriction sites are shown with their nucleotide posistion in the vector relative to the origin.

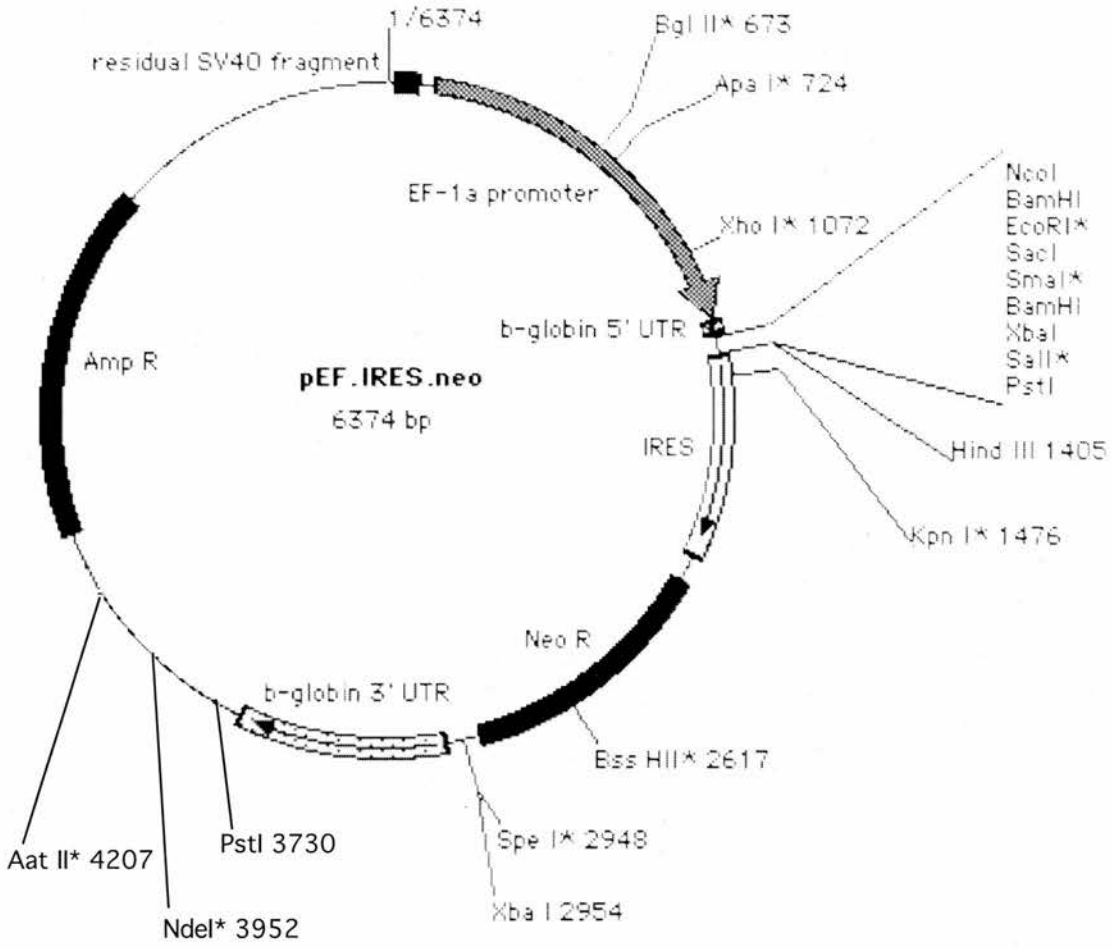


Figure 12: Schematic diagram of pEF.IRES.neo mammalian expression construct

Vector diagram of pEF.IRES.neo expression vector showing approximate positions of the EF-1α promoter, multiple cloning site, IRES element, neomycin and ampicillin resistance genes. Restriction sites within the vector are shown, but only those marked with an asterisk are unique.

V ORFs were amplified from V or P templates using PCR with a proofreading polymerase to preserve the fidelity of the DNA sequence. Specific forward and reverse primers were designed for each V protein, the forward primer incorporating a 5´ *NcoI* restriction endonuclease site around the ATG codon used for translational initiation of V and the reverse primer incorporating a 3´ *XbaI* restriction endonuclease site downstream of the V stop codon. After the initial PCR amplification, a variety of cloning strategies were used to create the final V expression constructs, the full details of which are described in the following section.

i) Nipah V Geelong

The NiV V DNA was provided as a P ORF in the baculovirus vector pFAST-Bac-Hta. Mutagenic PCR from this template using 'NiV For' and 'NiV Rev' and internal 'G add For' and 'G add Rev' primers was used to insert an additional G residue at the RNA editing site, as shown in Fig. 13, generating a blunt-ended PCR product containing the NiV V ORF. This PCR product was then cloned into an intermediate vector, pGEX4T (linearised with a *SmaI* digest to create blunt ends for cloning). This intermediate NiV V construct was then digested with *NcoI/XbaI* to create a NiV V fragment for the final cloning step, which involved a 3-way ligation.

For this ligation, two vector fragments were required and these were prepared as follows; the pEF.myc.plink2 vector was digested with *NdeI/NcoI* to create a fragment containing the ampicillin resistance gene, EF-1α promoter, β-globin 5' UTR and myc tag and the pEF.IRES.neo vector was digested with *XbaI* (partial)/*NdeI* to create a fragment containing the IRES, neomycin resistance gene and β-globin 3' UTR (see Fig. 14 for an illustration of these fragments). These two vector fragments were ligated with the *NcoI/XbaI* digested NiV V fragment to create pEF.myc.NiV-V-Geelong.IRES.neo.

After cloning NiV V, the 3-way ligation process was modified as it was very inefficient. The pEF.myc.plink2 and pEF.IRES.neo were first digested with *NcoI* or *XbaI*(partial) respectively and these cut ends were dephosphorylated, after which both vectors were digested with *NdeI*, creating the same fragments as shown in Fig. 14.

Figure 13: Schematic representation of mutagenic PCR protocol

Panel A shows the first round of mutagenic PCR, which uses the plasmid DNA to be mutagenised as the template in two separate PCR reactions with four different primers. The two external primers ('For' and 'Rev') are partially complementary to the ends of the V ORF and also incorporate restriction endonuclease recognition sites (*NcoI* in 'For' primer and *XbaI* in 'Rev' primer) and the two internal primers ('mut F' and 'mut R') are completely complementary to each other and the V sequence, except at the nucleotide to be mutated where an alternative or additional base is added to the primer sequence. Two separate reactions are set up, one with the external 'For' primer and internal 'mut R' primer and the other with the internal 'mut F' primer and external 'Rev' primer. The resulting two PCR products contain the desired nucleotide change or addition and although they are not full-length compared to the template, they do cover the whole of the template sequence between them, with a small overlap.

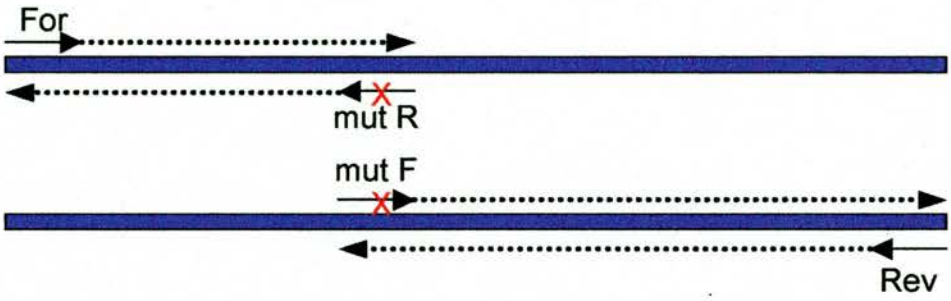
Panel B shows the use of *DpnI*, a restriction enzyme which cuts only methylated DNA, to remove the template DNA from the PCR reaction. Treatment of the completed first round PCR reactions with this enzyme leads to the degradation of the methylated, plasmid-derived template DNA but not the newly made mutant PCR products, so removing any possibility of contamination of the next round of PCR with wt template DNA. The PCR products are then cleaned up and used in the second round of PCR.

Panel C shows the second round PCR reaction, which uses the two slightly overlapping, mutated PCR products from the first round of PCR as the template for a second PCR reaction using only the external 'For' and 'Rev' primers. This reaction generates a full length copy of the original template, incorporating the mutation or nucleotide addition.

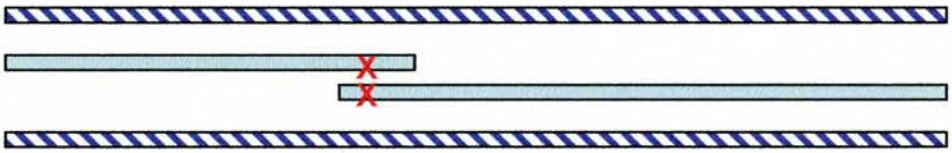
Panel D shows the full length mutant product after clean up of the second round PCR reaction to remove the truncated first round PCR products.

N.B. All PCR steps must be carried out with a proofreading DNA polymerase to prevent the introduction of random, unwanted mutations.

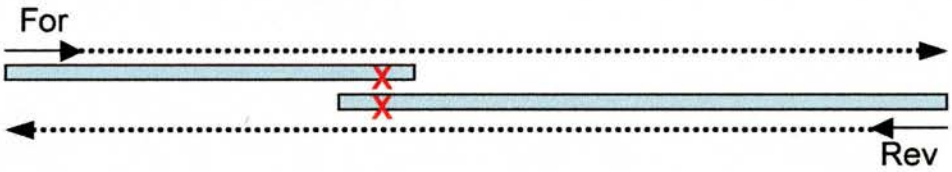
A First round PCR reactions



B *Dpn I* treatment of first round PCR reactions



C Second round PCR reaction



D Final full-length product including mutation

Key

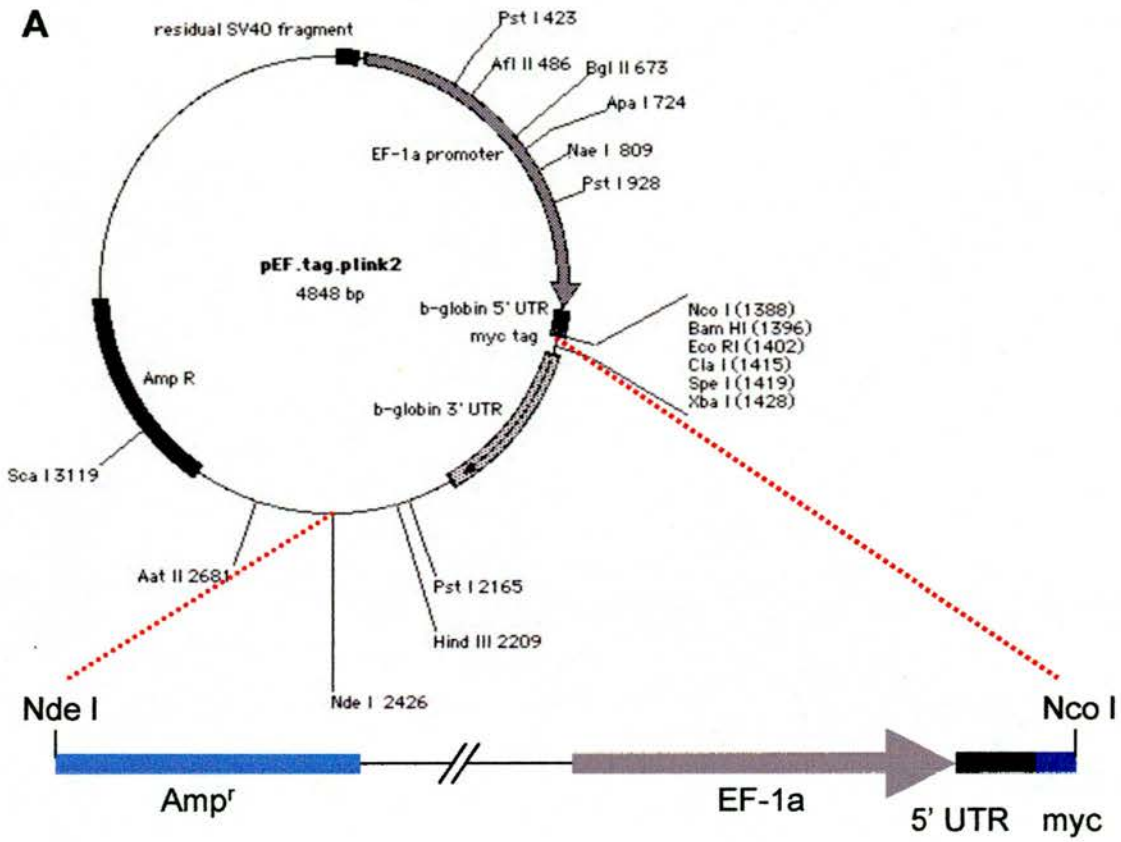
- Plasmid DNA template
- Degraded plasmid DNA template
- First round PCR products
- Full length second round PCR product
- X Mutated or additional nucleotide

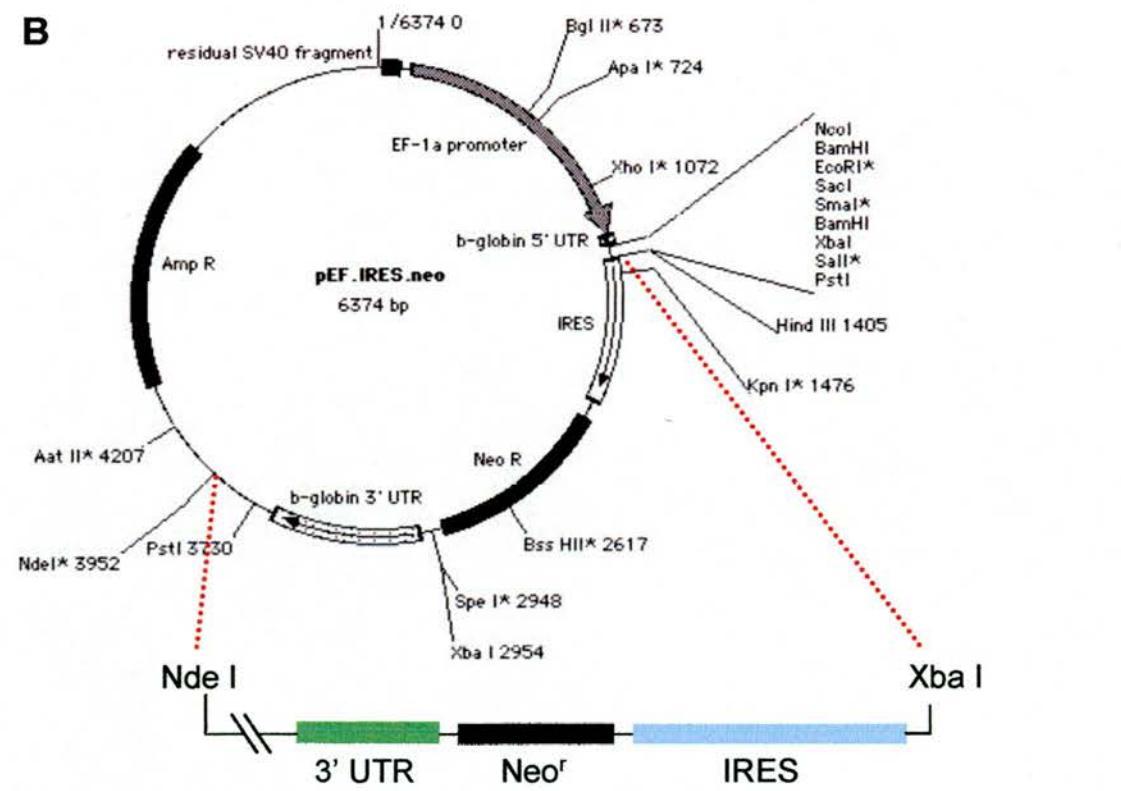
Figure 14: Schematic representation of vector fragments generated for 3-way cloning

Panel A shows pEF.myc.plink2 vector digested with NcoI/NdeI to generate the first part of the vector backbone for 3-way cloning, including the ampicillin resistance gene, EF-1α promoter, 5' UTR and myc epitope tag.

Panel B shows pEF.IRES.neo digested with XbaI(partial)/NdeI to create the other part of the vector backbone for 3-way cloning, including the IRES element, neomycin resistance gene and 3' UTR.

The initial cloning strategy combined these two vector fragments with the V ORF fragments in a 3-way ligation. However, this was fairly inefficient so later cloning strategies used the same two vector fragments but pre-ligated them together and then used them in a conventional 2-way ligation with the V protein fragments.


A
residual SV40 fragment
Pst I 423
Afl II 486
Bgl II 673
Apa I 724
EF-1a promoter
Nae I 809
Pst I 928
pEF.tag.plink2
4848 bp
b-globin 5' UTR
myc tag
Nco I (1388)
Bam HI (1396)
Eco RI (1402)
Cla I (1415)
Spe I (1419)
Xba I (1428)
Amp R
b-globin 3' UTR
Sca I 3119
Aat II 2681
Pst I 2165
Hind III 2209
Nde I 2426
Nde I
Nco I
Amp^r
EF-1a
5' UTR
myc


B
1/6374 0
residual SV40 fragment
Bgl II* 673
Apa I* 724
EF-1a promoter
Xho I* 1072
NcoI
BamHI
EcoRI*
SacI
SmaI*
BamHI
XbaI
SalI*
PstI
b-globin 5' UTR
Amp R
pEF.IRES.neo
6374 bp
IRES
Hind III 1405
Kpn I* 1476
Aat II* 4207
Neo R
b-globin 3' UTR
NdeI* 3952
PstI 3730
Bss HII* 2617
Spe I* 2948
Xba I 2954
Nde I
Xba I
3' UTR
Neo^r
IRES

These vector fragments were purified and ligated via their compatible *NdeI* restricted ends and after ligation the heterodimer products were purified. This was possible as the two fragments were of different sizes so homodimers and heterodimers of the two fragments could be differentiated. The new vector backbone, pEF.myc.IRES.neo, created by this ligation was then combined in a conventional 2-way ligation with the *NcoI/XbaI* digested V inserts.

ii) Porcine Rubulavirus V

PoRV V DNA was provided as a V gene PCR fragment and PCR from this template using LPMV For and LPMV Rev primers created a blunt-ended fragment, which was cloned into pCRBluntII-Topo. The *XbaI* site at the 3' end of PoRV was blocked by methylation but the cloning vector contained an *XbaI* site downstream of the MCS so this was used instead. Also, as PoRV V contains an internal *NcoI* site, a complete *XbaI* digest followed by a partial *NcoI* digest was used to excise the complete V ORF. This fragment was then ligated with the pEF.myc.IRES.neo backbone described above, to create pEF.myc.PoRV-V.IRES.neo.

iii) Salem V

Sal V DNA was provided as V ORF in a plasmid vector and PCR from this template with SalV For and SalV Rev primers created a blunt-ended fragment which was directly digested with an *AflIII/XbaI* digest (as the *XbaI* site would be blocked by methylation once cloned into a plasmid vector). *AflIII* was used as an alternative to *NcoI* as the amino acid sequence of Salem V did not accommodate the introduction of a *NcoI* site in the forward primer. The *AflIII/XbaI* fragment was then ligated with the pEF.myc.IRES.neo backbone to create pEF.myc.SalV-V.IRES.neo.

iv) Mapuera V

MapV V DNA was provided as a V gene PCR fragment and PCR from this template using MapV For and MapV Rev primers created a blunt-ended fragment which was directly digested with a *NcoI/XbaI* digest (as the *XbaI* site would be blocked by methylation once cloned into a plasmid vector). This fragment was then ligated with the pEF.myc.IRES.neo backbone to create pEF.myc.MapV-V.IRES.neo.

v) Tioman V

TiV V DNA was provided as a V ORF cloned into the vector pRSET-B. PCR from this template using TiV For and TiV Rev primers generated a blunt-ended fragment, which was directly digested with *NcoI/XbaI* and initially ligated with the pEF.myc.IRES.neo backbone to create pEF.myc.TiV-V.IRES.neo. However, sequencing of this construct revealed a nucleotide change, G602T, presumably introduced during PCR, which created a stop codon at residue 201. This residue lies within the V-unique region of TiV V and the mutation creates a TiV V protein with a deletion of 31 residues from the C-terminus, a region that contains five of the seven conserved cysteine residues in the carboxy terminus and the construct was re-named pEF.myc.TiV-VΔC31.IRES.neo. To obtain a full-length TiV V, the initial PCR was repeated and the blunt-ended product generated was directly digested with *NcoI/XbaI* and cloned into pEF.myc.plink2 digested with *NcoI/XbaI* to create pEF.myc.TiV-V.plink2.

vi) Hendra V

HeV V DNA was provided as a P ORF in the vector pRSET-A. Mutagenic PCR from this template using HeV For and HeV Rev and internal HeV G add For and HeV G add Rev primers was used to insert an additional G residue at the P gene RNA editing site (method outlined in Fig. 13). The resulting PCR product encoding the HeV V ORF was directly digested with *NcoI/XbaI* and cloned into pEF.myc.plink2 digested with *NcoI/XbaI* to create pEF.myc.HeV-V.plink2.

vii) Menangle V

MenV V DNA was provided as a V ORF cloned into the vector pCR4Blunt-Topo. PCR from this template using MenV For and MenV Rev primers generated a blunt-ended fragment, which was directly digested with *NcoI/XbaI* and cloned into pEF.myc.plink2 digested with *NcoI/XbaI* to create pEF.myc.MenV-V.plink2.

viii) Mumps V

MuV V DNA was provided as a V ORF from the vaccine strain Jeryl Lynn, cloned into the vector pCG. PCR from this template using MuV For and MuV Rev primers generated a blunt-ended fragement, which was directly digested with *NcoI/XbaI* and

cloned into pEF.myc.plink2 digested with *NcoI/XbaI* to create pEF.myc.MenV-V.plink2.

ix) Nipah V Mutant Panel

Using pEF.myc.NiV.IRES.neo as a template, mutagenic PCR using external primers NiV For and NiV Rev and various pairs of internal primers containing the desired nucleotide changes created full-length, mutated NiV V fragments (method outlined in Fig. 13). The blunt-ended PCR products generated by this reaction were directly digested with *NcoI/XbaI* and cloned into pEF.myc.plink2 digested with *NcoI/XbaI* to create the various mutant pEF.myc.NiV-V.plink2 constructs.

V truncation construct cloning

In order to study the properties of the amino and carboxy regions of various V proteins, truncation constructs were made that encoded either the amino P/V shared domain of V or the carboxy V-unique domain. This was carried out for NiV, MapV and MuV and all sets of truncations were cloned into the pEF.myc.plink2 vector, giving them amino-terminal myc epitope tags.

PCR was carried out using the newly created full-length V constructs as templates and both the existing 'For' and 'Rev' primers specific for each V, as well as newly designed internal primers complementary to the sequences immediately up and downstream of the RNA editing site ('N Rev' and 'C For'). The 'N Rev' primers incorporated an *XbaI* site and a stop codon immediately after the last amino terminal codon and the 'C For' primers incorporated a *NcoI* site around a start codon introduced immediately before the first carboxy terminal codon. Amino terminal PCR products were generated using 'For' and 'N Rev' primer combinations and the carboxy terminal PCR products were generated using 'C For' and 'Rev' primer combinations.

i) Nipah V truncations

As the NiV V Geelong, NiV V NIH and NiV V E125G constructs all differed in their amino terminal sequence, all three were used as templates to create three NiV Vn constructs. Each template was used to create a NiV Vn PCR product using 'NiV For'

and 'NiV N Rev' primers and a proofreading polymerase. The resulting blunt-ended PCR product was directly digested with *NcoI/XbaI* and ligated with pEF.myc.plink2 digested with *NcoI/XbaI* to create pEF.myc.NiV-Vn-Geelong.plink2, pEF.myc.NiV-Vn-NIH.plink2 and pEF.myc.NiV-Vn-E125G.plink2.

As all of the NiV V constructs had identical C-termini, the NiV NIH construct was used as a template to generate a NiV Vc PCR product using 'NiV C For' and 'NiV Rev' primers and a proofreading polymerase. The resulting blunt-ended PCR product was directly digested with *NcoI/XbaI* and ligated with pEF.myc.plink2 digested with *NcoI/XbaI* to create pEF.myc.NiV-Vc.plink2.

ii) Mapuera V truncations

The full-length MapV V construct was used as a template for PCR using combinations of 'MapV For' and 'MapV N Rev' primers to create a MapV Vn PCR product and 'MapV C For' and 'MapV Rev' primers to create a MapV Vc PCR product. These PCR products were directly digested with *NcoI/XbaI* and ligated with pEF.myc.plink2 digested with *NcoI/XbaI* to create pEF.myc.MapV-Vn.plink2 and pEF.myc.MapV-Vc.plink2.

iii) Mumps V truncations

The full-length MuV V construct was used as a template for PCR using combinations of 'MuV For' and 'MuV N Rev' primers to create a MuV Vn PCR product and 'MuV C For' and 'MuV Rev' primers to create a MuV Vc PCR product. These PCR products were directly digested with *NcoI/XbaI* and ligated with pEF.myc.plink2 digested with *NcoI/XbaI* to create pEF.myc.MuV-Vn.plink2 and pEF.myc.MuV-Vc.plink2.

GST-Mapuera V cloning

In the laboratory a vector for the expression of GST fusion constructs had been created in a modified pGEX-4T-3 vector, pGEX4T*, incorporating a TeV protease site between the GST and MCS and an additional *NcoI* site at the start of the MCS to allow sub-cloning of V proteins (see Fig. 15). To create a Mapuera V GST fusion construct, pEF.myc.MapV-V.IRES.neo was digested with *NcoI/SalI* and ligated into

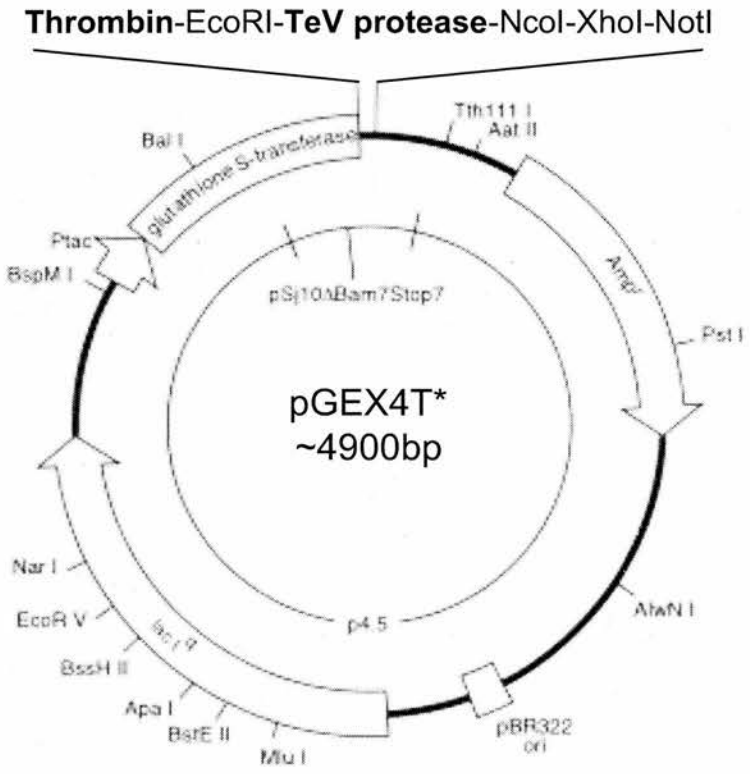


Figure 15: A schematic representation of the modified pGEX-4T-3 vector, pGEX4T*, used to create GST fusion proteins

Vector diagram of pGEX4T* showing positions of the promoter, GST ORF, MCS (with modifications) and ampicillin resistance gene. This vector is modified from the parent by addition of a TeV protease site between the GST coding region and the MCS, allowing cleavage of GST fusion proteins using TeV protease. Restriction sites downstream of the protease site are available for cloning.

pGEX4T* digested with *NcoI/XhoI*. However, as pEF.myc.MapV-V.IRES.neo contained multiple *NcoI* sites, the construct was first digested with *ApaI/SalI,* the fragment containing MapV V purified and then digested with *NcoI*, now a unique site. This MapV V fragment was then ligated into the pGEX4T* backbone to create pGEX.MapV-V.

Nipah V T7 promoter cloning

In order to create NiV V Geelong and NiV V E125G constructs under the control of a T7 promoter, the yeast-2-hybrid vector pGBKT7 was used as this has a T7 promoter and a MCS with an upstream myc epitope tag (see Fig. 16). The general strategy used was to cut the vector with *NcoI/BamHI*, but to blunt the *BamHI* cut end as the inserts could only be excised with *NcoI/XbaI* digests, the *XbaI* site being blunted also. The pGBKT7 *NcoI/BamHI*-blunt fragment was prepared in two parts; initially pGBKT7 was digested in two separate reactions with *NcoI* and *BamHI*. The *BamHI* digest was then blunt-ended using a Klenow fill-in reaction and both reactions were digested with *PvuI*, giving two vector fragments, one with *NcoI* and *PvuI* ends and one with a blunt end and a *PvuI* end.

i) Nipah V Geelong

The pEF.myc.NiV-V-Geelong.IRES.neo construct used as the source of NiV V Geelong for the T7 cloning had multiple *NcoI* sites, so in order to generate the *NcoI/XbaI*-blunt fragment required for the 3-way ligation the construct was first digested with *XbaI*, generating two fragments, both of which were blunted using a Klenow fill-in reaction. The larger fragment containing NiV V Geelong was purified and then digested with *NcoI*, now a unique site. This NiV V Geelong fragment was combined with the two pGBKT7 vector fragments in a 3-way ligation to create pGBKT7.NiV-V-Geelong.

ii) Nipah V E125G

The NiV V E125G fragment was obtained by digesting pEF.myc.NiV-V-E125G.plink2 with *XbaI*, blunting with a Klenow fill-in reaction and then digesting with *NcoI*. This fragment was then combined with the two pGBKT7 vector fragments in a 3-way ligation to create pGBKT7.NiV-V-E125G.

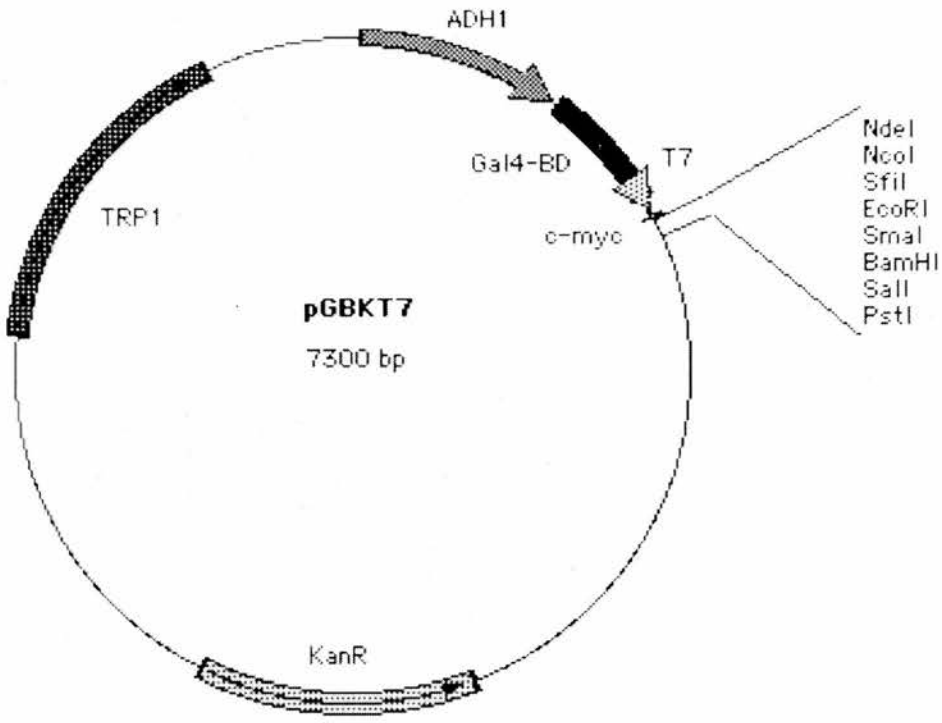


Figure 16: A schematic representation of the pGBKT7 vector

Vector diagram of pGBKT7 showing positions of the yeast ADH1 promoter, Gal4 DNA binding domain, T7 promoter, c-myc tag, MCS and kanamycin resistance gene.

ORFs cloned into the MCS and expressed from the T7 promoter have an amino terminal myc tag, but are not Gal4 DNA binding domain fusions.

RESULTS

SECTION ONE

The experiments described in this section were carried out in order to address questions both arising from current work in our laboratory and from published data on IFN evasion by SV5 V and MuV V.

I. SIMIAN VIRUS 5

Previous work in our laboratory had demonstrated that truncations of SV5 V were unable to block IFN signalling, but it was not clear whether this was due to a loss of interaction with a cellular protein, normally mediated by the deleted section of the protein, or a disruption of the normal structure and therefore function of the V protein. It was thought possible that the amino and carboxy terminal domains of V might be able to *trans*-complement each other and enable a block of IFN signalling, perhaps by bringing together other, cellular proteins with which each domain was interacting.

SV5 V Truncations and IFN Signalling

To investigate this possibility, amino and carboxy-terminal SV5 V constructs were assessed for their ability to block IFN signalling both individually and in combination.

Amino and carboxy-terminal portions of SV5 V cannot block IFN signalling, even when co-expressed

As shown in Fig. 17, expression of full-length SV5 V blocks IFN signalling, even when 50% less DNA is used in combination with pEF.plink2 empty vector. However, the amino and carboxy-terminal sections of SV5 V are unable to block IFN signalling, even when expressed together.

II. MUMPS VIRUS

It has been known for some time that MuV V can antagonise both IFNα/β and IFNγ signalling via the targeted degradation of STAT1 and it is thought that this is achieved in a similar way to STAT1 degradation by SV5 V. However, an early report in the literature claimed that the C-terminus of MuV, MuV Vc, had a similar ability to full-length MuV V to antagonise the IFN response and that MuV Vc alone could

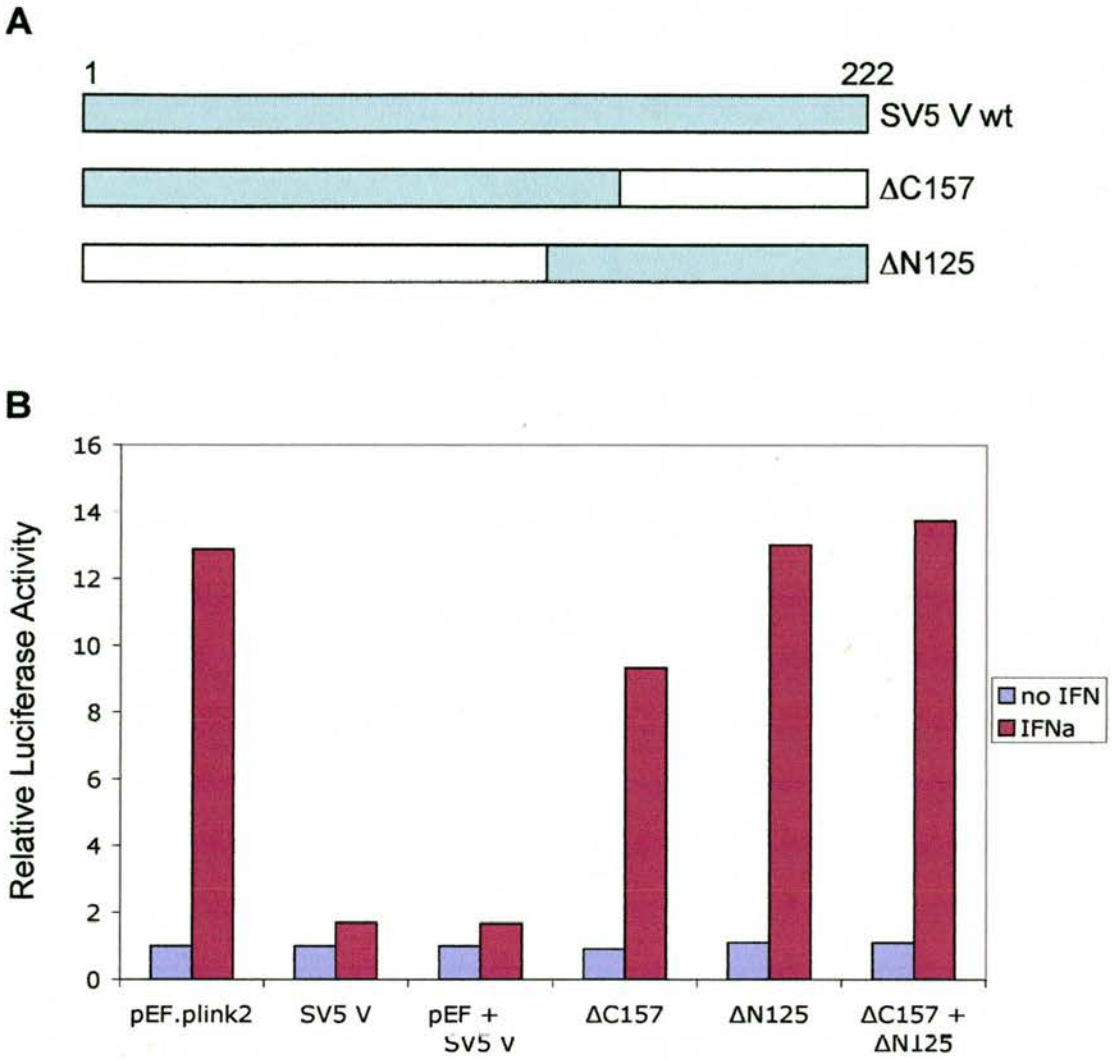


Figure 17: IFNα/β signalling assay in HeLa cells: SV5 V truncations

Panel A shows a schematic representation of full length SV5 V, ΔC157 and ΔN125 expression constructs, not drawn to scale.

Panel B shows the results of an IFNα/β signalling assay where HeLa cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V full-length and truncated expression constructs singly and in combination (same total amount of DNA in each transfection). 48h post transfection cells were stimulated or not with IFNα, 1.8×10^4 IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

antagonise IFN signalling (Kubota et al. 2001). This is in direct contradiction to our results obtained with SV5 V truncations so it was of interest to investigate the ability of full-length and truncated MuV V constructs to antagonise the IFN response using the IFN signalling assay used for other V proteins.

A clone of MuV V from the Jeryl Lynn vaccine strain was obtained from Bert Rima, Queen's University, Belfast and sub-cloned into an expression vector with an N-terminal myc epitope tag. Similarly tagged amino and carboxy terminal MuV V constructs were also generated (for full details see Cloning Strategies section).

Mumps V and IFN signalling

Full-length and amino and carboxy-terminal truncations of MuV V were assayed for their ability to antagonise IFNα/β signalling in various cell types.

Mumps V blocks IFNα/β signalling in simian, human and murine cells

Fig. 18 shows that full-length MuV V blocks IFNα/β signalling to a similar extent as SV5 V in Vero cells. A subsequent assay in murine BF cells, shown in Fig. 19, indicated that MuV V also blocks IFNα/β signalling in these cells, as has previously been demonstrated for murine NIH 3T3 cells. MuV V also blocks IFNα/β signalling in human cells as shown in Fig. 20.

Mumps V N and C-terminal truncations do not block IFNα/β signalling

Fig. 20 shows the results of an IFNα/β signalling assay using both full-length and truncated MuV V constructs in HeLa cells. It is clear from this data that neither MuV Vn nor MuV Vc are capable of blocking IFNα/β signalling when expressed alone, in contrast with previously published data which suggested that the C-terminus of MuV V alone could block IFNα/β signalling and target STAT1 for degradation.

Mumps V and IFN production

Recent studies using SV5 have shown that its V protein antagonises the IFN response not only by blocking IFN signalling but also by blocking the production of IFNβ. This is achieved by preventing the activation of the IFNβ promoter, something which can be assayed using a luciferase reporter under the control of the dsRNA-responsive

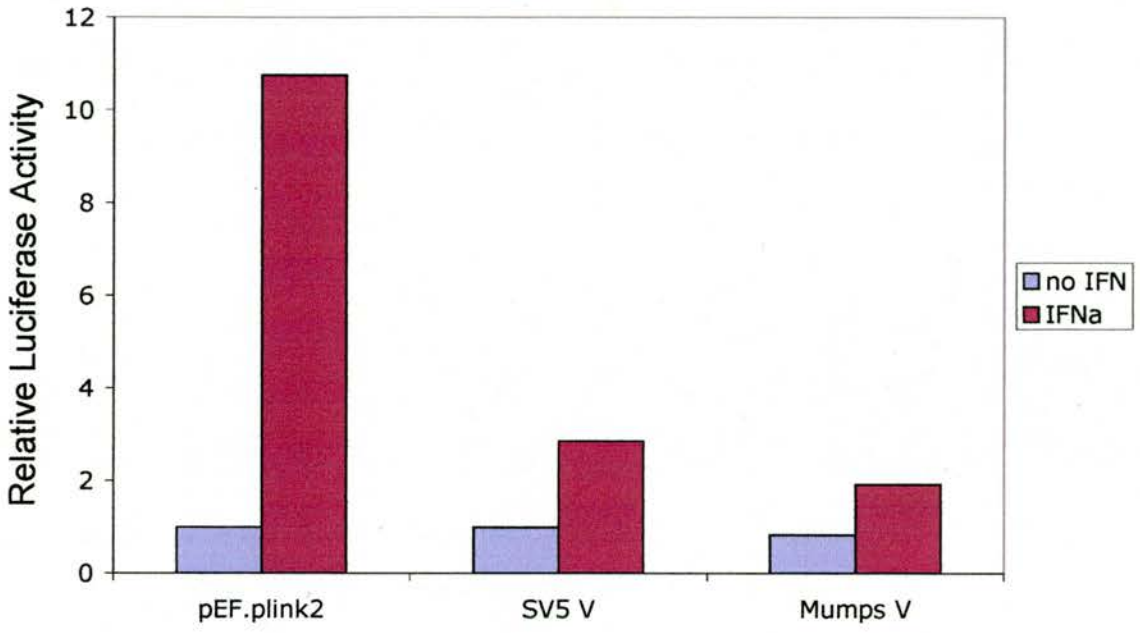


Figure 18: IFNα/β signalling assay in Vero cells: Mumps V construct

Vero cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Mumps V expression constructs. 48h post transfection cells were stimulated or not with IFNα, $1.8x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

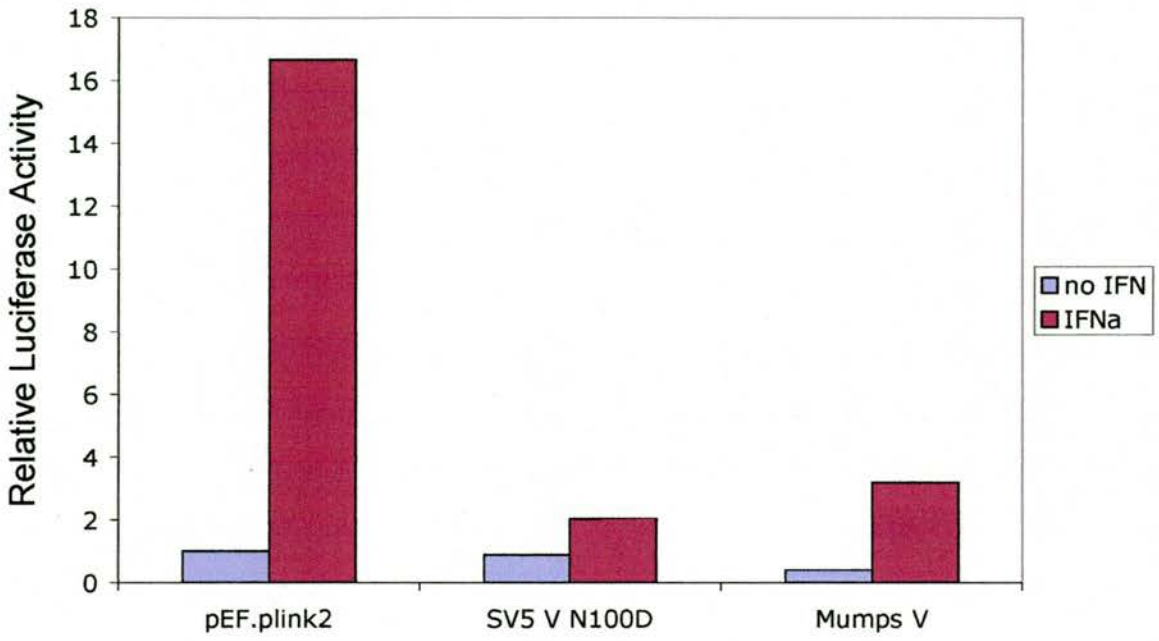


Figure 19: IFNα/β signalling assay in BF cells: Mumps V construct

BF cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Mumps V expression constructs. 48h post transfection cells were stimulated or not with 'Universal Type I IFN', $1.0x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

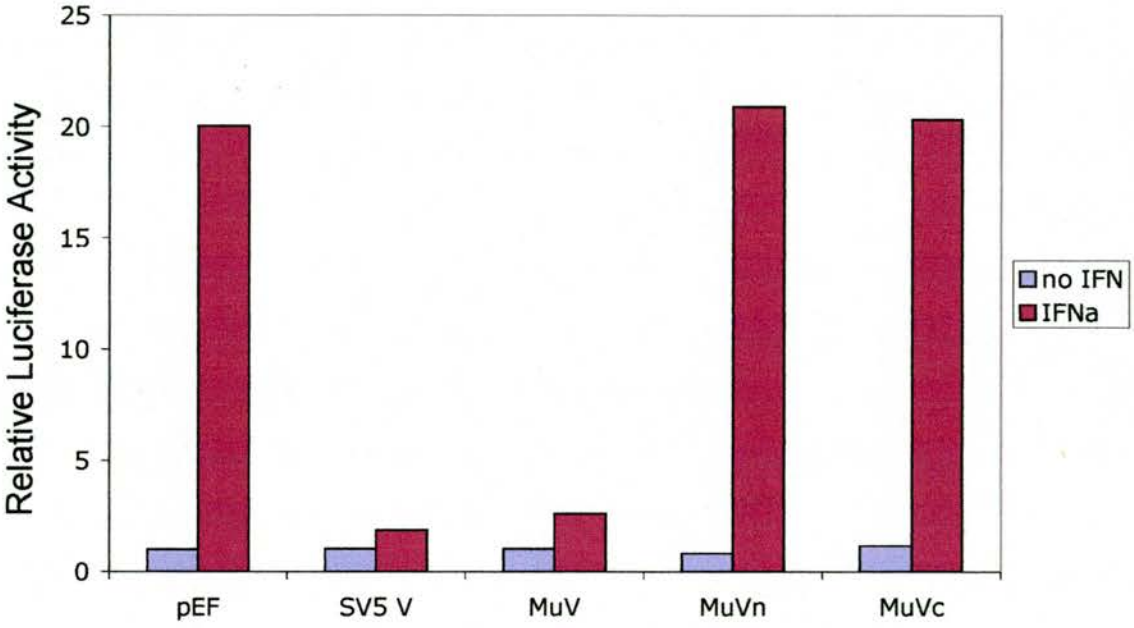


Figure 20: IFNα/β signalling assay in HeLa cells: Mumps V truncations

HeLa cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Mumps V full-length and truncated expression constructs. 48h post transfection cells were stimulated or not with IFNα, $1.8x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

IFNβ promoter, stimulated by the transfection of poly(I):poly(C) artificial dsRNA into cells. This property of SV5 V has been localised to its cysteine-rich C-terminus, which is highly conserved in other paramyxovirus V proteins, including MuV V (see Fig. 21). Hence MuV V may also be capable of blocking dsRNA signalling to the IFNβ promoter and to investigate this both the full-length and truncated MuV V constructs were assayed using a luciferase reporter gene under the control of the dsRNA-responsive IFNβ promoter.

Mumps V blocks the activation of the IFNβ promoter via its cysteine-rich C-terminus

The IFNβ promoter activation assay, shown in Fig. 22, demonstrates that MuV V blocks the activity of the IFNβ promoter in response to dsRNA to a similar degree as SV5 V. The assay also shows that whilst expression of the N-terminus of MuV V has no effect on dsRNA signalling to the IFNβ promoter, the C-terminus of MuV V alone is sufficient to suppress IFNβ promoter activation.

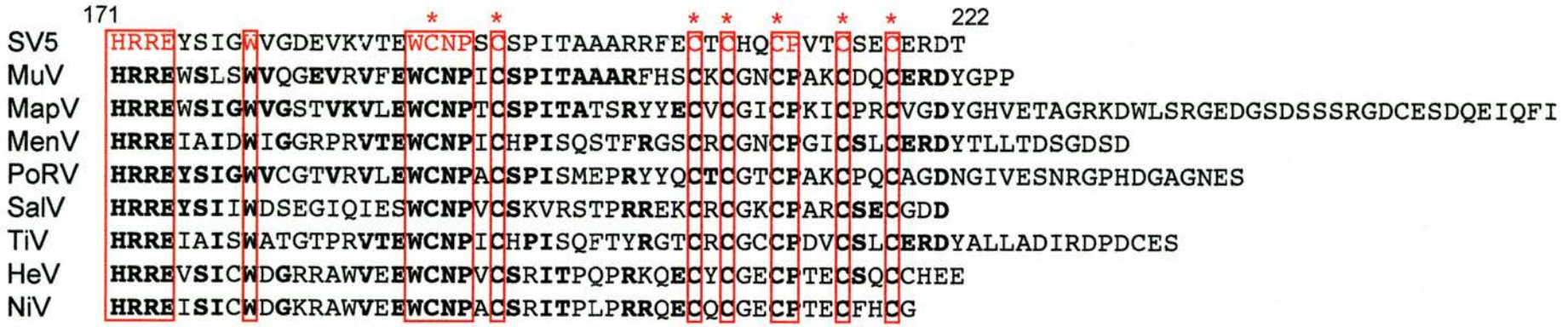


Figure 21: Sequence alignment of C-terminal regions of various paramyxovirus V proteins relative to SV5 V C-terminus

The C-terminal regions of all V proteins included in this study have been aligned to the SV5 V sequence from a conserved HRRE motif 7aa downstream of the RNA editing site to the end of the protein.

Residues in bold type are identical to the SV5 V sequence, residues in red type and enclosed by boxes are completely conserved between all nine V proteins and asterisks indicate the positions of the seven conserved cysteine residues. Amino acid positions refer to the SV5 V protein.

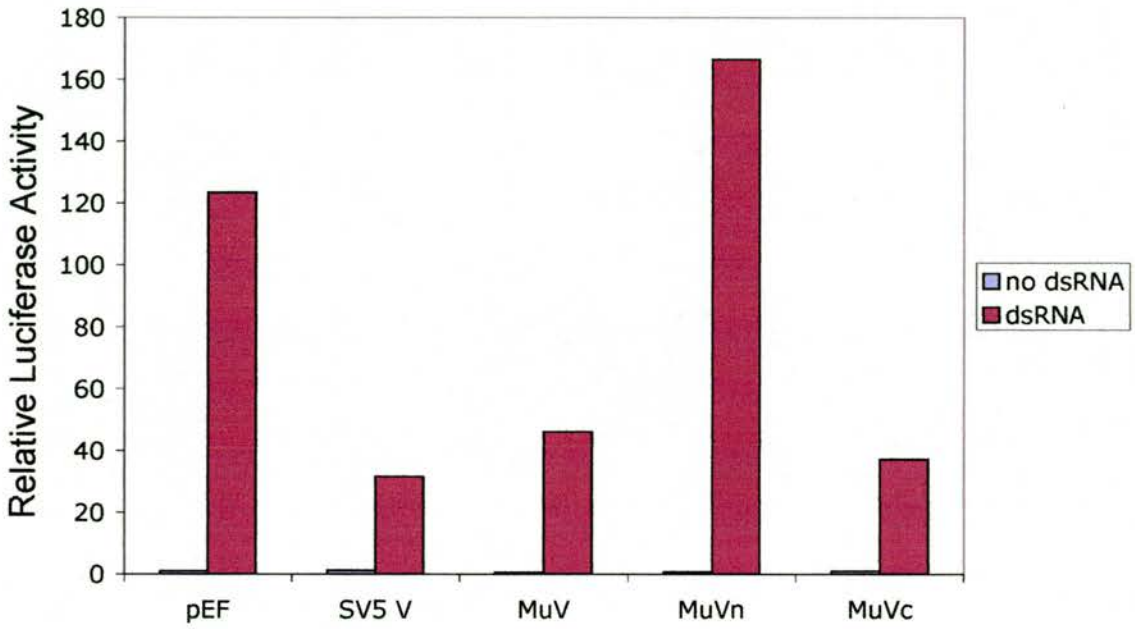


Figure 22: IFNβ promoter activation assay in Vero cells : Mumps V constructs

Vero cells were seeded in 6-well plates and transfected with a dsRNA-responsive IFNβ promoter-luciferase reporter, a β-gal control plasmid and and either pEF.plink2 empty vector, SV5 V or Mumps V full-length and truncated expression constructs. 48h post transfection cells were stimulated or not with 2.5μg poly(I):poly(C) per well, 12h @37°C.

Cells were harvested in 200μl each Luciferase buffers A and B and 300μl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

SECTION TWO

In recent years, novel paramyxoviruses have been the cause of outbreaks of severe disease both in humans and animals and it was of considerable interest to investigate whether these newly emerging viruses and others previously isolated from animal hosts have IFN evasion mechanisms, as demonstrated for other members of the *Paramyxoviridae* such as SV5 and SeV. The V proteins of these viruses were of particular interest as they share the same seven conserved cysteine residues found in other paramyxovirus V proteins and therefore may have a similar function in IFN evasion as the V proteins of viruses such as SV5, MuV and MeV (see Fig. 21 for an alignment of the carboxy termini of various V proteins). This section will begin by discussing work on the V proteins of the recently discovered Nipah and Hendra viruses and then will discuss work on other paramyxovirus V proteins isolated from animal hosts.

All of the V proteins described were obtained as P/V gene clones or PCR products from various laboratories and were cloned into mammalian expression vectors with N-terminal myc epitope tags as no anti-sera were available for detection of the cloned proteins. Fig. 23 shows that transient expression of the cloned proteins could be detected by western blots of total cell lysates with an α-myc MAb and that the V proteins expressed were of the expected sizes (data for all V constructs not shown).

I. NIPAH AND HENDRA VIRUSES

Although the P genes of both NiV and HeV encode multiple products, the V proteins were of particular interest in this study due to their carboxy terminal identity with other paramyxovirus V proteins (see Fig. 21). NiV and HeV P gene clones were obtained from Lin-Fa Wang at CSIRO in Geelong, Australia and mutagenic PCR was used to add an additional non-templated G residue at the editing site of both P constructs, creating V ORFs. These were then cloned into mammalian expression constructs with an N-terminal myc epitope tag (for full details see Cloning Strategies section).

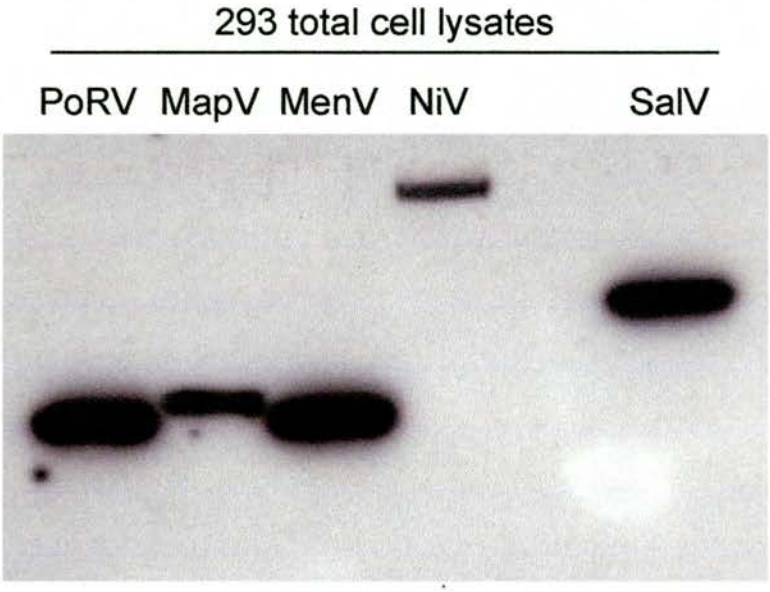


Figure 23: Anti-myc western blot of 293 cell lysates transiently expressing myc-tagged V constructs

293 cells in 6-well plates were transiently transfected with 2μg either PoRV V, MapV V, MenV V, NiV V Geelong or SalV V expression constructs using Fugene-6 transfection reagent. 48h post transfection cells were harvested in disruption buffer and 1/5th each sample was separated by 10% SDS-PAGE and transferred to PVDF.

Samples were western blotted with α-myc MAb and α-mouse-HRP secondary Ab and detected with ECL reagents.

The predicted sizes of these V proteins according to their amino acid sequences were: PoRV V 26.1 kDa; MapV V 27.5 kDa; MenV V 25.1 kDa; NiV V 50.4 kDa; SalV V 32.8 kDa.

Nipah V and IFN signalling

The cloned NiV V was assayed for its ability to antagonise IFN signalling in human cells using IFN-responsive luciferase reporter constructs.

Nipah V Geelong does not block IFNα/β signalling

IFNα/β signalling assays carried out in HeLa cells clearly show that the NiV V protein obtained from CSIRO, referred to as Nipah V Geelong (NiV V Geelong), does not block IFNα/β signalling (Fig. 24). However, a paper subsequently appeared in the literature describing a similar assay system, which demonstrated that Nipah V could block both IFNα/β and IFNγ signalling in human cells (Rodriguez et al. 2002). Comparison of NiV V Geelong and the Nipah V used in the published study (referred to here as NiV V NIH) revealed four nucleotide differences in the V ORF, three of which result in differences in V amino acid sequence. These three amino acids are all in the N-terminal, P/V common domain of NiV V, at positions 125, 248 and 280.

In order to determine which of the amino acid differences are important in NiV V function, a panel of mutants based on NiV V Geelong was made, incorporating combinations of the amino acid differences between NiV V Geelong and NiV V NIH. These mutants were generated by PCR mutagenesis using NiV V Geelong as the initial template and were cloned into the pEF.myc.plink2 mammalian expression vector (see Fig. 25 and Cloning Strategies section for full details).

Nipah V mutants and IFN signalling

The NiV V mutants were assayed for their ability to antagonise both IFNα/β and IFNγ signalling using the IFN-responsive luciferase reporters described previously.

E125G mutation enables NiV V Geelong to block IFNα/β signalling

Fig. 26 shows the results of an IFNα/β signalling assay in Vero cells and demonstrates that as reported in the literature, NiV V NIH blocks IFNα/β signalling and as previously demonstrated in HeLa cells, NiV V Geelong does not. Of the three single mutants, only NiV V E125G blocks type IFNα/β signalling to the same extent as NiV V NIH and of the three double mutants, only those which contain the E125G amino acid change block IFNα/β signalling. This data suggests that it is only the

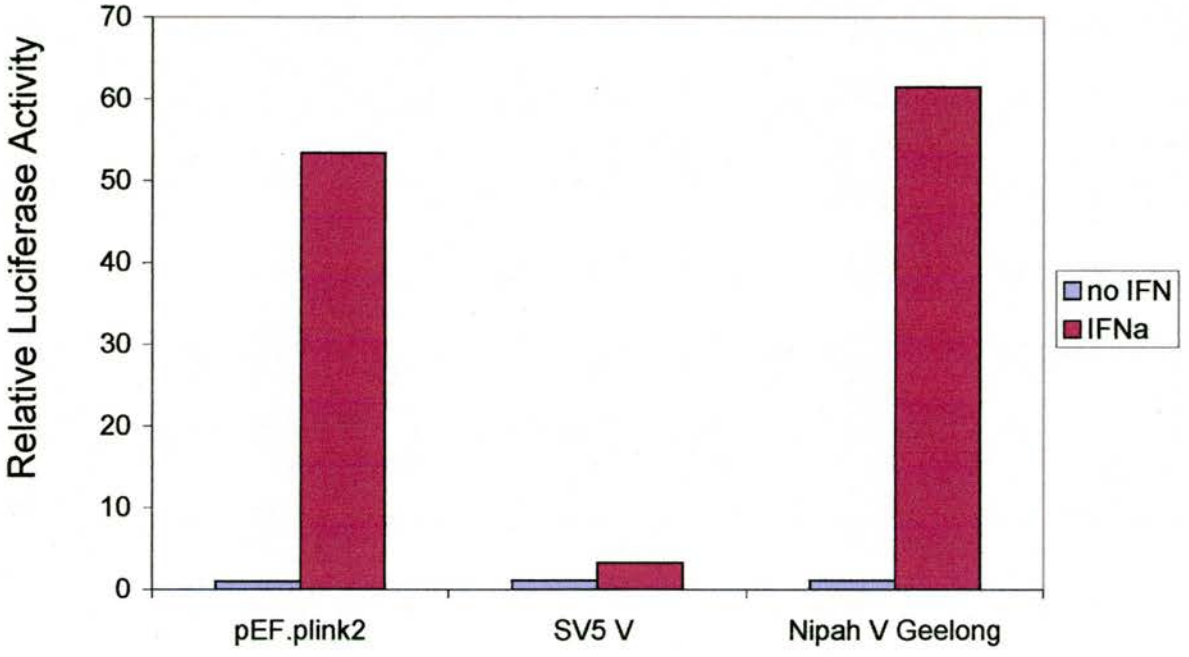


Figure 24: IFNα/β signalling assay in HeLa cells: Nipah V Geelong construct

HeLa cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Nipah V expression constructs. 48h post transfection cells were stimulated or not with IFNα, $1.8x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

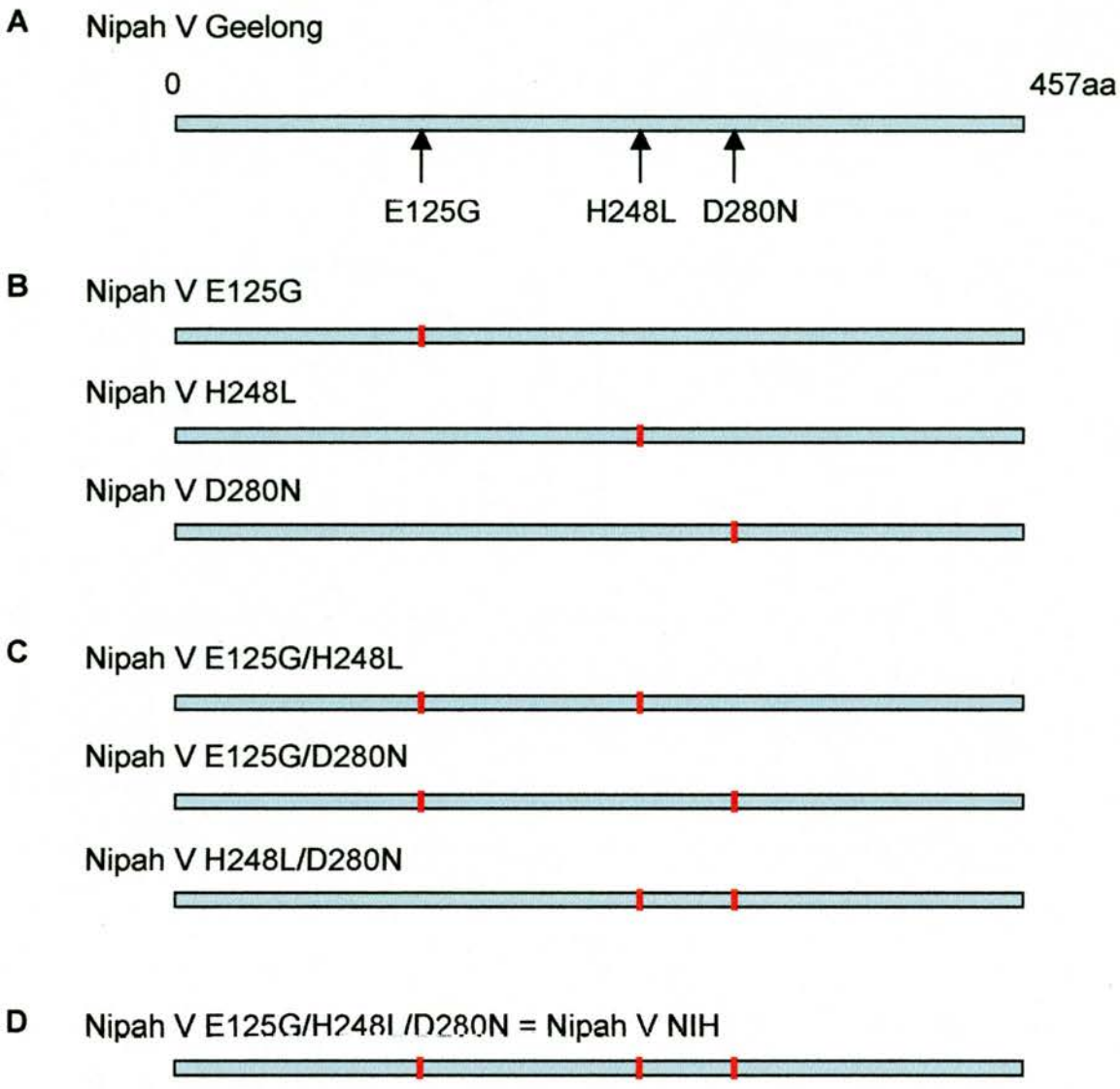


Figure 25: Construction of mutant panel based on Nipah V Geelong

The original Nipah V Geelong construct (Panel A with aa differences from Nipah V NIH shown) was used as a template in mutagenic PCR with different sets of primers to create Nipah V E125G, Nipah V H248L and Nipah V D280N single mutants (Panel B).

Nipah V E125G was then used as a template to create the double mutants Nipah V E125G/H248L and Nipah V E125G/D280N and Nipah V H248L was used as a template to create Nipah V H248L/D280N (Panel C).

Finally, Nipah V E125G/H248L was used as a template to create Nipah V E125G/H248L/D280N which is identical to Nipah V NIH (Panel D).

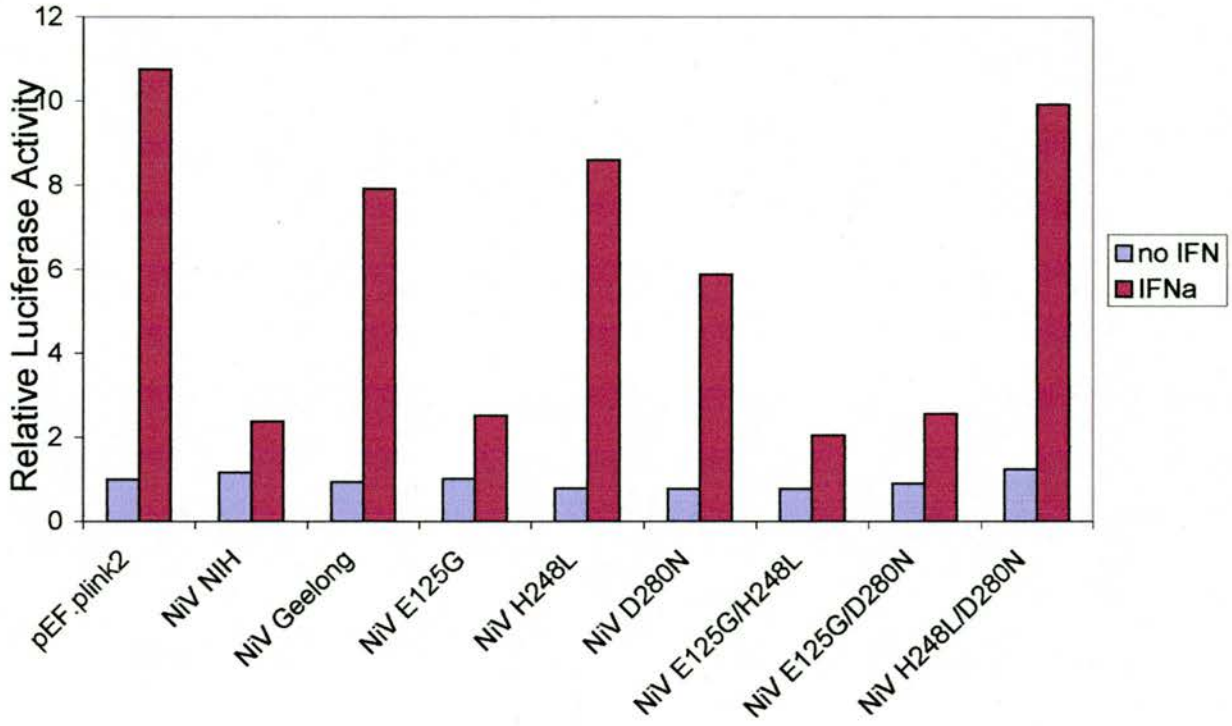


Figure 26: IFNα/β signalling assay in Vero cells: Nipah V mutant constructs

Vero cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Nipah V expression constructs. 48h post transfection cells were stimulated or not with IFNα, $1.8x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

difference in amino acid sequence between NiV V NIH and NiV V Geelong at residue 125 that is important for the antagonism of IFNα/β signalling.

E125G mutation enables NiV V Geelong to block IFNγ signalling

The results of IFNγ signalling assays with these mutants were broadly similar to the IFNα/β signalling results as can be seen in Fig. 27. NiV V NIH blocks IFNγ signalling whereas NiV V Geelong is unable to do so and the mutants also behave in a similar way except that the single mutant NiV V E125G seems to be not quite as effective at blocking IFNγ signalling as IFNα/β signalling. However, both of the double mutants containing the E125G residue change block IFNγ signalling to the same extent as NiV V NIH and it is not clear whether the apparent difference in NiV V E125G activity is significant.

Nipah V truncations and IFN Signalling

The regions of NiV V required for function had not been elucidated and simple amino and carboxy terminal truncations of NiV V were constructed in order to investigate this (see Fig. 28 and Cloning Strategies section for details). The NiV Vn construct expresses a protein equivalent to the shared domain of P and V whereas the NiV Vc construct expresses the V-unique, cysteine-rich region.

Nipah V N-terminus but not C-terminus blocks IFNα/β signalling

Fig. 29 shows the results of an IFNα/β signalling assay in Vero cells expressing NiV V truncation constructs in which, as already demonstrated, NiV V NIH blocks IFNα/β signalling to the same extent as SV5 V. Of the N-terminal constructs, both NiV NIH Vn and NiV E125G Vn block signalling to the same extent as full-length NiV V NIH whereas NiV Geelong Vn does not block signalling. The C-terminal construct NiV Vc also does not block IFN signalling, suggesting that it is not required for this function of NiV V and that IFN signalling can be blocked by NiV Vn alone provided that it has the correct residue at position 125.

Nipah V and IFN Production

As already mentioned, recent studies have shown that the V proteins of various paramyxoviruses including SV5 and SeV V block the production of IFNβ via

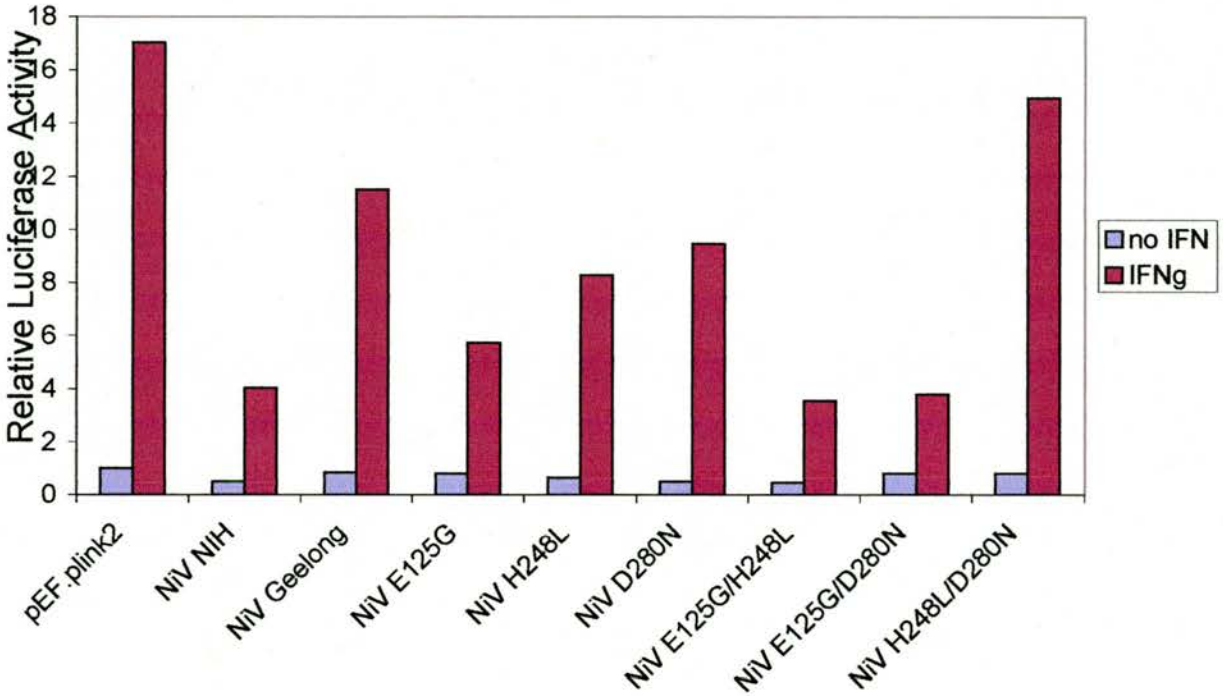


Figure 27: IFNγ signalling assay in Vero cells: Nipah V mutant constructs

Vero cells were seeded in 6-well plates and transfected with an IFNγ-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Nipah V expression constructs. 48h post transfection cells were stimulated or not with IFNγ, $1.0x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

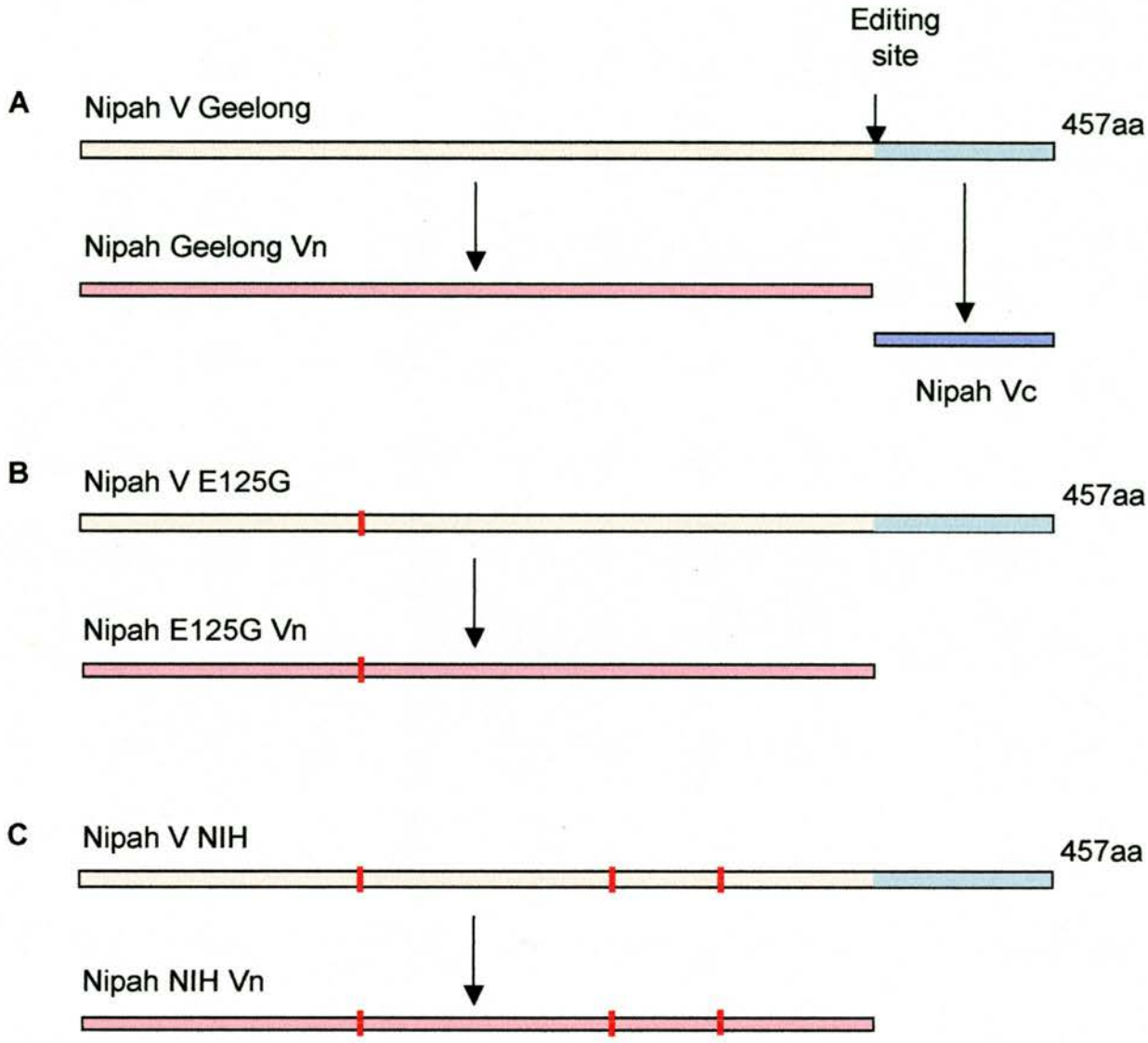


Figure 28: Construction of Nipah V truncations

The original Nipah V Geelong construct was used as a PCR template to create Nipah Geelong Vn and Nipah Vc constructs (Panel A).

Nipah V E125G was used as a PCR template to create Nipah E125G Vn (Panel B) and Nipah V NIH was used to create Nipah NIH Vn (Panel C).

All of the full-length Nipah V constructs have the same sequence after residue 280 so it was not necessary to create more than one Vc construct.

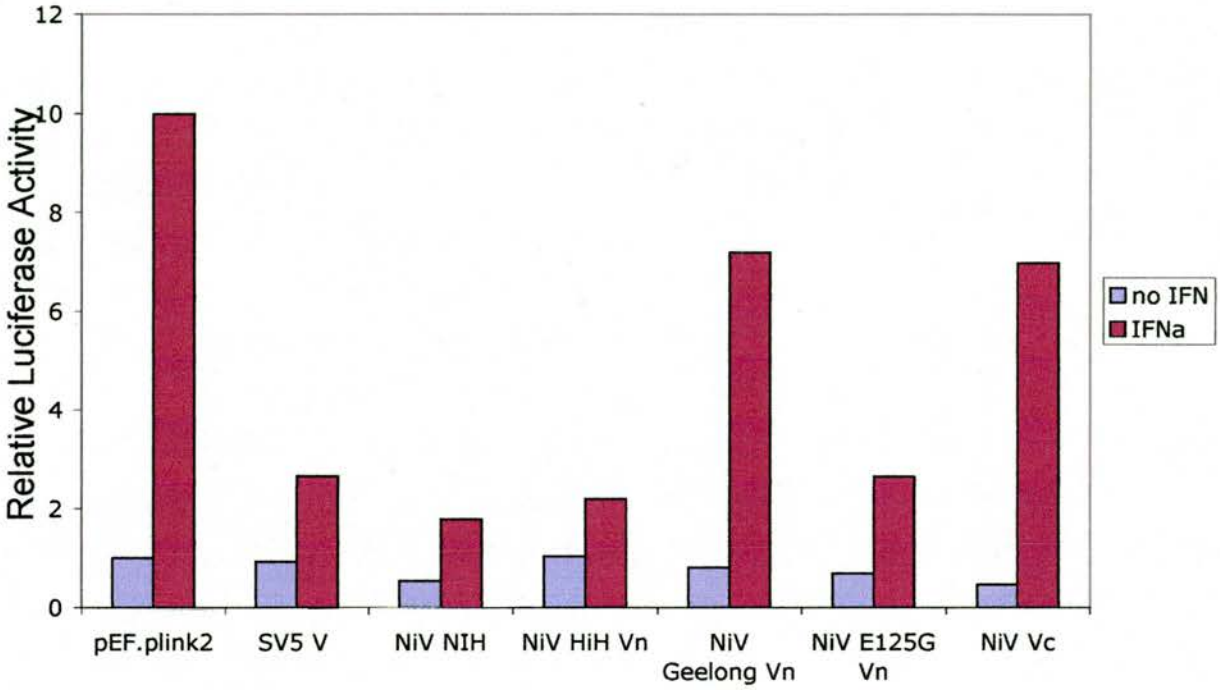


Figure 29: IFNα/β signalling assay in Vero cells: Nipah V truncation constructs

Vero cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Nipah V full-length and truncated expression constructs. 48h post transfection cells were stimulated or not with IFNα, $1.8x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

suppressing the activation of the IFNβ promoter. This property has been localised to the cysteine-rich carboxy terminus of SV5 V, which is highly conserved in other paramyxovirus V proteins, including NiV V. Although NiV V is twice as long as SV5 V, this difference in size is almost all to be found in the N-terminus of the protein and the unique C-terminus of NiV is almost the same length as that of SV5 V and contains the same seven cysteine residues with conserved spacing, as well as an additional cysteine residue only found in NiV and HeV (see Fig. 21).

Nipah V blocks IFNβ promoter activation by dsRNA

NiV V NIH, NiV V Geelong and NiV V E125G constructs were assayed for their ability to block the activation of the IFNβ promoter by dsRNA and the results are shown in Fig. 30. This shows that all three NiV V constructs block dsRNA signalling to the IFNβ promoter to a similar extent as SV5 V, regardless of their ability to block IFN signalling. All sequence differences between the three constructs are in the N-terminal region of NiV V protein, suggesting that, as for SV5 V, this region is not important for suppressing the activation of the IFNβ promoter.

Nipah V C-terminus but not N-terminus blocks IFNβ promoter activation by dsRNA

In order to further investigate the roles of the N and C-terminal regions of NiV V in the suppression of IFNβ promoter activation, the NiV V NIH truncations already used in IFN signalling assays were used in the dsRNA signalling assay. The results of this experiment can be seen in Fig. 31, which shows that the C-terminus of NiV V NIH alone is sufficient to block dsRNA signalling to the IFNβ promoter and that expression of the N-terminus of NiV V NIH does not affect this signalling pathway.

<u>Nipah V and STAT distribution: Immunofluorescence in 2fTGH cells</u>

The same publication which showed that NiV V NIH blocks IFN signalling also demonstrated that in cells expressing NiV V NIH, STAT1 and STAT2 do not translocate to the nucleus in response to IFN and instead appear to co-localise in the cytoplasm with NiV V, suggesting that the mechanism by which NiV V blocks IFN signalling is binding to STAT1 and STAT2 and sequestering them in the cytoplasm (Rodriguez et al. 2002). It was therefore of interest to examine the distribution of STAT1 and STAT2 in cells expressing NiV V Geelong and the various NiV V

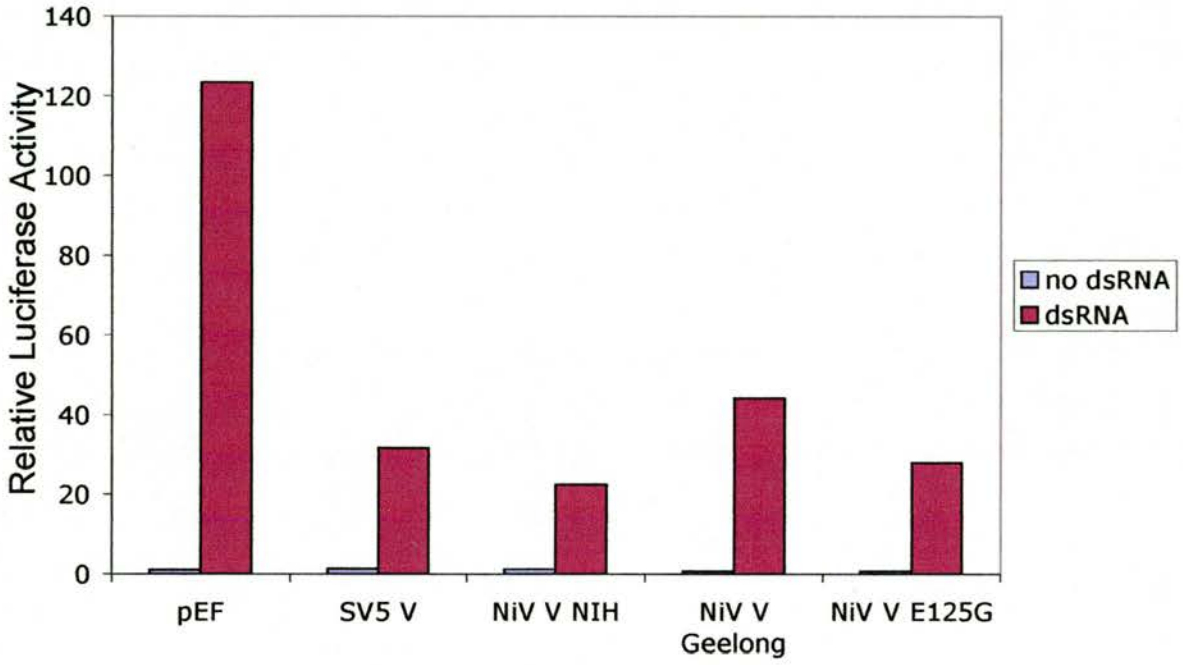


Figure 30: IFNβ promoter activation assay in Vero cells: Nipah V constructs

Vero cells were seeded in 6-well plates and transfected with a dsRNA-responsive IFNβ promoter-luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Nipah V expression constructs. 48h post transfection cells were stimulated or not with 2.5μg poly(I):poly(C) per well, 12h @37°C.

Cells were harvested in 200μl each Luciferase buffers A and B and 300μl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

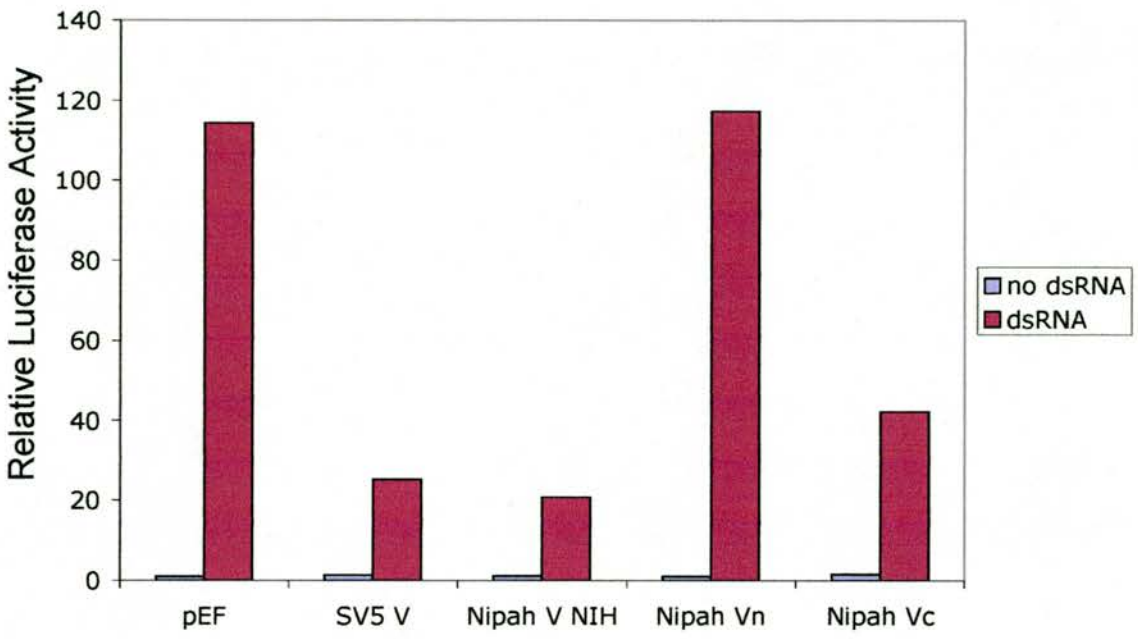


Figure 31: IFNβ promoter activation assay in Vero cells: Nipah V truncation constructs

Vero cells were seeded in 6-well plates and transfected with a dsRNA-responsive IFNβ promoter-luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Nipah V full-length and truncated expression constructs. 48h post transfection cells were stimulated or not with 2.5μg poly(I):poly(C) per well, 12h @37°C.

Cells were harvested in 200μl each Luciferase buffers A and B and 300μl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

Geelong mutants, to see if there were any effects of these NiV V constructs on STAT1 and STAT2 distribution in response to IFN. It might be expected that NiV V proteins that are unable to block IFN signalling such as NiV V Geelong would also be unable to block the nuclear translocation of STAT1 and STAT2.

2fTGH cells were used in the immunofluorescence experiments due to their high constitutive levels of STAT1 and STAT2. Fig. 32 shows the results of STAT1 and STAT2 immunofluorescence in naïve 2fTGH cells stimulated or not with IFNα. In the absence of IFN stimulation both STAT1 and STAT2 have a cytoplasmic distribution and the nuclei of the cells appear to be dark and empty, but once stimulated with IFNα the distribution of STAT1 and STAT2 changes dramatically and most of the STAT fluorescence is in the nuclei of the cells with much less in the cytoplasm. After a few hours without further IFN stimulation, both STAT1 and STAT2 translocate out of the nucleus to the cytoplasm (data not shown).

i) <u>STAT1 Immunofluorescence in cells expressing Nipah V</u>

2fTGH cells were transfected with myc-tagged NiV V expression constructs and stimulated with IFNα. Coverslips were harvested and fixed and immunofluorescence using α-myc and α-STAT1 antibodies was performed.

Nipah V NIH blocks the nuclear translocation of STAT1 in response to IFNα

The top panel of Fig. 33 shows that in cells expressing NiV V NIH the V protein is distributed throughout the cytoplasm and the IFN-stimulated translocation of STAT1 to the nucleus is blocked, resulting in a cytoplasmic distribution of STAT1. This is in contrast to neighbouring cells not expressing NiV V NIH in which the STAT1 clearly translocates to the nucleus in response to IFNα. This confirms observations made in the literature using a similar NiV V construct.

Nipah V Geelong has no effect on STAT1 distribution in response to IFNα

The middle panel of Fig. 33 shows that NiV V Geelong has an almost exclusively cytoplasmic distribution, which looks identical to that of NiV V NIH and thus the lack of function of NiV V Geelong cannot be explained by a difference in the subcellular localisation of the V protein. It can also be seen that cells expressing NiV V Geelong

Figure 32: STAT1 and STAT2 indirect immunofluorescence in naïve 2fTGH cells

2fTGH cells were seeded onto coverslips in 24 well plates and when 80% confluent were stimulated or not with $1.8x10^4$ IU/ml IFNα, 1h @37°C.

Coverslips were harvested, fixed and permeabilised and indirect immunofluorescence was carried out using α-STAT1 MAb (upper panels) or α-STAT2 PAb (lower panels) with α-mouse or α-rabbit-Texas red conjugated secondary antibodies. Slides were examined using a Nikon Microphot-FXA microscope.

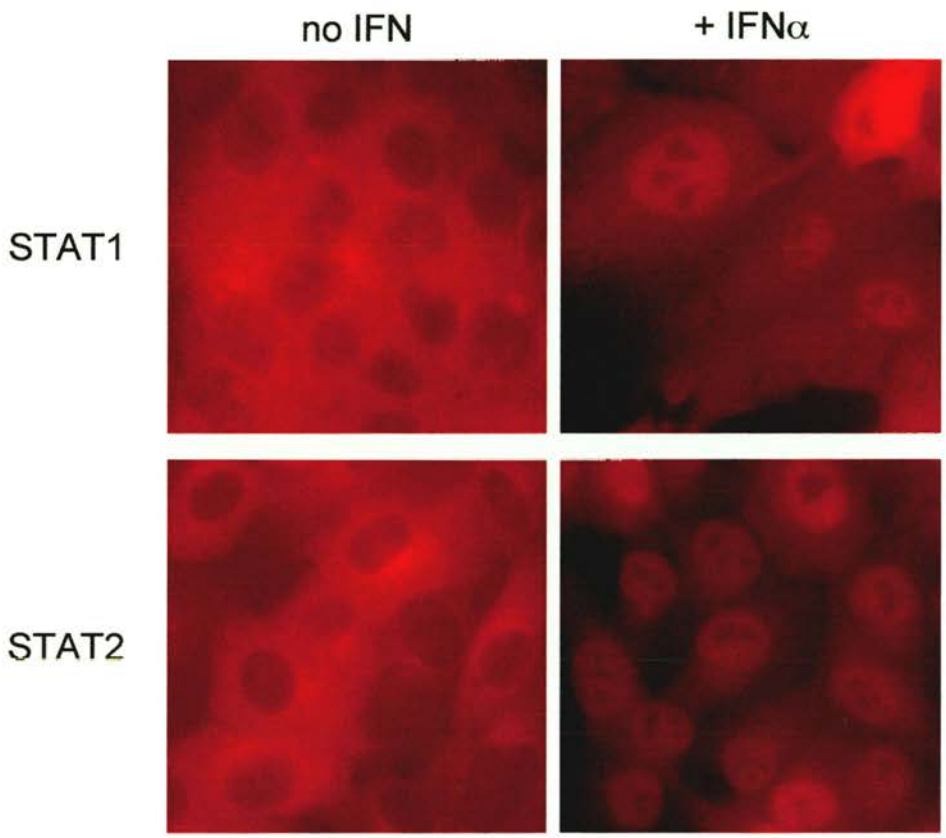
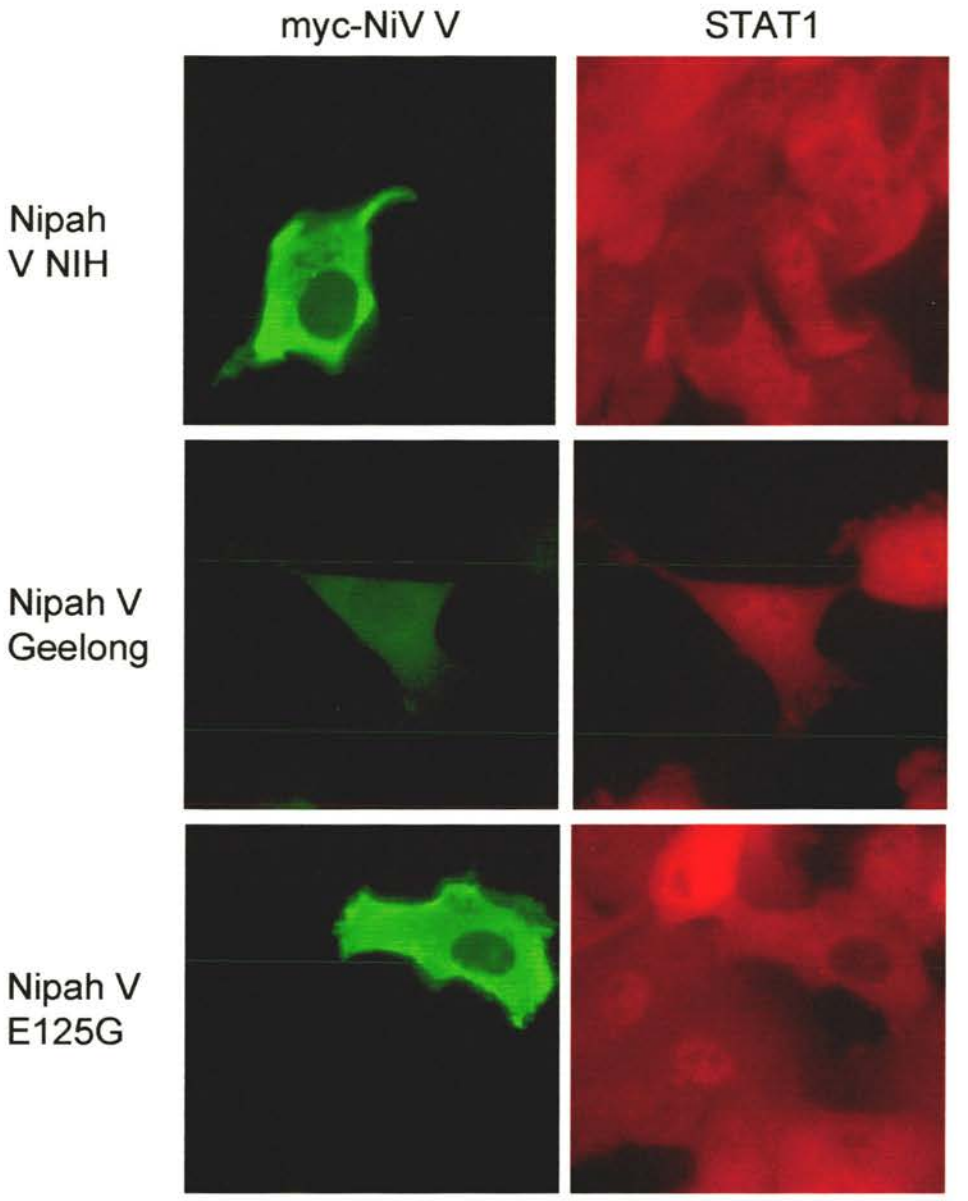

no IFN
+ IFNα
STAT1
STAT2

Figure 33: STAT1 and Nipah V indirect immunofluorescence in 2fTGH cells transfected with Nipah V constructs

2fTGH cells were seeded on coverslips in 24 well plates and once 50% confluent were transfected with either Nipah V NIH, Nipah V Geelong or Nipah V E125G myc-tagged expression constructs. 24h post transfection the cells were stimulated with $1.8x10^4$ IU/ml IFNα, 1h @37°C.

Coverslips were harvested, fixed and permeabilised and indirect immunofluorescence was carried out using α-STAT1 MAb and α-myc PAb with α-mouse-Texas red and α-rabbit-FITC conjugated secondary antibodies. Slides were examined using a Nikon Microphot-FXA microscope.


myc-NiV V
STAT1
Nipah V NIH
Nipah V Geelong
Nipah V E125G

behave as naïve 2fTGH cells in that stimulation with IFNα results in the translocation of STAT1 to the nucleus and no alteration of the normal subcellular distribution of STAT1 as can be seen by comparing the transfected cells to neighbouring untransfected cells, in contrast with NiV V NIH.

Nipah V E125G blocks the nuclear translocation of STAT1 in response to IFNα

The lower panel of Fig. 33 shows the effect on STAT1 distribution of NiV V E125G expression. The localisation of NiV V E125G is predominantly cytoplasmic and like NiV V NIH it appears to prevent the translocation of STAT1 to the nucleus in response to IFNα.

Nipah V H248L can in some cases block the nuclear translocation of STAT1 in response to IFNα but Nipah V D280N cannot

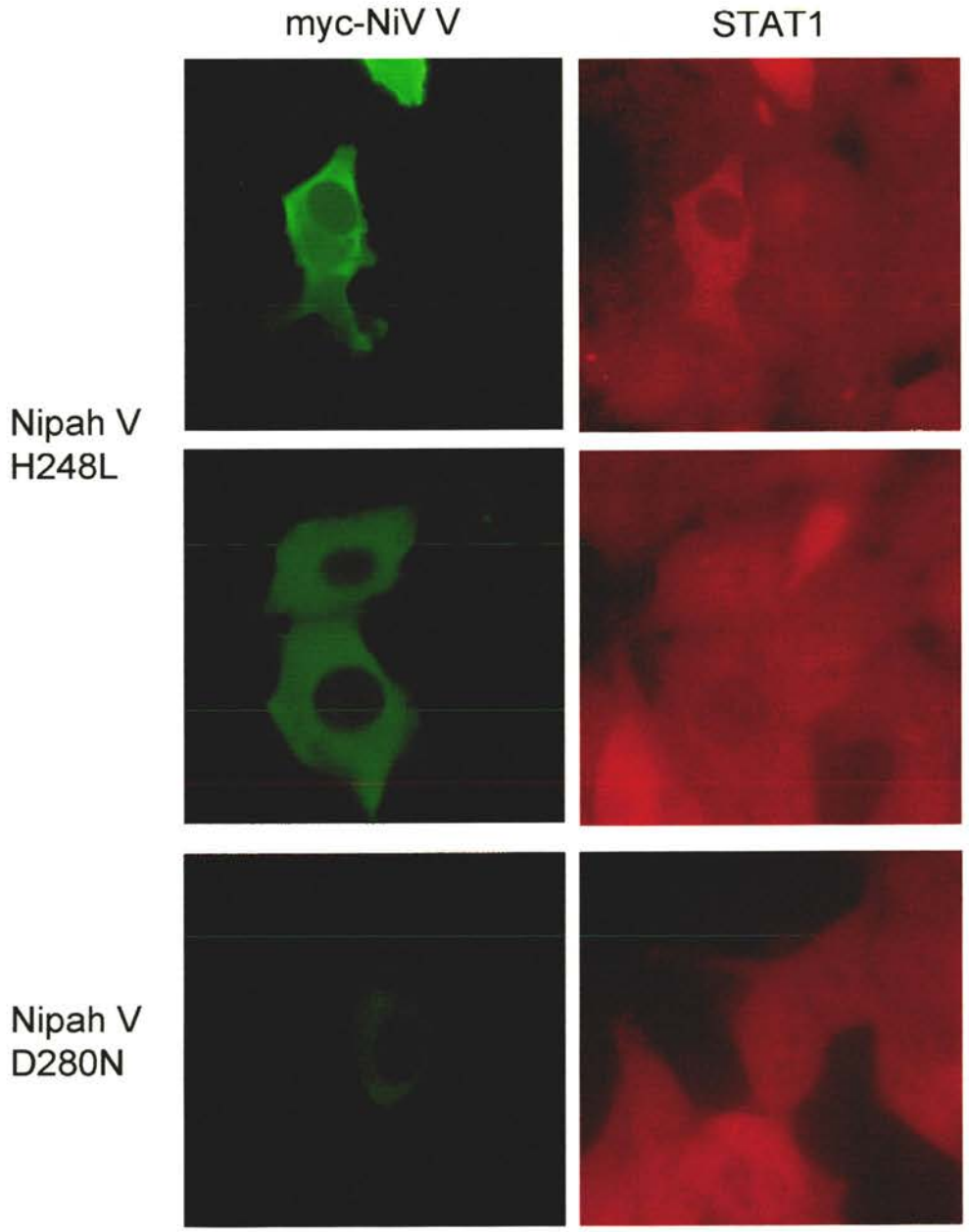
Fig. 34 shows the effects of the two other NiV V Geelong single mutants on the distribution of STAT1. As for NiV V Geelong and NiV V E125G, both NiV V H248L and NiV V D280N have a mainly cytoplasmic distribution, indicating that neither of the amino acid changes affects the localisation of NiV V protein. As seen in cells expressing NiV V Geelong, NiV V D280N expression has no effect on the translocation of STAT1 to the nucleus in response to IFNα. However, cells expressing NiV V H248L show a mixed pattern of STAT1 distribution in response to IFNα, with some cells having a similar distribution of STAT1 to neighbouring untransfected cells and other cells having only cytoplasmic STAT1 after IFNα treatment, suggesting that NiV V H248L can in some cases disrupt the normal distribution of STAT1, although it does not appear to block IFN signalling in the assays described previously.

Thus it appears that both NiV V NIH and NiV V E125G, which are able to block IFN signalling are also able to block the translocation of STAT1 to the nucleus in response to IFNα, suggesting a correlation between the ability to block IFN signalling and the ability to affect STAT1 distribution. However, there is also evidence of a block of STAT1 translocation by the 'non-functional' NiV V H248L in some cells, suggesting that the ability of NiV V to alter the distribution of STAT1 in response to IFNα may not be absolutely correlated with the ability of V to block IFN signalling.

Figure 34: STAT1 and Nipah V indirect immunofluorescence in 2fTGH cells transfected with Nipah V constructs

2fTGH cells were seeded on coverslips in 24 well plates and when 50% confluent were transfected with either Nipah V H248L or Nipah V D280N myc-tagged expression constructs. 24h post transfection the cells were stimulated with $1.8x10^4$ IU/ml IFNα, 1h @37°C.

Coverslips were harvested, fixed and permeabilised and indirect immunofluorescence was carried out using α-STAT1 MAb and α-myc PAb with α-mouse-Texas red and α-rabbit-FITC conjugated secondary antibodies. Slides were examined using a Nikon Microphot-FXA microscope.


myc-NiV V
STAT1
Nipah V
H248L
Nipah V
D280N

ii) STAT2 Immunofluorescence in cells expressing Nipah V

Similar immunofluorescence experiments using α-myc and α-STAT2 antibodies were carried out in 2fTGH cells to investigate the subcellular localisation of STAT2 in cells expressing NiV V proteins.

Nipah V NIH blocks the nuclear translocation of STAT2 in response to IFNα

The top panel of Fig. 35 shows that NiV V NIH is able to disrupt the normal subcellular localisation of STAT2, as in cells expressing this protein there is a lack of nuclear translocation of STAT2 in response to IFNα. This supports observations made in the literature using a similar NiV V construct.

Nipah V Geelong can in some cases block the nuclear translocation of STAT2 in response to IFNα

The lower two panels of Fig. 35 show that most cells expressing NiV V Geelong have a normal, predominantly nuclear distribution of STAT2 in response to IFNα. However, a significant subset of transfected cells show an altered distribution of STAT2 with either no STAT2 or very small amounts of STAT2 in the nucleus and instead an accumulation of STAT2 in the cytoplasm, a similar distribution to that seen in cells expressing NiV V NIH. There is some indication that this may be related to the level of NiV V Geelong in transfected cells as higher levels of α-myc fluorescence seem to correlate with cytoplasmic retention of STAT2.

Nipah V E125G blocks the nuclear translocation of STAT2 in response to IFNα whereas Nipah V H248L and Nipah V D280N do not

Fig. 36 shows the localisation of STAT2 in cells transfected with the three single mutants of NiV V Geelong. Only NiV V E125G disrupts the localisation of STAT2, which remains in the cytoplasm in cells expressing this construct, whereas cells expressing the H248L and D280N mutants have the same distribution of STAT2 as untransfected cells.

As seen for STAT1, there seems to be a general correlation of the ability of NiV V to disrupt STAT2 localisation with its ability to block IFN signalling. However, similar to the effects on STAT1 distribution in cells expressing NiV V H248L, some cells

Figure 35: STAT2 and Nipah V indirect immunofluorescence in 2fTGH cells transfected with Nipah V constructs

2fTGH cells were seeded on coverslips in 24 well plates once 50% confluent were transfected with either Nipah V NIH or Nipah V Geelong myc-tagged constructs expression constructs. 24h post transfection the cells were stimulated with $1.8x10^4$ IU/ml IFNα, 1h @37°C.

Coverslips were harvested, fixed and permeabilised and indirect immunofluorescence was carried out using α-STAT2 PAb and α-myc MAb with α-rabbit-Texas red and α-mouse-FITC conjugated secondary antibodies. Slides were examined using a Nikon Microphot-FXA microscope.

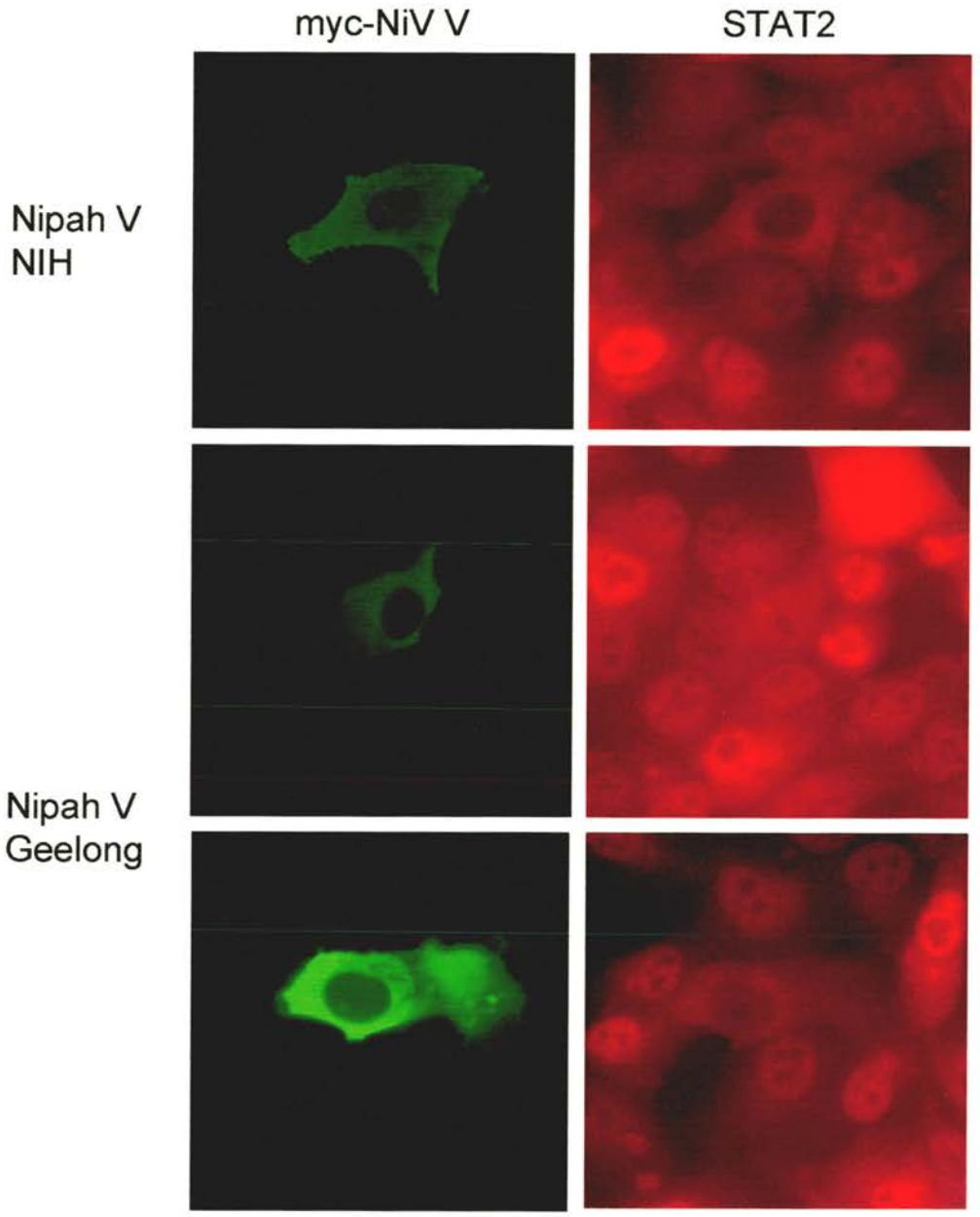
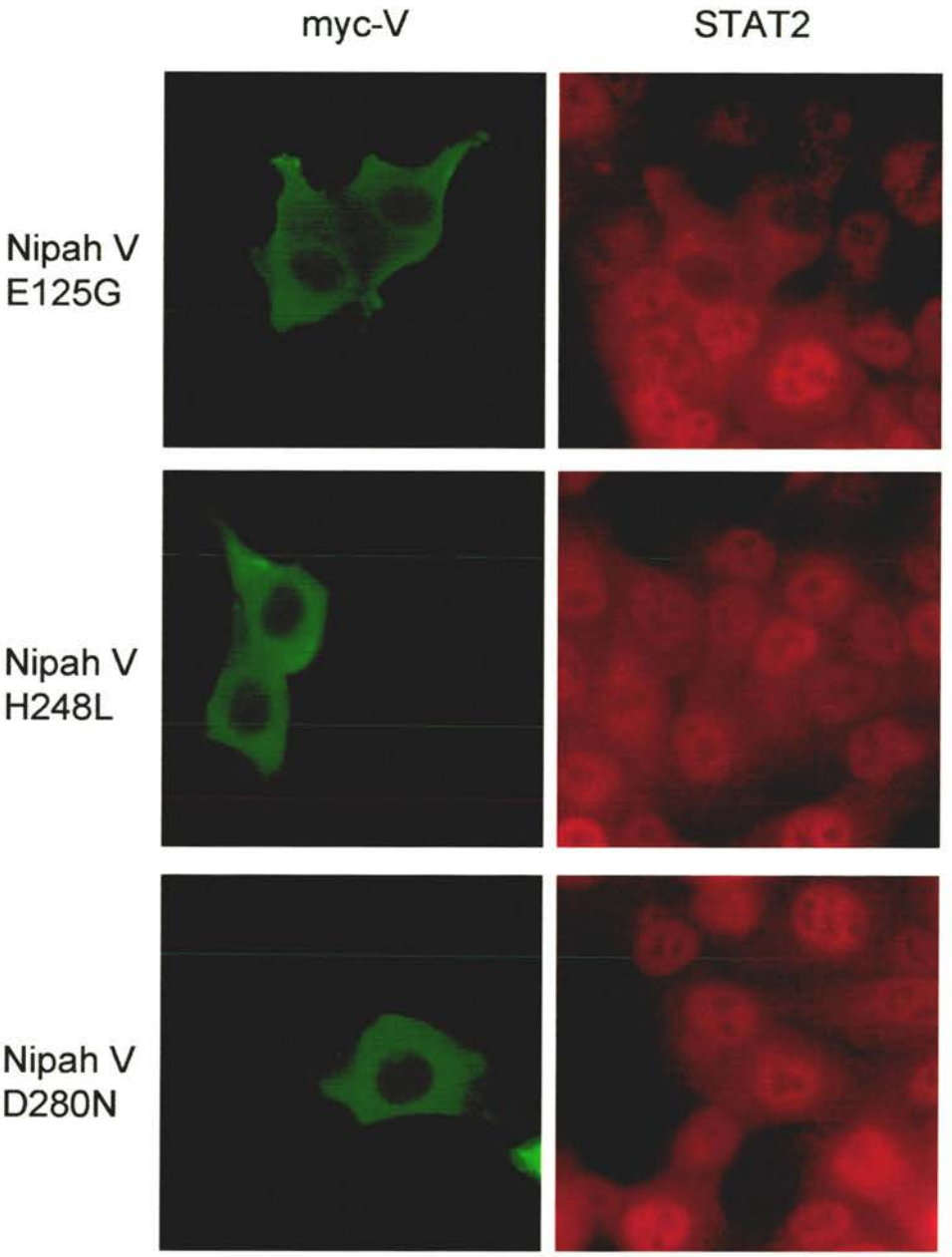

myc-NiV V
STAT2
Nipah V
NIH
Nipah V
Geelong

Figure 36: STAT2 and Nipah V indirect immunofluorescence in 2fTGH cells transfected with Nipah V constructs

2fTGH cells were seeded on coverslips in 24 well plates and when 50% confluent were transfected with either Nipah V E125G, Nipah V H248L or Nipah V D280N myc-tagged expression constructs. 24h post transfection the cells were stimulated with $1.8x10^4$ IU/ml IFNα, 1h @37°C.

Coverslips were harvested, fixed and permeabilised and indirect immunofluorescence was carried out using α-STAT2 PAb and α-myc MAb with α-rabbit-Texas red and α-mouse-FITC conjugated secondary antibodies. Slides were examined using a Nikon Microphot-FXA microscope.


myc-V
STAT2
Nipah V
E125G
Nipah V
H248L
Nipah V
D280N

expressing NiV V Geelong seem to be able to disrupt STAT2 distribution, possibly in a concentration-dependent manner. Thus it may not be possible to directly correlate the effects of NiV V proteins on STAT2 distribution with their ability to block IFN signalling.

Interactions between Nipah V and STAT1 & STAT2

Recent publications (Rodriguez et al. 2002; Park et al. 2003) have suggested that NiV V interacts directly with both STAT1 and STAT2, sequesters them in the cytoplasm and so blocks both IFNα/β and IFNγ signalling. If this is indeed the mechanism of action of NiV V, it would be logical to hypothesise that NiV V proteins able to block IFN signalling are able to bind STAT1 and STAT2 but that NiV V proteins not able to block IFN signalling have some kind of defect in STAT binding. In order to test this hypothesis, a number of experiments were performed to test the interactions of NiV V NIH, NiV Geelong and NiV V E125G with STAT1 and STAT2.

i) Immune Precipitation of endogenous STAT1 and STAT2 from 293 cells transiently transfected with Nipah V constructs

NiV V-STAT interactions were initially investigated by the immune precipitation of extracts prepared from 293 cells transiently transfected with myc-tagged NiV V constructs. Extracts were immune precipitated with α-myc MAb and the precipitated proteins were separated by SDS-PAGE and western blotted for either STAT1 or STAT2.

Nipah V does not co-immune precipitate STAT1 from transfected 293 cells

Fig. 37 shows the result of a STAT1 western blot of α-myc immune precipitates from 293 cells expressing NiV V constructs. There is no evidence of immune precipitated STAT1 in any of the samples, despite the presence of myc-NiV V (data not shown). Although repeated many times and with a range of immune precipitation buffers of differing stringencies, no evidence of immune precipitation of STAT1 by NiV V was seen. Thus as a result of these experiments it appears that there is no direct interaction between any of the NiV V constructs and STAT1.

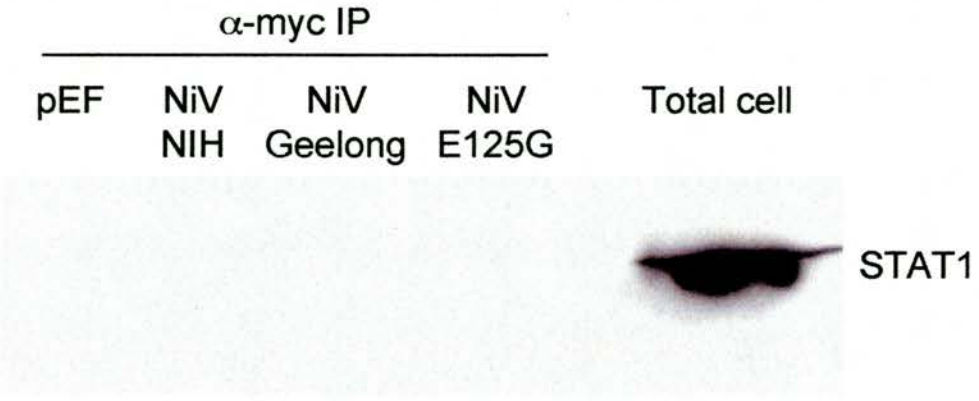


Figure 37: Immune Precipitation of Nipah V constructs from 293 cells: STAT1 Western Blot

293 cells were seeded in 60mm dishes and transfected with either NiV V NIH, NiV V Geelong or NiV V E125G myc-tagged expression constructs or empty pEF.plink2 vector. 48h post transfection cells were washed in ice-cold PBS and lysed in 250μl Immune Precipitation Buffer (IPB).

Lysates were cleared by centrifugation and mixed with α-myc MAb, 1h @4°C. 10μl 50% Protein G-sepharose suspension in IPB was added to each sample and incubated with mixing o/n @ 4°C. Beads were pelleted and washed 7 times with 1ml IPB. All s/n was removed and beads were resuspended in 35μl Disruption Buffer.

Samples were separated by 10% SDS-PAGE with a positive control cell lysate and transferred to PVDF. The samples were western blotted with α-STAT1 MAb and α-mouse HRP conjugated secondary antibody and detected using ECL reagents.

Nipah V NIH and Nipah V E125G efficiently co-immune precipitate STAT2 from transfected 293 cells whereas Nipah V Geelong does not

Fig. 38 shows the result of a STAT2 western blot of α-myc immune precipitates from 293 cells expressing NiV V. It is clear from this data that both NiV V NIH and NiV V E125G co-immune precipitate STAT2 whereas NiV V Geelong does not. Other experiments and longer western blot exposures (data not shown) suggested that there may be some binding of NiV V Geelong to STAT2 but this was always at a much lower level than that seen with NiV V NIH and NiV V E125G and not much above the background levels seen in precipitates from cells transfected with empty pEF.plink2 vector. It is possible that NiV V Geelong has a weak interaction with STAT2 and it may be that this interaction is not sufficient to enable NiV V Geelong to block IFN signalling.

The immune precipitation data agrees with the hypothesis that the ability to bind STAT2 is correlated with the ability to block IFN signalling as the two isolates that clearly precipitate STAT2 are also the ones capable of blocking IFN signalling. However, there is no support for the hypothesis that the ability to bind STAT1 is correlated with the ability to antagonise IFN signalling as none of the NiV V constructs studied precipitated STAT1 from transfected 293 cells.

ii) Immune precipitation of NiV V and STAT proteins from mixtures of *in vitro* transcribed-translated proteins

The published data showing an interaction between NiV V and STAT1 seemed very strong so it was decided to try a different approach to study the interactions between NiV V and STAT proteins. NiV V Geelong and NiV V E125G were sub-cloned into a vector which has a T7 promoter driving expression of a N-terminal myc-tagged insert (for details, see Cloning Strategies section). FLAG-tagged STAT1 and STAT2 constructs under the control of a T7 promoter were already available in the lab and these four constructs were *in vitro* transcribed, translated and labelled with ^{35}S-Met, Fig. 39 showing that labelled proteins of the expected sizes for STAT1, STAT2, NiV V Geelong and NiV V E125G were produced. These proteins were then mixed together to allow any interactions to occur and immune precipitated with either α-myc

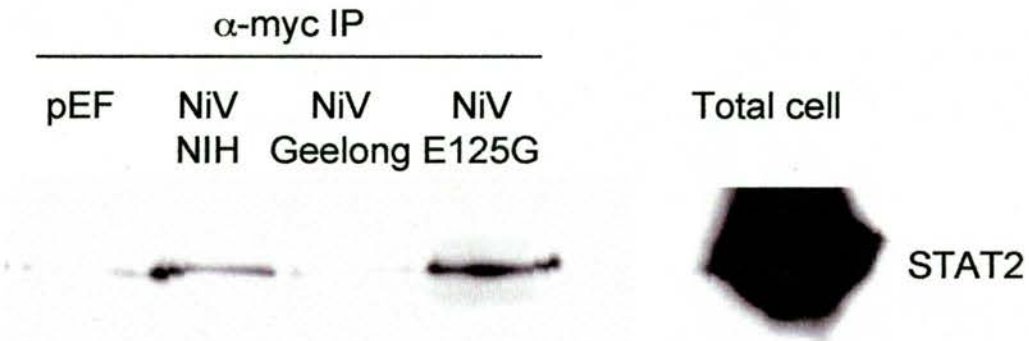


Figure 38: Immune Precipitation of Nipah V constructs from 293 cells : STAT2 Western Blot

293 cells were seeded in 60mm dishes and transfected with myc-Nipah V expression constructs or empty vector. 48h post transfection cells were washed in ice-cold PBS and lysed in 250μl Immune Precipitation Buffer (IPB).

Lysates were cleared by centrifugation and bound to anti-myc 9E10 MAb, 1h @ 4°C. 10μl 50% Protein G-sepharose suspension in IPB was added to each sample and incubated with mixing o/n @ 4°C.

Beads were pelleted and washed 7 times with 1ml IPB. All s/n was removed and beads were resuspended in 35μl 4x Disruption Buffer.

Samples were separated on a 10% SDS-PAG with a positive control cell lysate and transferred to PVDF. The membrane was western blotted with anti-STAT2 PAbs (Santa Cruz, 1:2,000) and anti-rabbit HRP 2° Ab. HRP was detected by ECL method.

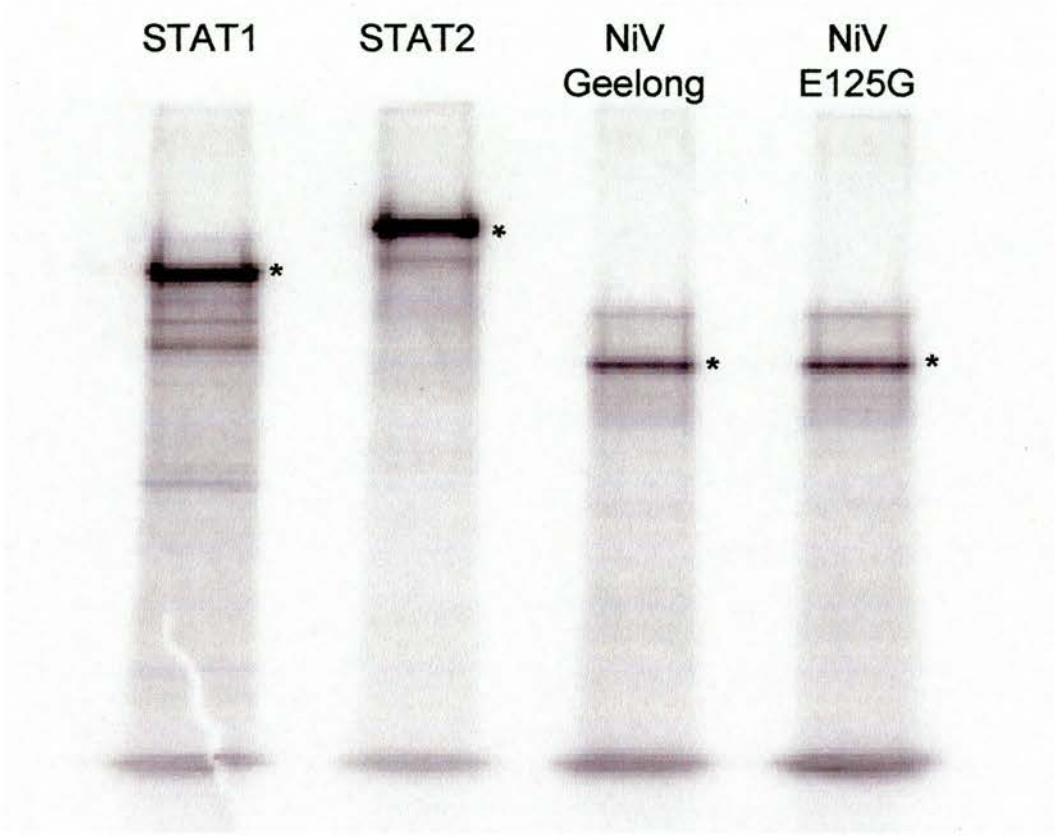


Figure 39: *In vitro* transcribed-translated STAT1, STAT2, Nipah V Geelong and Nipah V E125G

STAT1, STAT2, Nipah V Geelong and Nipah V E125G under the control of T7 promoters were *in vitro* transcribed-translated and labelled with ^{35}S-Met using a TNT kit (Promega).

1/10th of the products of the four reactions were separated by 10% SDS-PAGE and the gel was stained, destained, dried and exposed to a phosphorimager plate o/n.

MAb to pull down NiV V, α-FLAG MAb to pull down STAT1 or STAT2 or a non-specific α SV5 P MAb as a negative control.

Neither Nipah V Geelong nor Nipah V E125G interact with STAT1 in vitro

Fig. 40 shows the results of mixing and subsequent immune precipitation of *in vitro*-transcribed and translated myc-NiV V Geelong, myc-NiV V E125G and FLAG-STAT1. Neither NiV V construct is pulled down by the anti-FLAG MAb precipitations and neither of the anti-myc MAb precipitations pull down STAT1. It therefore appears from this experiment that there is no direct interaction between NiV V Geelong and STAT1 or NiV V E125G and STAT1, as already suggested by immune precipitations from 293 cells.

Nipah V E125G but not Nipah V Geelong interacts with STAT2 in vitro

Fig. 41 shows the results of mixing and subsequent immune precipitation of *in vitro*-transcribed and translated myc-NiV V Geelong, myc-NiV V E125G and FLAG-STAT2. It seems that while there is no evidence of an interaction between NiV V Geelong and STAT2, there is an interaction between NiV V E125G and STAT2 as judged by the presence of a band of the same size as STAT2 in the sample precipitated with α-myc MAb, suggesting that the NiV V E125G pulled down with α-myc MAb is associated with STAT2. It is not clear whether the band of approximately the same size as NiV V E125G in the α-FLAG MAb precipitation (lane 4) is NiV V E125G as there was a band of a similar size in the total STAT2 run-off (data not shown), making it possible that this band is a non-specific species. As a band of the same size can also be seen in the α-FLAG MAb precipitation from the NiV V Geelong mixture it seems likely that this is the case.

These data confirm the findings of the immune precipitations from transiently transfected 293 cells in that there is no evidence for a direct interaction between any of the NiV V proteins studied and STAT1 but there does appear to be an interaction between STAT2 and both NiV V NIH (in 293 immune precipitations) and NiV V E125G (in 293 and *in vitro* immune precipitations). This suggests that the initial hypothesis was at least partially correct in that binding to STAT2, if not STAT1, correlates with the ability of NiV V to antagonise IFN signalling.

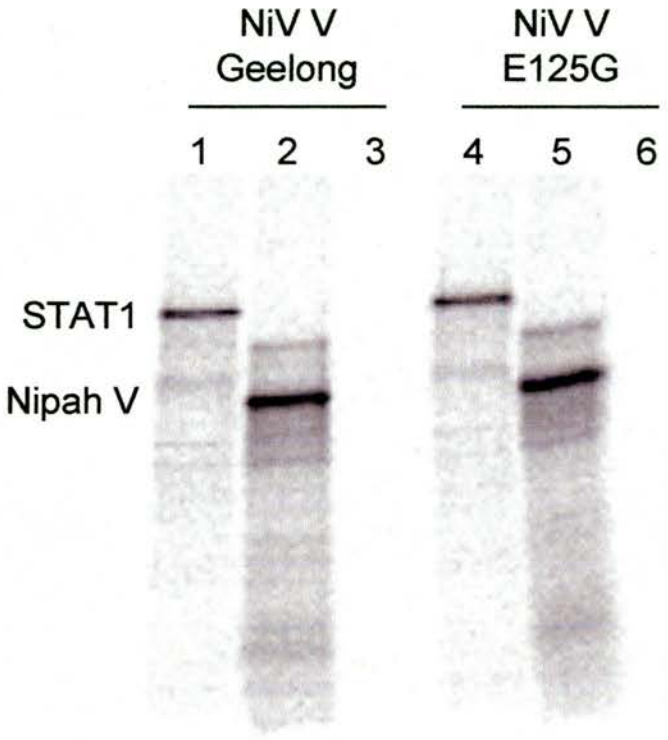


Figure 40: Pull-down of *in vitro*-transcribed and translated STAT1 mixed with Nipah V Geelong and Nipah V E125G

FLAG-STAT1, myc-NiV Geelong and myc-NiV E125G were *in vitro*-transcribed and translated and ^{35}S-Met labelled using a TNT kit (Promega).

Mixtures of STAT1/NiV Geelong and STAT1/NiV E125G were made and incubated on ice, 30min. These mixtures were then divided between three tubes containing either α-FLAG MAb, α-myc 9E10 MAb or α-SV5 P Mab. These protein/antibody mixes were incubated on ice, 30min. 10μl 50% Protein G-sepharose suspension was then added to each tube and these were incubated with mixing @4°C, 30min. The beads were then washed 7 times with IPB and all supernatant removed after the final wash. The bound proteins were eluted in 4xDB, boiled, separated by 10% SDS-PAGE and analysed using a phosphorimager.

Lanes 1&4: α-FLAG MAb IP, lanes 2&5: α-myc MAb IP, lanes 3&6: αSV5 P MAb IP (-ve control).

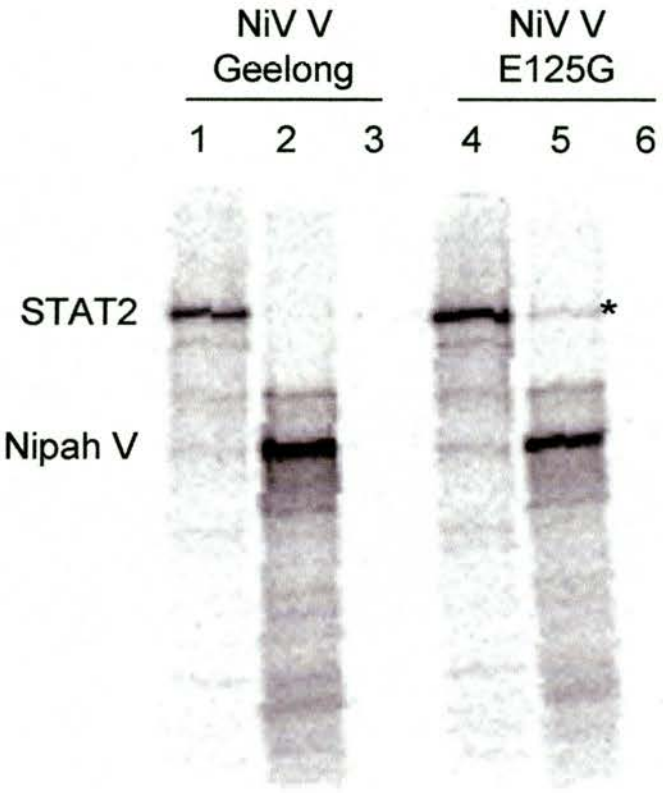


Figure 41: Pull-down of *in vitro*-transcribed and translated STAT2 mixed with Nipah V Geelong and Nipah V E125G

FLAG-STAT1, myc-NiV Geelong and myc-NiV E125G were *in vitro*-transcribed and translated and ^{35}S-Met labelled using a TNT kit (Promega).

Mixtures of STAT2/NiV Geelong and STAT2/NiV E125G were made and incubated on ice, 30min. These mixtures were then divided between three tubes containing either α-FLAG MAb, α-myc 9E10 MAb or α-SV5 P Mab. These protein/antibody mixes were incubated on ice, 30min. 10μl 50% Protein G-sepharose suspension was then added to each tube and these were incubated with mixing @4°C, 30min. The beads were then washed 7 times with IPB and all supernatant removed after the final wash. The bound proteins were eluted in 4xDB, boiled, separated by 10% SDS-PAGE and analysed using a phosphorimager.

Lanes 1&4: α-FLAG MAb IP, lanes 2&5: α-myc MAb IP, lanes 3&6: αSV5 P MAb IP (-ve control). Putative STAT2 band marked with an asterisk.

Hendra V and IFN signalling

As HeV V is very similar to NiV V, it was expected that it would exhibit similar IFN evasion properties. The cloned and tagged HeV V was assayed for its ability to antagonise IFN signalling as already described for NiV V.

Hendra V blocks IFNα/β signalling

HeV V was found to block IFNα/β signalling to a similar extent as SV5 V in Vero cells, as can be seen in Fig. 42. Subsequently this result was confirmed by a publication that also showed that HeV V blocks IFNγ signalling (Rodriguez et al. 2003).

II. MAPUERA VIRUS

MapV was isolated on a single occasion from a bat in the Brazilian rainforest and although classified as a member of the *Rubulavirus* genus, little is known about its molecular pathogenicity. It was of interest for this study as its sequence homology to SV5 and other rubulaviruses makes it likely to have some form of IFN evasion strategy, probably involving the viral V protein. A MapV V PCR fragment was obtained from Lin-Fa Wang at CSIRO in Geelong, Australia and cloned into a mammalian expression vector with an N-terminal myc epitope tag (see Cloning Strategies section for details).

Mapuera V and IFN signalling

The ability of the cloned MapV V to block IFN signalling was examined using assays based on the transient expression of MapV V and IFN-responsive luciferase reporters.

Mapuera V blocks IFNα/β signalling

Fig. 43 shows that expression of MapV V in Vero cells also transfected with an IFNα/β-responsive luciferase reporter results in a block of IFNα/β signalling to a similar extent as expression of SV5 V. MapV V also blocks IFNα/β signalling in HeLa cells (shown in Fig. 46).

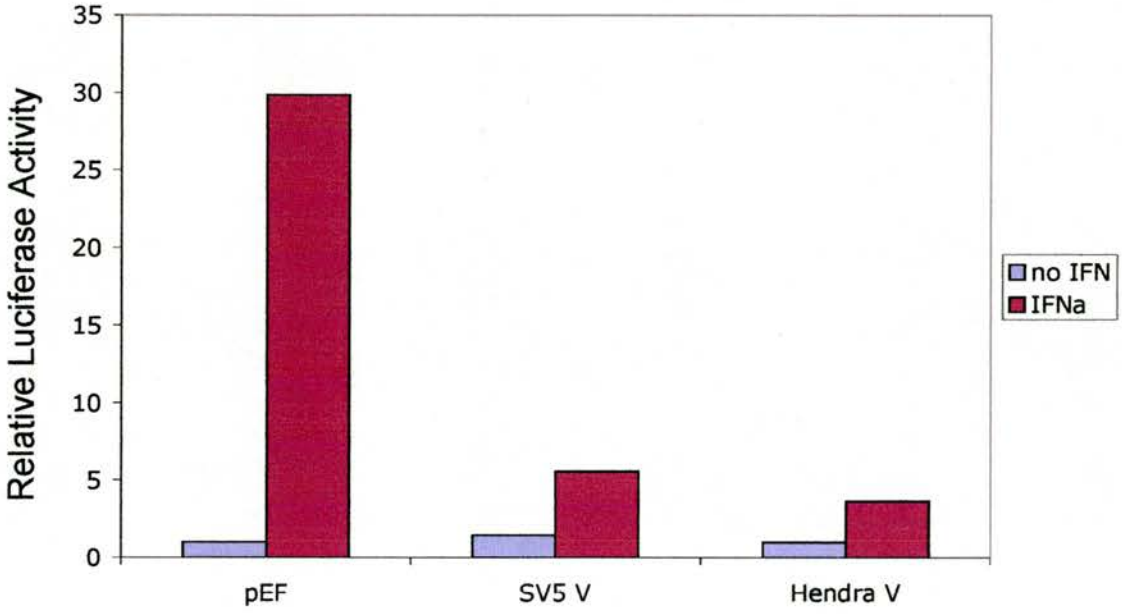


Figure 42: IFNα/β signalling assay in Vero cells: Hendra V construct

Vero cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Hendra V expression constructs. 48h post transfection cells were stimulated or not with IFNα, $1.8x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

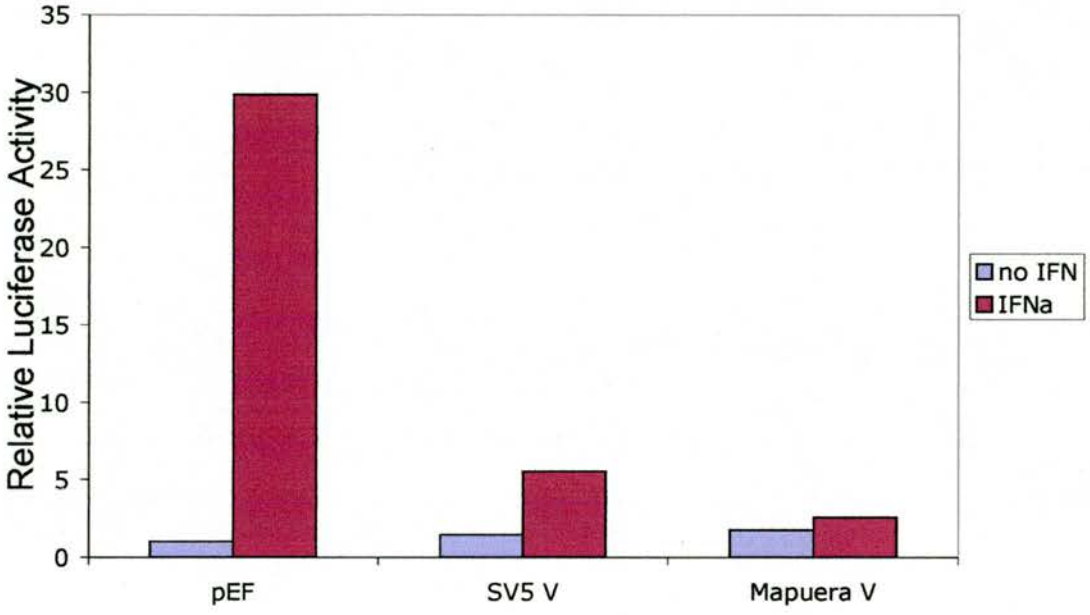


Figure 43: IFNα/β signalling assay in Vero cells: Mapuera V construct

Vero cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Mapuera V expression constructs. 48h post transfection cells were stimulated or not with IFNα, $1.8x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

Mapuera V blocks IFNγ signalling

Expression of MapV V also blocks IFNγ signalling in Vero cells expressing an IFNγ-responsive luciferase reporter to a degree comparable to SV5 V as can be seen in Fig. 44.

Mapuera V does not block IFN signalling in murine cells

In contrast to the effects seen in Vero cells, transfection of murine BF cells with MapV V does not result in a block of IFNα/β signalling, unlike expression of SV5 V^{N100D}, shown in Fig. 45.

Neither Mapuera V N-terminus or C-terminus block IFNα/β signalling

Truncations of MapV V were constructed so that the N-terminal portion (Vn) contained the shared P/V region and the C-terminal portion (Vc) contained the V-unique region downstream of the editing site (see Cloning Strategies section for details). Both constructs were assayed in HeLa cells for their ability to block IFNα/β signalling and Fig. 46 shows that neither truncation is capable of blocking IFNα/β signalling compared to the full-length MapV V or SV5 V.

Mapuera V and IFN Production

As already mentioned, SV5 V has been found to block the activation of the IFNβ promoter in response to dsRNA and this property depends on its cysteine-rich C-terminus. As MapV V contains the same seven cysteine residues as well as number of other conserved residues in comparison to SV5 V (see Fig. 21), the ability of MapV V to block the activation of the IFNβ promoter was assayed.

Mapuera V blocks the activation of the IFNβ promoter

IFNβ promoter activation assays in Vero cells, seen in Fig. 47, show that MapV V blocks the activation of the IFNβ promoter by dsRNA to the same extent as SV5 V. Expression of the MapV V truncations in the same assay shows that MapV Vc expressed alone effectively blocks IFNβ promoter activation, whereas MapV Vn does not, indicating that it is the residues in the C-terminal unique region of MapV V which are required and sufficient to block IFN production.

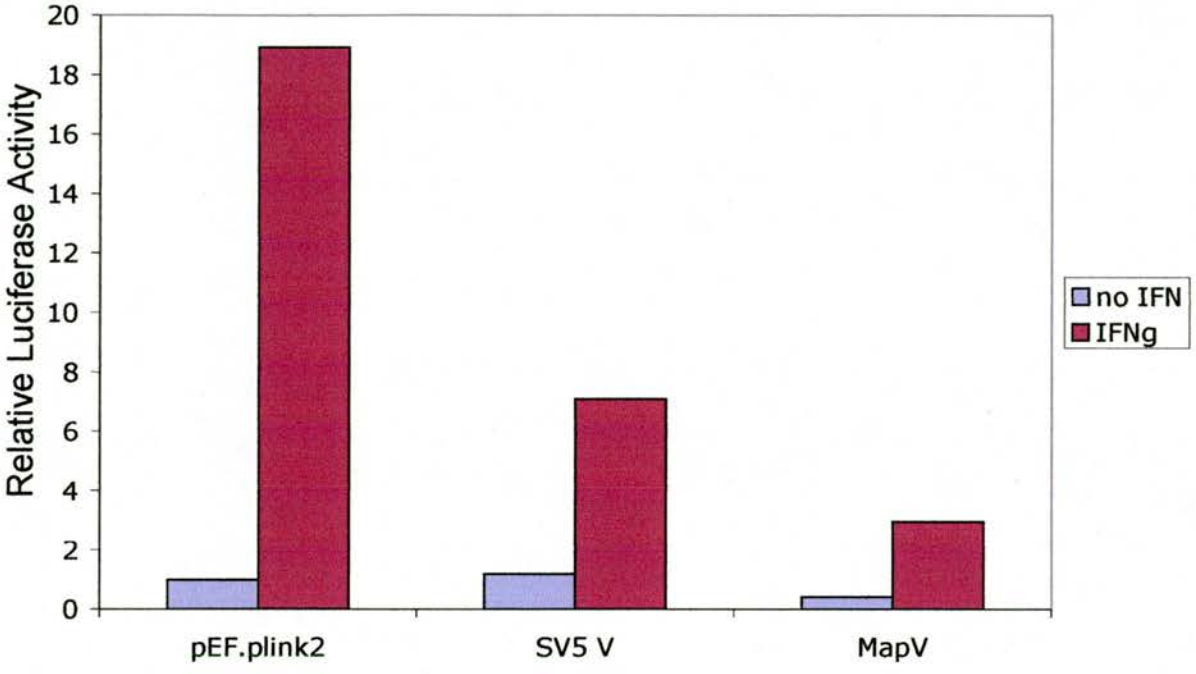


Figure 44: IFNγ signalling assay in Vero cells: Mapuera V construct

Vero cells were seeded in 6-well plates and transfected with an IFNγ-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Mapuera V expression constructs. 48h post transfection cells were stimulated or not with IFNα, $1.0x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

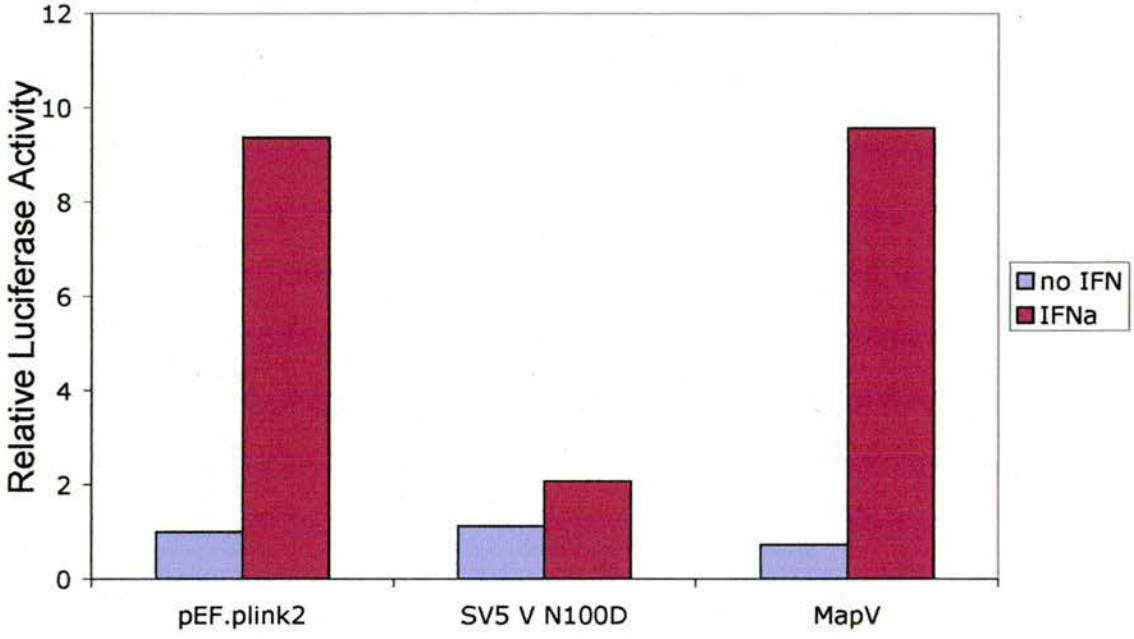


Figure 45: IFNα/β signalling assay in BF cells: Mapuera V construct

BF cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V^{N100D} or Mapuera V expression constructs. 48h post transfection cells were stimulated or not with 'Universal Type I IFN', $1.0x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

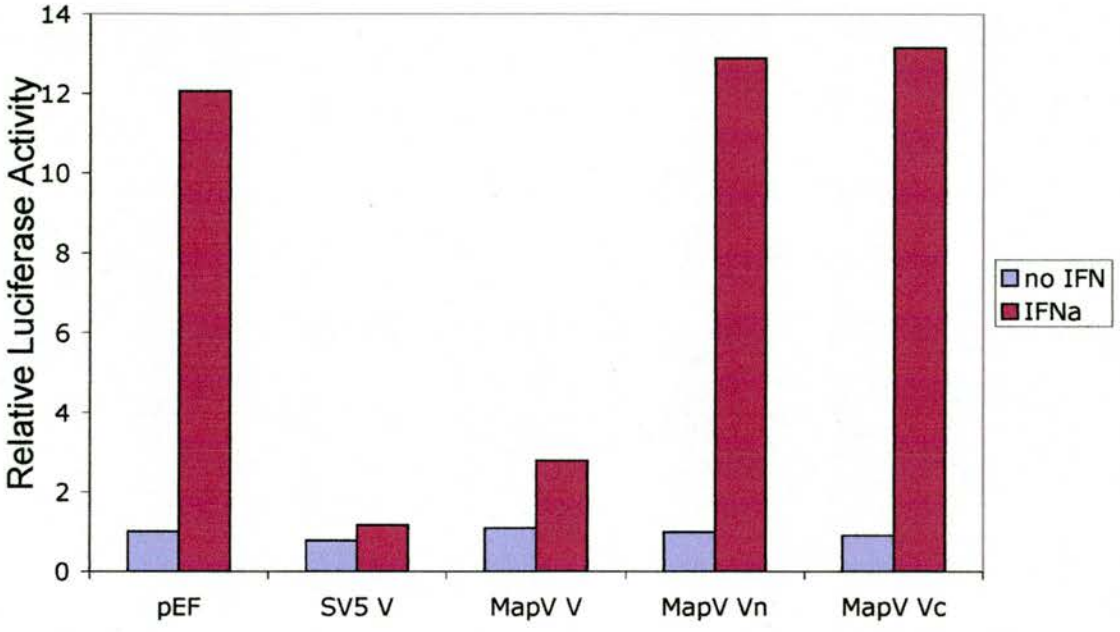


Figure 46: IFNα/β signalling assay in HeLa cells: Mapuera V truncations

HeLa cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Mapuera V full-length and truncated expression constructs. 48h post transfection cells were stimulated or not with IFNα, $1.8x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

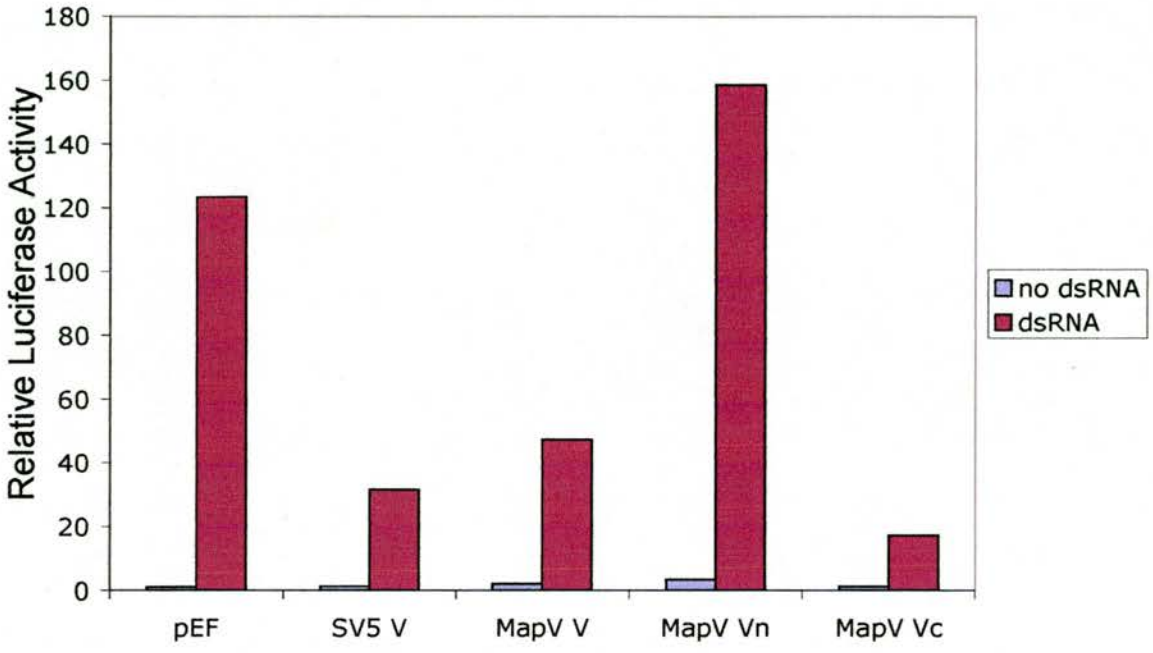


Figure 47: IFNβ promoter activation assay in Vero cells: Mapuera V construct

Vero cells were seeded in 6-well plates and transfected with a dsRNA-responsive IFNβ promoter-luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Mapuera V expression constructs. 48h post transfection cells were stimulated or not with 2.5μg poly(I):poly(C) per well, 12h @37°C.

Cells were harvested in 200μl each Luciferase buffers A and B and 300μl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

Interaction of Maperua V with STAT1 and STAT2

It has been reported that SV5 V interacts with both STAT1 and STAT2 and that these interactions are involved in its function as an IFN antagonist (Parisien et al. 2002b; Ulane and Horvath 2002). In order to investigate whether MapV V interacts with these proteins, an α-myc MAb was used to precipitate myc-MapV V and any associated proteins from 293 cells transiently expressing MapV V. These precipitates were separated by SDS-PAGE and western blotted with antibodies specific to STAT1 and STAT2.

STAT1 does not co-immune precipitate with Mapuera V

Fig. 48 shows the results of an α-STAT1 western blot of samples immune precipitated from 293 cells transiently transfected with MapV V. STAT1 is not present in the precipitates from either MapV V expressing cells or those transfected with empty pEF.plink2 vector when compared to the total cell lysate. Despite numerous attempts with a variety of immune precipitation conditions, STAT1 was never seen in association with MapV V, indicating that these two proteins do not directly interact.

STAT2 is co-immune precipitated with Mapuera V

Fig. 49 shows that immune precipitates from 293 cells expressing MapV V, but not those from cells transfected with empty pEF.plink2 vector, contain STAT2, indicating an interaction between MapV V and STAT2 in these cells.

Interaction of GST-Mapuera V with DDB1

Several paramyxovirus V proteins have been shown to interact with DDB1 and it has been demonstrated that the interaction of SV5 V with DDB1 is essential for STAT1 degradation and IFN evasion in V expressing cells (Andrejeva et al. 2002a). Hence it was of interest to discover whether MapV V interacts with DDB1. In order to investigate this, a GST-MapV V fusion protein was constructed and expressed in bacterial cells alongside GST alone and GST-SV5 V, purified on a glutathione column and subsequently bound to glutathione beads. These beads were incubated with ^{35}S-Met labelled, *in vitro* transcribed and translated DDB1 and used to precipitate the GST fusions and any associated proteins.

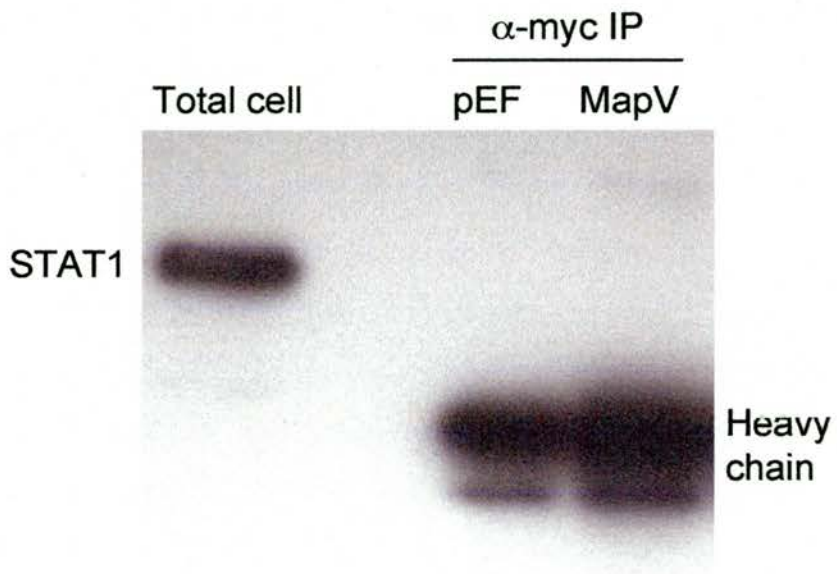


Figure 48: Immune Precipitation of Mapuera V from 293 cells: STAT1 Western Blot

293 cells were seeded in 60mm dishes and once 50% confluent were transfected with either pEF.plink2 empty vector or a myc-tagged Mapuera V expression construct. 48h post transfection cells were washed in ice-cold PBS and lysed in 250μl Immune Precipitation Buffer (IPB). Lysates were cleared by centrifugation and mixed with α-myc MAb, 1h @4°C. 10μl 50% Protein G-sepharose suspension in IPB was then added to each sample and incubated with mixing o/n @ 4°C. The beads were pelleted and washed 7 times with IPB and all s/n was removed after the final wash. The beads were then resuspended in 35μl Disruption Buffer and boiled to elute the bound proteins.

The precipitates were separated by 10% SDS-PAGE with a positive control total cell lysate and transferred to PVDF. The samples were western blotted with α-STAT1 MAb and α-mouse-HRP conjugated secondary antibody and detected using ECL reagents.

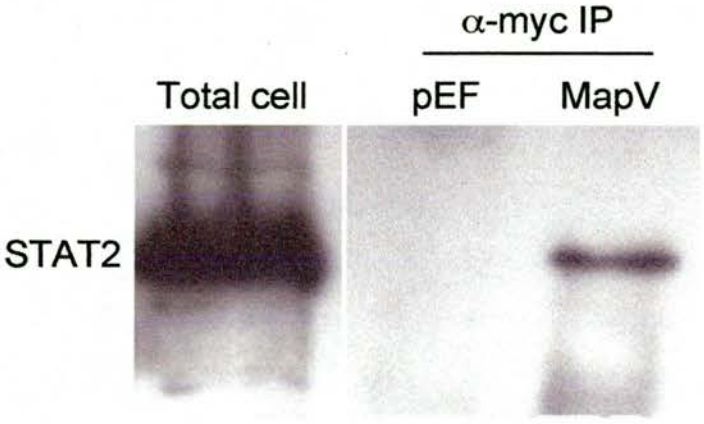


Figure 49: Immune Precipitation of Mapuera V from 293 cells: STAT2 Western Blot

293 cells were seeded in 60mm dishes and once 50% confluent were transfected with either pEF.plink2 empty vector or a myc-tagged Mapuera V expression construct. 48h post transfection cells were washed in ice-cold PBS and lysed in 250μl Immune Precipitation Buffer (IPB). Lysates were cleared by centrifugation and mixed with α-myc MAb, 1h @4°C. 10μl 50% Protein G-sepharose suspension in IPB was then added to each sample and incubated with mixing o/n @ 4°C. The beads were pelleted and washed 7 times with IPB and all s/n was removed after the final wash. The beads were then resuspended in 35μl Disruption Buffer and boiled to elute the bound proteins.

The precipitates were separated by 10% SDS-PAGE with a positive control total cell lysate and transferred to PVDF. The samples were western blotted with α-STAT2 PAb and α-rabbit-HRP conjugated secondary antibody and detected using ECL reagents.

Mapuera V does not interact with DDB1 in vitro

Fig. 50 shows that the glutathione-agarose pulldown of the GST fusion proteins was successful and that approximately equal amounts of GST, GST-SV5 V and GST-MapV V were precipitated as judged by Coomassie Blue staining. The phosphorimage of the same gel in Fig. 51 shows that DDB1 is clearly present in the GST-SV5 V precipitate but not in the GST alone or GST-MapV V precipitates, suggesting that MapV V and DDB1 do not interact. This was also suggested by the results of α-myc immune precipitations from ^{35}S-Met labelled Cos7 cells expressing MapV V, which failed to show any evidence of a co-precipitating species of the correct size for DDB1 (data not shown).

Mechanism of Mapuera V Action

As MapV is a member of the *Rubulavirus* genus it may have a similar IFN evasion mechanism to other members of the genus such as SV5, MuV, hPIV2 and SV41, all of which antagonise IFN signalling via the degradation of either STAT1 or STAT2. As both IFNα/β and IFNγ signalling are blocked by MapV V, the obvious candidate for degradation by MapV V is STAT1, which is common to both pathways.

To study the effects of MapV V on STAT levels, a cell line constitutively expressing MapV V was required as it is difficult to obtain very high transfection efficiencies using transient transfections and for this type of experiment an expression efficiency of near to 100% is required, as any cells in the sample not expressing MapV V would contain normal levels of STAT proteins and thus could mask any degradation occurring in the MapV V-expressing cells. A stable cell line was created by transfection of HeLa cells with a pEF.myc.MapV-V.IRES.neo construct and selection with the antibiotic G418. The MapV V construct has a poliovirus IRES between the myc-tagged MapV V gene and the G418^R (neo^R) gene which allows the expression of both proteins from a single mRNA, which in theory ensures that all cells selected by the G418 treatment also express MapV V. A number of positive clones were identified by α-myc western blots (data not shown), one of which was selected for use in further experiments and is referred to here as H/MapV_V.

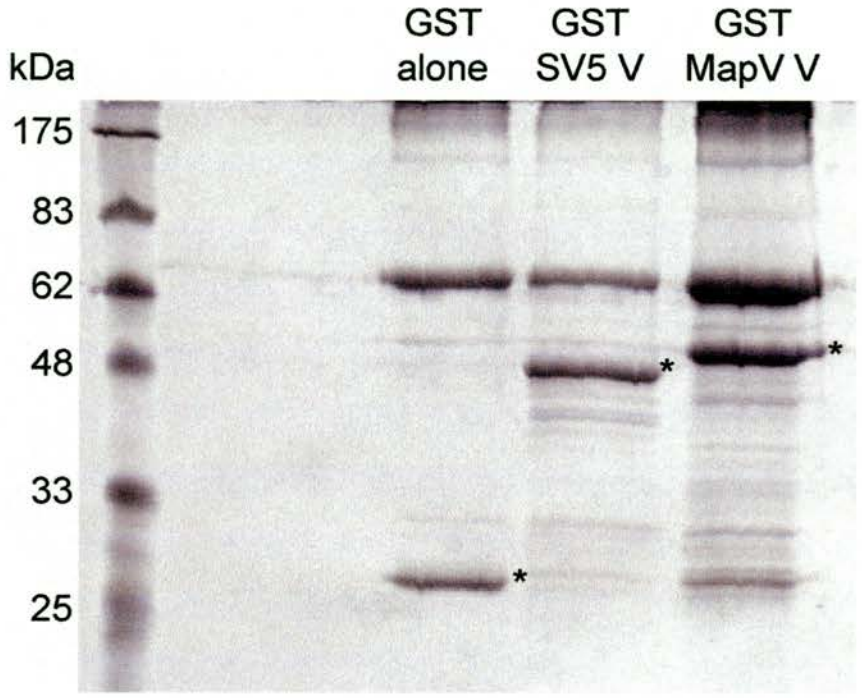


Figure 50: Pulldown of *in vitro*-transcribed and translated DDB1 with GST-SV5 V and GST-Mapuera V: Coomassie Blue stained gel

^{35}S-Met labelled DDB1 was *in vitro*-transcribed and translated using a TNT kit (Promega). The resulting protein was mixed with either GST alone, GST-SV5 V or GST-MapV pre-bound to glutathione-agarose and incubated, 30min @4°C. The beads were subsequently pelleted and washed and the resulting protein complexes were dissociated in Disruption Buffer and separated by 10% SDS-PAGE. The resulting gel was stained with Coomassie Blue and dried.

Asterisks mark the positions of GST, GST-SV5 V and GST-MapV V.

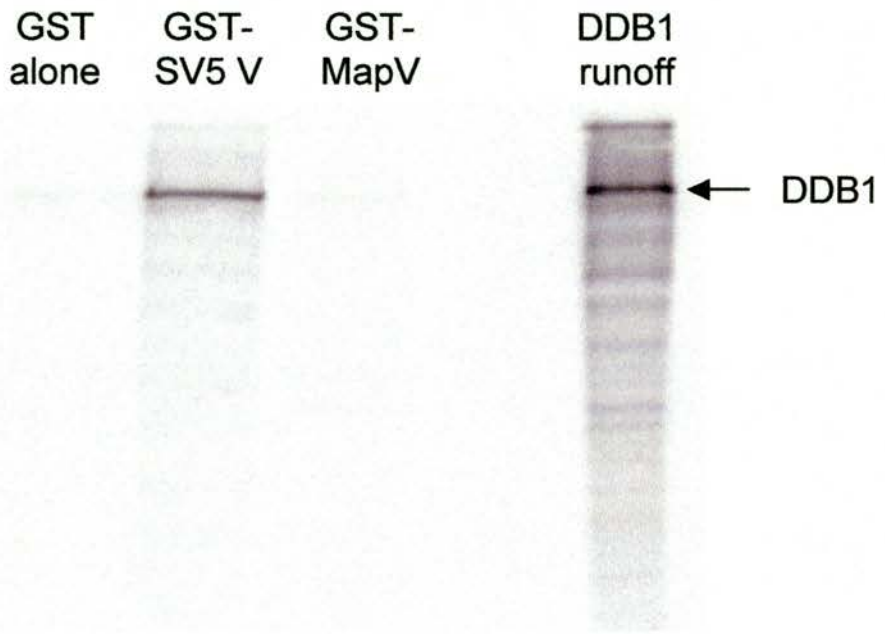


Figure 51: Pulldown of *in vitro*-transcribed and translated DDB1 with GST-SV5 V and GST-Mapuera V: Phosphorimage

^{35}S-Met labelled DDB1 was *in vitro*-transcribed and translated using a TNT kit (Promega). The resulting protein was mixed with either GST alone, GST-SV5 V or GST-MapV pre-bound to glutathione-agarose and incubated, 30min @4°C. The beads were subsequently pelleted and washed and the resulting protein complexes were dissociated in Disruption Buffer and separated by 10% SDS-PAGE. The resulting gel was stained with Coomassie Blue, dried and exposed to a phosphorimager screen.

H/MapV_V cell line expresses Mapuera V protein in all cells

The upper panel of Fig. 52 shows the results of α-myc immunofluorescence of naïve HeLa and H/MapV_V cells. In the H/MapV_V cells there is distinct nuclear fluorescence in contrast to the naïve cells in which only a small amount of cytoplasmic fluorescence is seen, indicating that myc-MapV V is expressed in the nuclei of H/MapV_V cells, a distribution similar to that of stably expressed SV5 V in similar cell lines. The lower panels show the same cells stained with DAPI, which is specific for nucleic acids and confirms that all of the H/MapV_V cells in the field of view are positive for myc-MapV expression.

STAT1, STAT2, STAT3 and p48 are not degraded in the H/MapV_V cell line

Western blots of both naïve HeLa and H/MapV_V cell lysates shown in Fig. 53 show that STAT1, STAT2, STAT3 and p48 are all present in both cell types, indicating that MapV V expression does not result in the degradation of any of these components of the IFN signalling pathway. A further examination of STAT1 levels in response to IFNα treatment, the results of which are shown in Fig. 54, demonstrates that although STAT1 is present in H/MapV_V cells it is not upregulated in response to IFNα in contrast to naïve HeLa cells, indicating that although STAT1 is not degraded, IFNα/β signalling is blocked in H/MapV_V cells.

Mapuera V does not degrade exogenous STAT1 in transiently transfected 293 cells

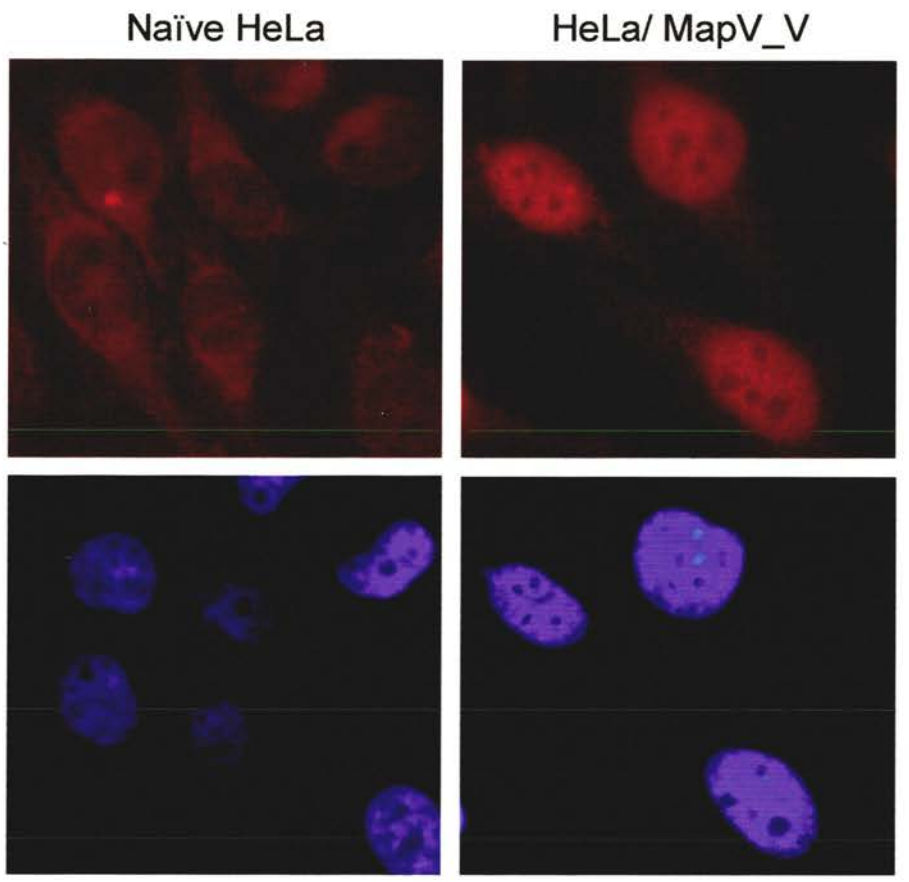
Another approach previously used to demonstrate that SV5 V requires the expression of both exogenous STAT1 and STAT2 to bring about the degradation of the exogenous STAT1 was used for MapV V. 293 cells were transiently transfected with combinations of myc-STAT1, STAT2, SV5 V and myc-MapV V and Fig. 55 shows that whilst in cells expressing SV5 V the exogenous STAT1 is degraded in the presence of exogenous STAT2, in cells expressing MapV V STAT1 is not degraded either with or without exogenous STAT2.

To conclude, it is clear that MapV V blocks both IFNα/β and IFNγ signalling but as yet the mechanism for this block is not known. The experiments outlined above suggest that this IFN antagonism is not achieved via the degradation of STAT1, in contrast with other rubulaviruses such as SV5 and MuV. It also appears that other

Figure 52: Indirect immunofluorescence of naïve and MapV V-expressing HeLa cells

Naïve HeLa and HeLa/MapV_V cells were seeded onto coverslips in 24-well plates. Once 80% confluent the coverslips were harvested, fixed and permeabilised before indirect immunofluorescence with α-myc MAb and α-mouse-Texas Red conjugated secondary antibody with DAPI. Slides were examined using a Nikon Microphot-FXA microscope.

The top panels show α-myc immunofluorescence and bottom panels show DAPI-stained nuclei.


Naïve HeLa
HeLa/ MapV_V

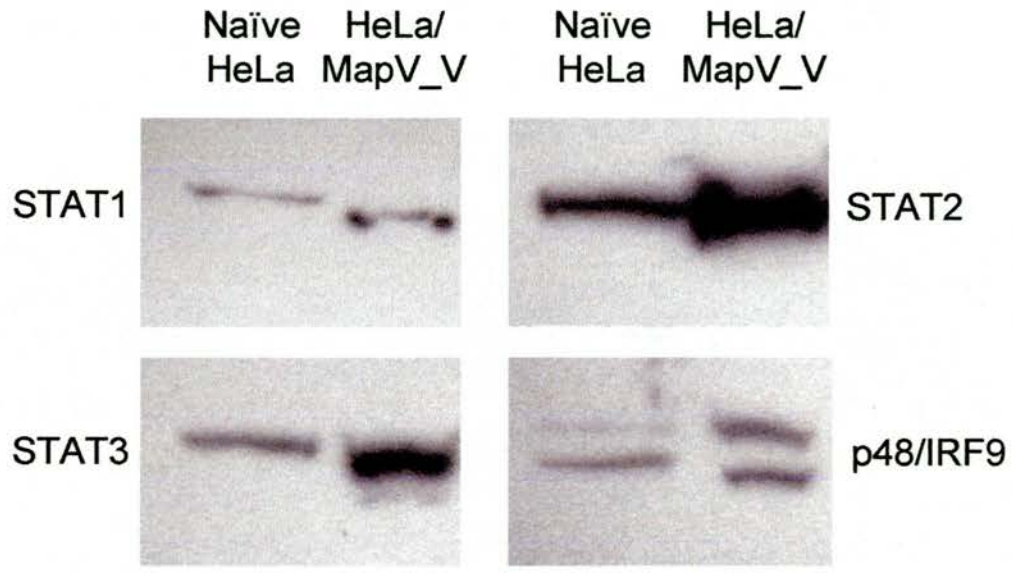


Figure 53: Anti-STAT1, STAT2, STAT3 and p48 western blots of naïve and MapV V-expressing HeLa cells

Naïve Hela and HeLa/MapV_V cells were seeded in 25cm^2 flasks. Once 90% confluent the cells were lysed in 300µl Disruption Buffer and four pairs of samples, each 1/20th of the total volume of lysate were separated by 10% SDS-PAGE and transferred to PVDF.

The samples were western blotted with either α-STAT1 MAb, α-STAT2 PAb, α-STAT3 MAb and α-IRF-9 PAb and α-mouse-HRP or α-rabbit-HRP conjugated secondary antibody as appropriate and detected using ECL reagents.

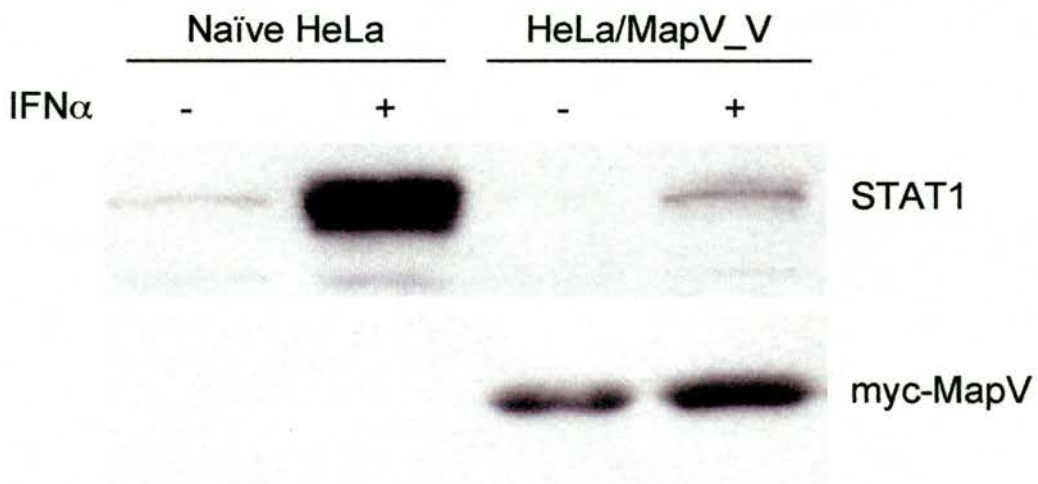


Figure 54: Stimulation of naïve HeLa and HeLa/MapV_V cells with IFNα: STAT1 and myc Western Blots

Naïve HeLa and HeLa/MapV_V cells were seeded into a 6-well plate and once 80% confluent were stimulated with IFNα, $1.8x10^3$ IU/ml, 22h @37°C. The cells were then lysed in 100µl Disruption Buffer and 1/5th each sample was separated by 10% SDS-PAGE and transferred to PVDF.

The samples were western blotted with α-STAT1 PAb and α-myc MAb and α-rabbit and α-mouse-HRP conjugated secondary antibodies as appropriate and detected using ECL reagents.

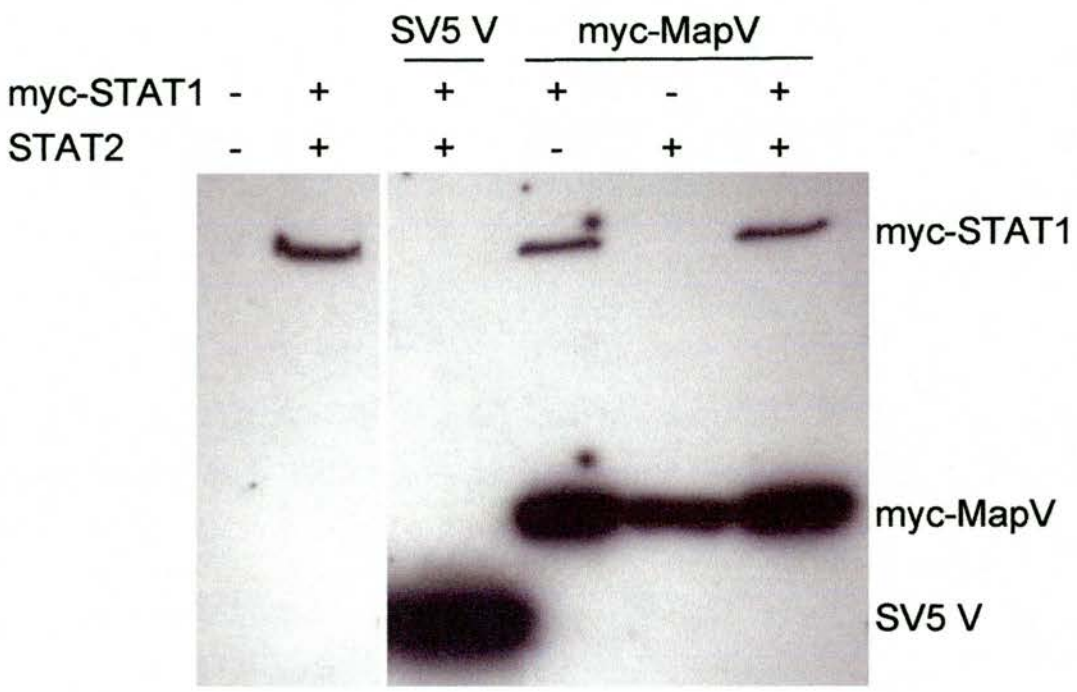


Figure 55: Degradation of myc-tagged STAT1 by SV5 V and Mapuera V

293 cells were seeded into 6-well plates and once 50% confluent were transiently transfected with various combinations of myc-STAT1, STAT2, SV5 V and myc-tagged Mapuera V expression constructs. 48h post transfection cells were lysed in 100µl Disruption Buffer and 1/5th of each sample was separated by 10% SDS-PAGE and transferred to PVDF.

Samples were western blotted with α-myc MAb, α-SV5 V MAb and α-mouse-HRP conjugated secondary antibody and detected using ECL reagents.

components of the IFN signalling pathway such as STAT2, STAT3 and p48 are similarly not targeted for degradation in cells expressing MapV V.

III. PORCINE RUBULAVIRUS, MENANGLE VIRUS, SALEM VIRUS AND TIOMAN VIRUS

The capacity of these viruses to antagonise the IFN response had not been investigated and due to their similarities to other paramyxoviruses, particularly rubulaviruses, it was thought that their V proteins might have a role in IFN antagonism. Clones of the viral V proteins were obtained from Lin-Fa Wang (PoRV, TiV) and Timothy Bowden (MenV) at CSIRO in Geelong, Australia and Randall Renshaw at Cornell University, USA (SalV). These V proteins were cloned into mammalian expression vectors with N-terminal myc epitope tags (see Cloning Strategies section for details).

IFN signalling

In order to assess the ability of the various V proteins to antagonise IFN signalling, the effects of transient expression of these proteins on the response of an IFNα/β-responsive promoter to IFNα was assayed.

The V proteins of Porcine Rubulavirus, Menangle virus, Salem virus and Tioman virus do not block IFNα/β signalling in human cells

Fig. 56 shows the results of a IFNα/β signalling assay in HeLa cells, indicating that none of the four V proteins block IFN signalling in these cells when compared to the suppression shown by SV5 V.

IFN production

Several paramyxovirus V proteins, including SV5 V, have been shown to block the activation of the IFNβ promoter in response to dsRNA and this is due to the presence of cysteine residues in the C-terminal, V-unique region of the protein. The V proteins of PoRV, MenV, SalV and TiV all share the same conserved cysteine residues as SV5 V (see Fig. 21 for an alignment) and so their ability to block IFNβ promoter activation was assayed.

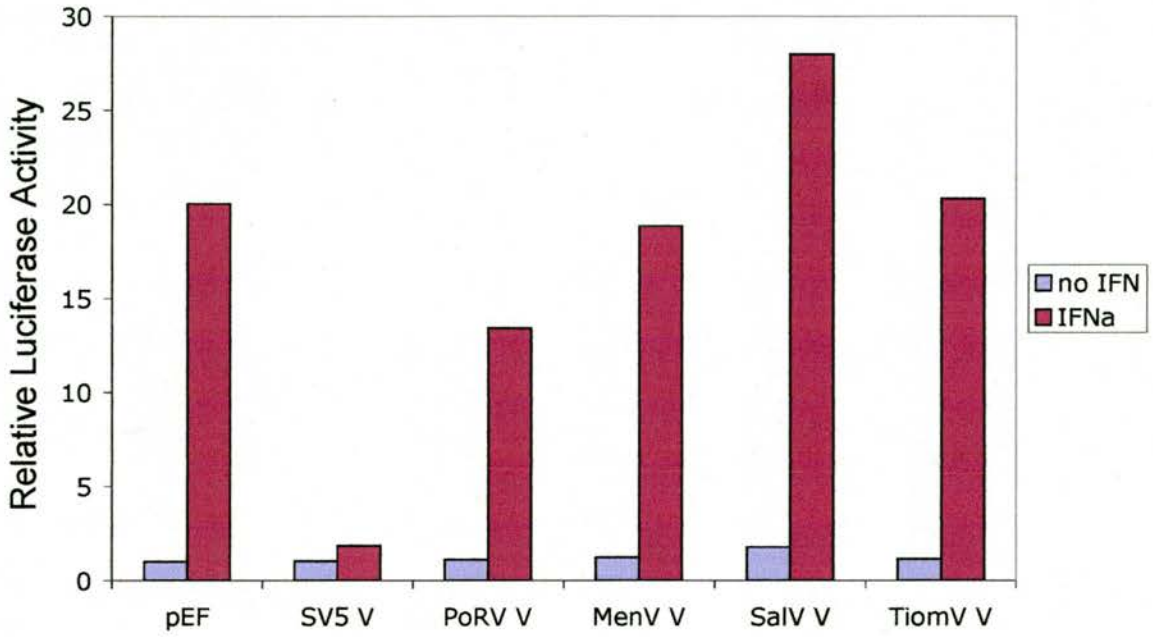


Figure 56: IFNα/β signalling assay in HeLa cells: Porcine Rubulavirus, Menangle virus, Salem virus and Tioman virus V constructs

HeLa cells were seeded in 6-well plates and transfected with an IFNα/β-responsive luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Porcine Rubulavirus, Menangle virus, Salem virus and Tioman virus constructs. 48h post transfection cells were stimulated or not with IFNα, $1.8x10^4$ IU/ml, 4h @37°C.

Cells were harvested in 200µl each Luciferase buffers A and B and 300µl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

The V proteins of Porcine Rubulavirus, Menangle virus, Salem virus and Tioman virus block IFNβ promoter activation

As shown in Fig. 57, all four V proteins block the activation of the IFNβ promoter to a degree comparable to SV5 V, as predicted from their conserved C-terminal sequences. SalV appears to block slightly less effectively, but it is not clear whether this result is significant.

The C-terminus of Tioman V protein is required for the block of IFNβ promoter activation

Due to a mutation introduced during PCR from the TiV V template, a stop codon was introduced in the TiV V ORF, downstream of the editing site at amino acid position 201. This stop codon is downstream of the first two conserved cysteine residues and upstream of the remaining five and in encodes a TiV V protein truncated by 31 amino acids and lacking the full cysteine-rich C-terminus, referred to as TiV VΔC31. When the ability of this construct to antagonise dsRNA signalling to the IFNβ promoter was assayed, as shown in Fig. 58, it was found not to suppress IFNβ promoter activation, unlike the full-length TiV V construct. This suggests that, as found for SV5 V, MuV V, NiV V and MapV V, the conserved cysteine residues in the C-terminus of TiV V are required to enable the protein to block the activation of the IFNβ promoter.

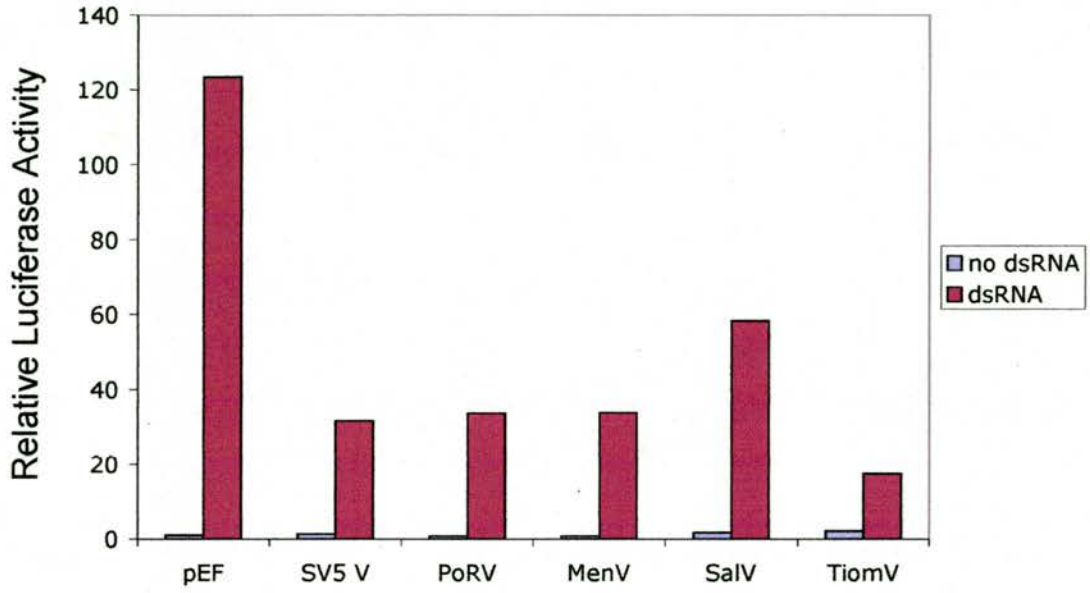


Figure 57: IFNβ promoter activation assay in Vero cells: Porcine Rubulavirus, Menangle virus, Salem virus and Tioman virus V constructs

Vero cells were seeded in 6-well plates and transfected with a dsRNA-responsive IFNβ promoter-luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Porcine Rubulavirus, Menangle virus, Salem virus and Tioman virus V expression constructs. 48h post transfection cells were stimulated or not with 2.5μg poly(I):poly(C) per well, 12h @37°C.

Cells were harvested in 200μl each Luciferase buffers A and B and 300μl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

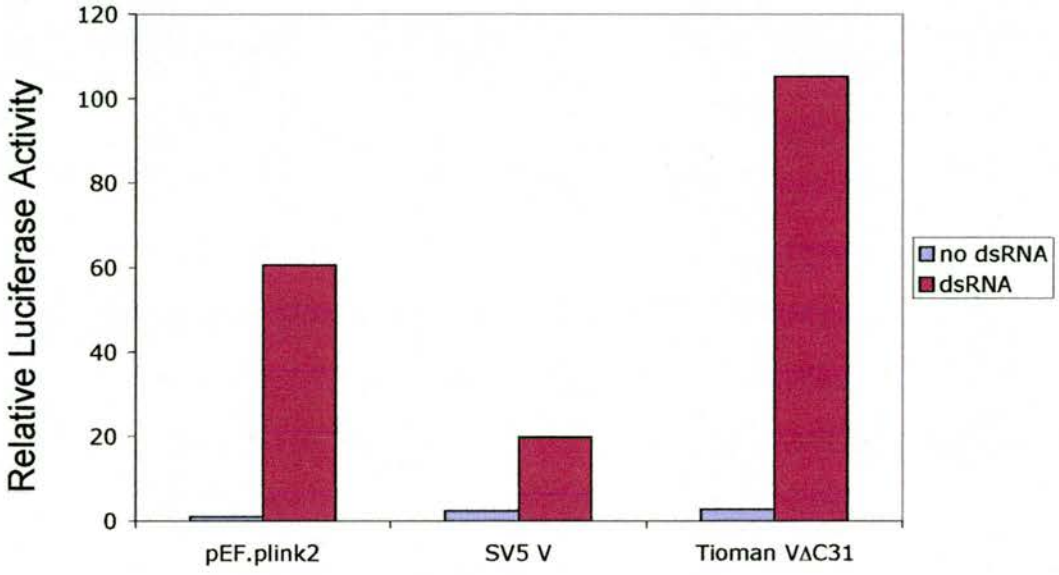


Figure 58: IFNβ promoter activation assay in Vero cells: Tioman virus VΔC31 construct

Vero cells were seeded in 6-well plates and transfected with a dsRNA-responsive IFNβ promoter-luciferase reporter, a β-gal control plasmid and either pEF.plink2 empty vector, SV5 V or Tioman virus VΔC31 expression constructs. 48h post transfection cells were stimulated or not with 2.5μg poly(I):(C) per well, 12h @37°C.

Cells were harvested in 200μl each Luciferase buffers A and B and 300μl cleared lysates were assayed for luciferase and β-gal activity.

Relative light unit readings from the luminometer were divided by A_{420} β-gal readings to give a measure of relative luciferase activation and this data was adjusted to the unstimulated negative control value to give a measure of relative luciferase activity.

This data represents averaged values from at least three equivalent assays.

Simian virus 5

The theory that truncations of SV5 V, when co-expressed, could *trans*-complement each other and antagonise IFN signalling in the absence of a full-length V protein was disproved when a signalling assay in which truncated V constructs were expressed singly and in combination clearly showed that the truncated V proteins were not able to block IFN signalling, in any combination. This indicates that both regions of SV5 V are required to antagonise IFN signalling and that V function cannot be *trans*-complemented.

The *trans*-complementation theory was based on the notion that each domain of SV5 V might interact with different cellular proteins and bring them together, but it now seems more likely that both amino and carboxy terminal domains of SV5 V are involved in forming the proper tertiary structure of the protein, required for its interactions with cellular proteins such as DDB1 and STAT2. A study of the interaction of SV5 V with DDB1 showed that significant deletions from either end of the V protein cannot be made if the interaction with DDB1 and the ability to block IFN signalling are to be retained and this study and others have demonstrated that the function of SV5 V can be affected by point mutations in both its amino and carboxy termini (Young et al. 2001; Andrejeva et al. 2002; Chatziandreou et al. 2002). In addition, mutational analysis of hPIV2 V has suggested that the regions required for STAT2 degradation are discontinuous (Kozuka et al. 2003). It therefore seems likely that the tertiary structure of these V proteins is important for function and that this structure is influenced by residues in both amino and carboxy terminal domains.

As mentioned in the Introduction, it has been shown that the N-terminal common domain of the MeV P/V protein is natively unstructured and a similar predictive model has suggested an unstructured state for part of the N-terminus of SV5 V (He et al. 2002; Karlin et al. 2002). It has been suggested that the interactions of SV5 V with other proteins, both viral and cellular, could influence its tertiary structure and that V may be able to adopt different tertiary structures depending on the nature of these interactions, allowing what is a relatively small protein to have several different

functions. This remains to be confirmed, but current work aiming to co-crystallise SV5 V with one or more of its protein partners may give further insight into this.

Mumps virus

The preliminary IFN signalling assays carried out with the cloned MuV V construct demonstrated that it could block IFNα/β signalling in both human and murine cells, as previously reported in the literature. However, similar assays using amino and carboxy-terminal truncations of MuV V, MuV Vn and MuV Vc, showed that neither truncation construct was capable of blocking IFNα/β signalling when expressed alone. This appears to contradict previously published data, which suggested that the C-terminus of MuV V alone is sufficient to antagonise the IFN signalling pathway (Kubota et al. 2001). However, assays carried out with a dsRNA-responsive IFNβ promoter reporter construct demonstrated that both full-length MuV V and MuV Vc were capable of blocking dsRNA signalling to the IFNβ promoter and therefore presumably suppressing the production of IFNβ in infected cells, as previously demonstrated for SV5 V (He et al. 2002; Poole et al. 2002).

The study of Kubota *et al* used the rescue of IFN-sensitive VSV from IFN primed cells expressing MuV V and MuV Vc as an indicator of IFN antagonism. This type of assay, widely used to study IFN antagonism by various viruses, is useful as it gives a clear indication of whether a particular virus or viral protein has a mechanism for IFN antagonism and can be used in as many cell types as will support the replication of the IFN-sensitive reporter virus. However, it is very difficult to distinguish between viruses or proteins which block IFN signalling and those that block IFN production using such assays and as many paramyxoviruses have been shown to antagonise both of these aspects of the IFN response, the results obtained can sometimes be misleading. However, the use of individual reporter assays for IFN signalling and IFN production, as described in this study, can identify which of the two pathways is antagonised by a particular virus or viral protein construct, so avoiding any of the potential confusion between the antagonism of IFN signalling and IFN production.

Nipah virus

As NiV is highly pathogenic in man, with high mortality rates associated with infection, it seemed likely that it would have some kind of mechanism for IFN antagonism. However, initial IFN signalling assays with the NiV V Geelong isolate indicated that NiV V did not antagonise IFN signalling. However, shortly after this it was demonstrated that NiV V blocked IFNα/β and IFNγ signalling in human cells (Rodriguez et al. 2002; Park et al. 2003b) and a comparison of the V sequence of the isolate used in these studies (referred to as NiV V NIH for the purposes of this thesis) with the NiV V Geelong isolate revealed four nucleotide differences in the V ORF, three of which result in amino acid differences in the encoded protein. All three of the amino acid changes were located in the P/V common domain of NiV V.

The construction of a panel of mutant NiV V proteins based on NiV V Geelong and with these three amino acid differences introduced singly, in pairs and finally in combination, to create a functional equivalent of NiV V NIH, demonstrated not only that the introduction of all three amino acids resulted in an equivalent to NiV V NIH that could block IFN signalling but also that a single amino acid change from glutamic acid (E) to glycine (G) at residue 125 reversed the phenotype of NiV V Geelong and enabled it to block IFN signalling. The other two amino acid changes were unable to allow NiV V to function as an IFN antagonist when introduced alone or in combination and it was only when introduced in combination with the E125G change that these NiV V proteins blocked IFN signalling. This data suggests that a point mutation leading to a single amino acid change in NiV V can switch the protein from non-functional to functional in terms of IFN antagonism and it is tempting to extrapolate this to the whole virus in which such a mutation could have the potential to switch the viral phenotype from IFN-sensitive to IFN-resistant, as seen for mci-2, a mouse-adapted strain of SV5 (Young et al. 2001).

Rodriguez *et al* also demonstrated that NiV V NIH interacts with STAT1 and STAT2 and blocks their translocation to the nucleus in response to IFN and suggested that the mechanism by which NiV V blocks IFN signalling is a novel one, involving the binding and sequestration of STAT1 and STAT2 in the cytoplasm of cells expressing NiV V via the interaction of NiV V with these proteins. It was therefore logical to

examine the interactions of the 'non-functional' NiV V Geelong and the functional NiV V NIH and NiV V E125G with STAT1 and STAT2. The data obtained showed that both NiV V NIH and NiV V E125G interact with STAT2 but that the 'non-functional' NiV V Geelong does not, suggesting that the ability of NiV V to interact with STAT2 can be correlated with its ability to block IFN signalling and that residue 125 in NiV V is critically involved in this interaction. However, no evidence of an interaction between NiV V and STAT1 was found either in cell extracts or *in vitro*, despite many attempts to reproduce the protocols described in Rodriguez *et al.* It is possible that the few differences in experimental procedure such as the epitope tag used to immune precipitate NiV V (c-myc rather than the FLAG tag used in the published study) contributed towards the difference between the results described in this thesis and those in the literature. However, it is clear that in order to block IFNγ signalling, NiV V NIH must have some mechanism to block the nuclear translocation of STAT1 homodimers and that in 'non-functional' isolates such as NiV V Geelong this mechanism is disrupted. It may be that NiV V Geelong and the other 'non-functional' NiV V proteins are unable to interact with STAT1, but with no evidence of an interaction between any of the NiV V proteins studied and STAT1, this remains to be investigated.

In addition to the interaction studies, the effects of the different NiV V proteins on STAT localisation in response to IFN stimulation was investigated and in general the ability of the various NiV V proteins to block the nuclear translocation of STAT1 and STAT2 reflected their ability to block IFN signalling, suggesting that the model of NiV V IFN antagonism proposed by Rodriguez *et al* may be correct. However, there was an exception to this rule whereby NiV V Geelong, classified as 'non-functional' in terms of its antagonism of IFN signalling, was found to block the nuclear translocation of STAT2 in some cells. NiV V Geelong appeared to have no effect on the nuclear translocation of STAT1, but in a significant number of cells observed using indirect immunofluorescence seemed to block the nuclear translocation of STAT2. It is difficult to quantify the amounts of proteins expressed in cells using indirect immunofluorescence, but it seemed as though the cells in which STAT2 translocation was blocked also had a higher level of NiV V fluorescence, suggesting that when more NiV V Geelong is present it is able to block the movement of STAT2

to the nucleus. It is possible that NiV V Geelong is not completely unable to bind STAT2 but rather has a much-reduced affinity when compared with NiV V NIH and NiV V E125G, something that was also suggested by anti-STAT2 western blots of immune precipitates from 293 cells expressing NiV V Geelong. Long exposures of these western blots showed that NiV V Geelong can immune precipitate STAT2, albeit at much lower levels than NiV V E125G or NiV V NIH and it may be useful to re-examine the interactions of NiV V Geelong with STAT2 to investigate the possibility of low-affinity binding to STAT2. This data suggests that the correlation between perturbation of STAT localisation and antagonism of IFN signalling is not always absolute, although it should be noted that the potential low affinity binding of NiV V Geelong to STAT2 did not appear to be sufficient to suppress IFNα/β signalling as measured by luciferase reporter assays.

The use of truncations of NiV V in IFN signalling assays showed that significant truncations of NiV V can be made without a concomitant loss of the ability to block IFN signalling, in contrast to data obtained using truncations of SV5 V, as discussed earlier. The assays showed that the amino-terminal, P/V common domain of NiV V (Vn) is sufficient to block IFN signalling, but only if the correct residues are present, so that while NiV Vn NIH and NiV Vn E125G block IFN signalling, NiV Vn Geelong does not, whereas the carboxy-terminal, V-unique domain of NiV V (Vc) has no ability to block IFN signalling. NiV V has a greatly extended N-terminus compared with that of other paramyxovirus V proteins and this may reflect the different mechanism by which NiV V antagonises IFN signalling. Assays using the rescue of an IFN-sensitive rNDV-GFP virus in cells expressing NiV proteins demonstrated that NiV V and also NiV W protein, which is amino co-terminal with V, antagonise the IFN response, but that NiV P, which contains the same amino-terminal domain as both V and W, does not (Park et al. 2003b). It is likely that NiV P forms a tetramer, as seen for other paramyxovirus P proteins, which would probably disrupt interactions with proteins such as STAT1 and STAT2 and prevent NiV P from antagonising the IFN response. It should also be noted that, similar to other paramyxovirus V proteins, the amino-terminal regions of NiV V and W are predicted to be natively unstructured, which may allow them to interact with a number of different proteins and carry out a number of functions.

Although it does not appear to be involved in the antagonism of IFN signalling, dsRNA signalling assays using full-length NiV V, NiV Vn and NiV Vc demonstrated that the carboxy terminus of NiV V is required and sufficient to block dsRNA signalling to the IFNβ promoter, presumably via its conserved cysteine residues previously shown to be essential for the block of IFNβ promoter activation by SV5 V (Poole et al. 2002). This activity is independent of the ability of NiV V to block IFN signalling as NiV V Geelong, which does not antagonise IFN signalling, is able to block dsRNA signalling to the IFNβ promoter. So, unlike other paramyxovirus V proteins such as SV5 V, the two aspects of NiV V IFN antagonism can be separated in terms of V protein domains. This separation of function in terms of the antagonism of IFN signalling and IFN production is also seen in SeV which, as discussed in the Introduction, uses its C protein to block IFN signalling and its V protein to block dsRNA signalling to the IFNβ promoter.

Returning to the fact that NiV V Geelong did not block IFN signalling, it is interesting to theorise about how such an isolate may have arisen. It is of course possible that the nucleotide changes in NiV V Geelong arose during the initial RT-PCR step used to generate the P gene DNA clone and it has proved difficult to confirm or deny this. However, it is also possible that the different sequence of NiV V Geelong reflects the sequence of a true IFN-sensitive isolate. Previous work on different strains of SV5 has suggested that persistent infections established by SV5 are the result of infection with IFN-sensitive viruses, which exist in a quiescent state in cytoplasmic inclusion bodies in persistently infected cells. These inclusion bodies have been observed both in infections of wt SV5 in murine cells, as SV5 is unable to antagonise the IFN response in these cells, and infections of the IFN-sensitive SV5 CPI- in canine cells (Fearns et al. 1994; Chatziandreou et al. 2002). The discovery that point mutations in the V proteins of both of these viruses could switch the viral phenotype from IFN-sensitive to IFN-resistant led to suggestions that viruses in inclusion bodies could mutate and as a consequence reactivate and spread to other cells (Young et al. 2001; Chatziandreou et al. 2002).

The NiV V Geelong isolate came from a human source, although it is not known whether the infection was fatal or if the patient recovered, something that could be

indicative of the IFN sensitivity of the isolate, but if NiV Geelong is IFN-sensitive it is possible that it had established a persistent infection. There is evidence suggesting that the closely related HeV can establish persistent infections in man and horses (reviewed in (Wang and Eaton 2001), so it is possible that IFN-sensitive NiV could persistently infect humans and be isolated from such individuals. There is also the possibility that NiV V Geelong is able to block IFN signalling in cells from other species and that the IFN-sensitivity of this isolate reflects a recent cross-over from an animal host. It has been demonstrated that SV5 V is unable to antagonise IFN signalling in murine cells but that after prolonged passage in such cells variants of SV5 arise that have a point mutation in V allowing them to block IFN signalling (Young et al. 2001) and a similar process may occur in NiV cross-species infections whereby viruses are initially IFN-sensitive but selection in the new host leads to IFN-resistant strains. In this regard it will be of interest to assay the ability of NiV V Geelong to block signalling in cells from other species such as pigs and fruit bats and also to study isolates of NiV from these other host species to look for any characteristic differences in V sequence and function.

In order to create the NiV V Geelong construct, mutagenic PCR was used to insert an additional G residue at the RNA editing site in the P ORF provided by CSIRO, to replicate the RNA editing process that normally occurs in NiV-infected cells in order to generate V mRNA from the P gene. When the nucleotide sequence of the NiV V Geelong construct was analysed and found to contain several differences from the NiV V NIH sequence, the first thought was that the changes had been introduced during the initial mutagenic PCR steps used to generate NiV V Geelong. With this in mind, the original P ORF template was sequenced and found to have the same nucleotide sequence as NiV V Geelong, indicating that the sequence changes were not introduced during the V cloning process. During this sequence analysis it was also noted that the P ORF contained more G residues than usual at the RNA editing site. NiV P mRNA usually contains three G residues at the editing site and it is only by RNA editing that an additional one or two G residues are added to create V or W mRNA respectively, but the Geelong P clone had a total of eleven G residues at this position and encoded a truncated P protein when expressed in *E. coli* (Lin-Fa Wang, personal communication). Subsequent sequencing of the NiV genomic RNA by direct

PCR showed the usual three G residues at the editing site of the P gene (Lin-Fa Wang, personal communication), suggesting that the P mRNA that was cloned was a product of RNA editing whereby an indiscriminate number of G residues were added rather than a faithful transcript of the P gene. This indiscriminate editing is not unknown in paramyxoviruses and is seen in the respirovirus hPIV3, the P mRNAs of which encode ORFs in all three reading frames by the addition of multiple G residues. This kind of editing mechanism has not previously been demonstrated for NiV, although one and two G residues are known to be added to access the V and W ORFs respectively and hence all three reading frames downstream of the editing site are used. A study of the editing sites of a number of populations of NiV P mRNAs may reveal whether NiV routinely uses this mechanism.

Mapuera virus

IFN signalling assays with MapV V showed that similar to SV5 V, MapV V blocks both IFNα/β and IFNγ signalling in both human and simian cells, but not murine cells. There is some experimental evidence for pathogenicity of MapV in mice, but as this was a result of intra-cranial inoculation, antagonism of the IFN system may not have been an important factor in the pathogenicity of the virus. It would be of interest to investigate whether the addition of human STAT2 to murine cells expressing MapV V could allow MapV V to antagonise IFN signalling, as has been demonstrated for SV5 V (Parisien et al. 2002). MapV appears to cause asymptomatic infection of bats and no pathogenicity in humans was observed during handling of the virus and MapV-infected cells, thus despite its ability to block IFN signalling in human cells, other factors may limit the ability of MapV to replicate and cause disease in man.

It was also demonstrated that truncations of MapV V are unable to block IFN signalling, as shown for SV5 V and MuV V, but in contrast to the more distantly related NiV V, suggesting that both amino and carboxy-terminal regions of MapV V are required to block IFN signalling. However, both full-length MapV V and MapV Vc were shown to block dsRNA signalling to the IFNβ promoter, indicating that similar to SV5 V, MuV V and NiV V, the carboxy-terminal cysteine residues in MapV V are required and sufficient to block this signalling pathway. Thus it seems

fair to assume that MapV infections would be able to suppress the production of IFNβ, similar to SV5 infections.

Despite the similar abilities of MapV V and SV5 V to antagonise IFN signalling, there is currently no evidence that MapV V achieves this via the targeted degradation of STAT1. Studies in a HeLa/MapV_V stable cell line showed no evidence for the degradation of STAT1 or indeed STAT2, STAT3 or p48/IRF9 and a GST-MapV V fusion protein did not appear to interact with DDB1, an interaction shown to be crucial for the degradation of STAT1 and antagonism of IFN signalling by SV5 V (Andrejeva et al. 2002). Experiments studying the degradation of exogenous, tagged STAT1 also found no evidence of degradation by MapV V and although IFN treatment of HeLa/MapV_V cell lines did not result in an upregulation of STAT1 levels, there was no decrease in STAT1 either, suggesting that IFN signalling was blocked, but that this was not achieved via the degradation of STAT1. Recent acquisition of a virus stock for MapV should enable further studies of infected cells and may reveal effects on other components of the IFN signalling cascade or possibly the phosphorylation status of STATs or their ability to transactivate IFN-stimulated promoters as demonstrated for SeV C protein (Komatsu et al. 2000; Young et al. 2000; Garcin et al. 2001; Takeuchi et al. 2001; Komatsu et al. 2002; Saito et al. 2002). It would also be of interest to confirm that MapV V does not interact with DDB1 by other techniques and to investigate the interactions of MapV V with others members of the 'V degradation complex' suggested to exist for SV5 V and MuV V (Ulane and Horvath 2002; Ulane et al. 2003).

Immune precipitation studies with MapV V revealed an interaction with STAT2, similar to that observed for SV5 V and NiV V, but no evidence was found for an interaction with STAT1. Several reports in the literature have suggested that SV5 V and NiV V interact with STAT1, but so far no evidence for such interaction has been found in our laboratory. As already discussed in terms of NiV V, the reasons for this apparent lack of interaction in the face of the published evidence are unclear, but currently we have no indication of a direct interaction between any of the V proteins studied and STAT1. Thus in summary, there is currently no molecular explanation for the mechanism of IFN evasion by MapV V, save that at present it appears that this

mechanism differs from that of other rubulaviruses such as SV5, MuV, hPIV2 and SV41 in that neither STAT1 or STAT2 appear to be targeted for degradation.

Porcine Rubulavirus, Menangle virus, Salem virus and Tioman virus

IFNα/β signalling assays with the V proteins of these viruses showed that none block IFN signalling in human cells. This is perhaps not surprising, as only MenV has been suggested to infect humans, the other three viruses having only been isolated from animal hosts. It would be of interest to test the abilities of these virus V proteins to block IFN signalling in cells from other species, including those from which the viruses were originally isolated such as pig, horse and bat cells as it may be that, as suggested for SV5 V, the ability to block IFN signalling is a determinant of host range. It is also possible that the methods used to isolate these viruses may have allowed the selection of IFN-sensitive viruses, as the initial amplification steps used to make virus stocks are usually carried out in Vero cells which are not IFN-competent. Thus any IFN-sensitive viruses in the population originally isolated would presumably form part of the final virus stock and their genomic RNA would be available as a template for the cloning of the P/V gene.

As already mentioned, previous work in our group has suggested that paramyxoviruses such as SV5 may switch from an IFN-resistant phenotype to an IFN-sensitive one and that this switching may be mechanism for persistence and reactivation. It may be the case that the virus isolates studied originated from persistently infected hosts, such as TiV which was isolated from an asymptomatic fruit bat, and are therefore IFN-sensitive, perhaps with the capacity to switch to an IFN-resistant phenotype via point mutations. It is particularly tempting to suggest that this is the case for Salem virus, which was isolated from a horse involved in an outbreak of respiratory disease but was subsequently found not to be the causative agent but rather an asymptomatic infection found in more than 50% of US horses sampled. It may be the case that SalV establishes persistent infections in horses and exists in an IFN-sensitive state and this could be further investigated by studying the ability of SalV V to block signalling in equine cells and its replication and protein distribution in these cells in the presence of IFN. In particular it would be of interest to examine whether SalV N and P proteins form cytoplasmic inclusion bodies in IFN-

treated cells as these are found as a consequence of IFN treatment in cells infected with IFN-sensitive SV5 strains and are thought to be related to the mechanism of viral persistence.

It may be that other viral proteins are involved in IFN antagonism, especially alternative products of the P gene, which may exist in these viruses. In the case of Salem virus, the viral P gene encodes a C protein in addition to P and V, the ORF of which has an alternative start site in the P mRNA and is likely to be accessed by ribosomal choice as in respiroviruses and morbilliviruses. It is possible that SalV C has a role in IFN evasion as demonstrated for SeV C, which blocks IFN signalling, primarily by altering the phosphorylation of STAT1. SalV C was cloned and preliminary signalling experiments indicated it did not block IFN signalling, but its expression in transfected cells could not be confirmed and it is possible that the protein was not expressed properly. Thus the properties of SalV C have not yet been ascertained and remain to be further investigated.

In contrast with their lack of ability to block IFN signalling, all four V proteins blocked the activation of the IFNβ promoter by dsRNA to a similar extent to SV5 V, presumably via their cysteine-rich carboxy termini. The contribution of this region was confirmed for TiV V at least, as a truncated TiV V generated by a PCR error, which lacked most of the V-unique C-terminus, was unable to block the activation of the IFNβ promoter whereas a full-length clone blocked effectively.

Suppression of IFNβ promoter activation by paramyxovirus V proteins

The fact that all of the V proteins examined in this study were able to block dsRNA signalling to the IFNβ promoter suggests a common mechanism for all V proteins involving the conserved carboxy-terminus. Currently the mechanism by which these proteins block the dsRNA signalling pathway is unknown, apart from the evidence suggesting that the block is upstream of both NFκB and IRF3 activation. The signalling cascade from internalised dsRNA to the IFNβ promoter remains to be elucidated and while it may share aspects of the pathway stimulated by external dsRNA, which involves the stimulation of cellular kinases by activated Toll-like receptors (TLRs), it seems to be distinct from this pathway as activation by external

dsRNA is not blocked by SV5 V (S. Goodbourn, unpublished data). Other viruses such as Influenza virus and Bovine diarrhoeal disease virus (BVDV) encode proteins that block IFN production and it is thought that these function by the binding and sequestration of dsRNA and while it is possible that paramyxovirus V proteins are dsRNA binding proteins, no evidence has been found to suggest any dsRNA binding by SV5 V.

In order to antagonise dsRNA signalling to the IFNβ promoter, it seems likely that V proteins will interact with at least one cellular protein. Currently we know that the well-documented interaction of various V proteins with DDB1 is not involved in the antagonism of IFN production, as V proteins that do not bind DDB1 such as SV5 CPI- V, MapV V and SeV V are able to suppress the activation of the IFNβ promoter. A possible alternative protein partner of paramyxovirus V proteins is an as yet unidentified 150kDa protein that is immune precipitated with the SV5 V protein and work is currently underway to identify this protein and examine whether it has any connection with the antagonism of IFN production.

Conclusions

A comparison of the V proteins analysed during this study reveals both similarities and differences in their IFN evasion strategies, summarised in Table 2. A common property of all V proteins studied is the ability to block the activation of the IFNβ promoter in response to dsRNA stimulation, which appears to be mediated via the cysteine-rich, conserved carboxy-terminus of V. This activity is seen in simian Vero cells and is unrelated to the ability of the V proteins to antagonise IFN signalling in these cells.

IFN signalling experiments using truncations of MuV V showed that similar to previous results obtained with SV5 V, the amino and carboxy-terminal domains of MuV V are unable to antagonise IFN signalling when expressed alone. However, it was also demonstrated that the carboxy-terminus of MuV V could block the activation of the IFNβ promoter, again similar to SV5 V, which can also block IFNβ promoter activation using the carboxy-terminus of V alone.

	MuV V	NiV V NIH	NiV V Geel.	HeV V	MapV V	MenV V	PoRV V	SalV V	TiV V
Blocks IFNα/β signalling: human/simian	✓	✓	✗	✓	✓	✗	✗	✗	✗
Blocks IFNγ signalling: human/simian	✓	✓	✗		✓				
Blocks IFNα/β signalling: murine	✓				✗				
Blocks IFNβ promoter activation	✓	✓	✓		✓	✓	✓	✓	✓
Binds STAT1		✗	✗		✗				
Binds STAT2		✓	✗		✓				
Binds DDB1					✗				
Blocks STAT1 nuclear translocation		✓	✗						
Blocks STAT2 nuclear translocation		✓	✗						
Targets STAT1,2,3 or IRF9 for degradation					✗				

Table 2: Properties of the various paramyxovirus V protiens studied in relation to IFN evasion.

Full-length V constructs and their properties as established in the study are shown in the table above. Not included are truncation and mutant constructs, but these are discussed in the text.

Although our initial NiV V isolate, NiV V Geelong, did not antagonise IFN signalling in human and simian cells, mutations made to alter the amino acid sequence of NiV V Geelong to that of a published isolate, NiV V NIH, enabled NiV V to block both IFNα/β and IFNγ signalling as previously observed for a variety of paramyxovirus V proteins. However, unlike SV5 V and MuV V, the amino-terminal domain of NiV V NIH was sufficient to block IFN signalling whilst the carboxy-terminal domain was sufficient to block IFNβ promoter activation and in this way the two IFN antagonist functions of NiV V could be separated, something not possible with previously investigated V proteins. NiV V NIH was also shown to interact with STAT2, similar to SV5 V and other V proteins but in addition, expression of NiV V NIH was demonstrated to block the nuclear translocation of STAT1 and STAT2, in contrast to the non-functional NiV V Geelong that did not, supporting the STAT sequestration model of NiV V function proposed by Rodriguez *et al.*

MapV V was initially found to be similar to the V protein of SV5, in that it antagonised both IFNα/β and IFNγ signalling in human and simian but not murine cells and that amino and carboxy-terminal truncations could not antagonise IFN signalling but the carboxy-terminus alone could block IFNβ promoter activation. MapV V also interacts with STAT2, also demonstrated for SV5 V. However, there was no evidence for the targeted degradation of STAT proteins by MapV V and it did not appear to interact with DDB1, so in these respects it is different from the other members of the *Rubulavirus* family so far investigated.

The remaining V proteins from MenV, PoRV, SalV and TiV behaved as other V proteins in that they all blocked the activation of the IFNβ promoter in response to dsRNA stimulation and this appeared to be due to the activity of the carboxy-terminus, at least in the case of TiV V. However, none of these V proteins antagonised IFN signalling in the cell lines used, something that may reflect their origins from pigs and bats. Further investigation of the ability of these V proteins to antagonise IFN signalling in a wider variety of cell lines will enable a more effective comparison with other paramyxovirus V proteins.

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APPENDIX 1: PRIMER SEQUENCES

Cloning

Name	Sequence (5'→3')	RE site
HeV For	GGGCCATGGACAAGTTGGATCTAG	NcoI
HeV Rev	GGGTCTAGATCATTCCTCGTGACAGCA	XbaI
MapV For	GGGCCATGGACCTCACCTTCTCTC	NcoI
MapV Rev	GGGTCTAGATCATTCTTGATCTGATTC	XbaI
MenV For	GGGCCATGGATAACCCACCCTCTG	NcoI
MenV Rev	GGGTCTAGATTAATCCGAGTCTCCAGA	XbaI
MuV For	GGGGCCATGGATCAATTTATAAAACAG	NcoI
MuV Rev	GGGTCTAGACTAAGGAGGTCCATAATC	XbaI
NiV For	GGGCCATGGATAAATTGGAACTAG	NcoI
NiV Rev	GGGTCTAGATTAACCGCAGTGGAAGCA	XbaI
PoRV For	GGGCCATGGCTAGTAGTTCGCTAA	NcoI
PoRV Rev	GGGTCTAGATCAACTTTCATTTCCAGC	XbaI
SalV For	GGGGACATGTCAGATGAAAATAGAAC	AflIII
SalV Rev	GGGTCTAGATCAGTCATCTCCACATTC	XbaI
TiV For	GGGCCATGGATCCTTCCCCGAGTG	NcoI
TiV Rev	GGGTCTAGATCAAGATTCGCAATCCGG	XbaI

Mutagenic PCR

Name	Sequence (5'→3')	Effect of mutation
G add For	ATTAAAAAGGGGCACAGACGC	Addition of non-templated G
G add Rev	GCGTCTGTGCCCCTTTTTAAT	"
A374G For	GGAGAATGTACCGGATATGGA	Amino acid change E125G
A374G Rev	TCCATATCCGGTACATTCTCC	"
C744T For	GCAGATCAGCTTGAATTCGAA	Amino acid change H248L
C744T Rev	TTCGAATTCAAGCTGATCTGC	"
G838A For	GGAAAACCCAATGAATCCATTG	Amino acid change D280N
G838A Rev	CAATGGATTCATTGGGTTTTCC	"

V Truncations

Name	Sequence (5'→3')	RE site
MapV C For	GTGCCATGGCAGACCTCACCAAG	NcoI
MapV N Rev	GGGTCTAGATCACCCCCTCTTAAAC	XbaI
MuV C For	GTGCCATGGCCGGGAGCGGCT	NcoI
MuV N Rev	GGGTCTAGACTACCCCCTCTTAAATTC	XbaI
NiV C For	GTGCCATGGGGCACAGACGCGAA	NcoI
NiV N Rev	GGGTCTAGATTACCCCTTTTT	XbaI

Sequencing

Name	Sequence (5'→3')	Description
pEF VECT	GCTTACATTTGCTTCTGAC	Anneals upstream of MCS in pEF.plink2

APPENDIX 2: PUBLICATIONS

He, B., R. G. Paterson, et al. (2002). "Recovery of paramyxovirus simian virus 5 with a V protein lacking the conserved cysteine-rich domain: the multifunctional V protein blocks both interferon-beta induction and interferon signaling." Virology **303**(1): 15-32.

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