Mutational analysis of the ability of the SV5 V protein to block IFN signalling

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Abstract

The main conclusion from the results of this study was that, although the carboxy-terminal domain of the V protein of SV5 is known to be essential for its anti-IFN function, point mutations in the amino-terminal domain of the protein can also alter dramatically the ability of the virus to overcome the host IFN response. Thus, mutations in the amino-terminal domain of V can change a virus from IFN-sensitive to IFN-resistant or vice versa. This result was obtained from two virus isolates, mci-2 and CPI-.

Previous findings have shown that SV5 is IFN-sensitive in murine cells, as it does not induce STAT1 degradation and, consequently, does not block IFN signalling in cells of this host species origin. In the present study, a single amino acid substitution in V (a.a. position 100) was shown to be sufficient to render SV5 IFN-resistant in murine cells. The isolate containing this mutation, termed mci-2, gained the ability to degrade STAT1 and block IFN signalling in these cells, without losing the (wild-type) ability to circumvent the IFN response in human cells.

CPI+ is a neurotropic strain of SV5 isolated from canine cerebrospinal fluid, whereas CPI- is an attenuated strain which was isolated from the brain tissue of a dog that had been experimentally infected with CPI+. Comparison of the abilities of these two closely related isolates to block IFN signalling reinforced that point mutations in the V protein can drastically alter the phenotype of the virus. CPI- V is different to CPI+ V at three amino acid residue positions (a.a 26, 50 and 102). It was shown that CPI+ V can block IFN signalling in human and canine cells, in contrast to CPI- V which fails to cause this effect. However, neither of the isolates can degrade STAT1 in murine cells, resembling W3. Mutational analysis of CPI+ and CPI- V revealed the relative importance of each of the three amino acid differences, indicating that, of the three mutations, a.a. 50 has the most dramatic effect on the ability of V to block IFN signalling. Further observations confirmed that CPI+ is similar to W3, establishing productive infections in human and canine cells, but not in murine cells. In contrast, CPI- is an IFN-sensitive virus that is restricted in these cells, with a phenotype that resembles that of W3 in murine cells.

More SV5 strains of human, canine and porcine origin have been previously isolated and, to determine whether they are IFN-sensitive, their ability to degrade STAT1 was examined in human and canine cells. STAT1 could not be detected in extracts from infected cell monolayers (>95% infection). Furthermore, sequence analysis of their V/P and F genes revealed low sequence variation (max. 2.7% and 2.4%, respectively). The significance of these results in terms of paramyxovirus persistence is discussed.

Declarations

(i) I, Nikolaos Chatziandreou, hereby certify that this thesis, which is approximately 44,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for higher degree.

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(ii) I was admitted as a research student in September 1998 and 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 1998 and 2002.

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(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.

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Αυτή η Διδακτορική Θέση είναι αφιερωμένη στους γονείς μου Δημοσθένη και Βασιλική
This PhD Thesis is dedicated to my parents Dimosthenis and Vassiliki

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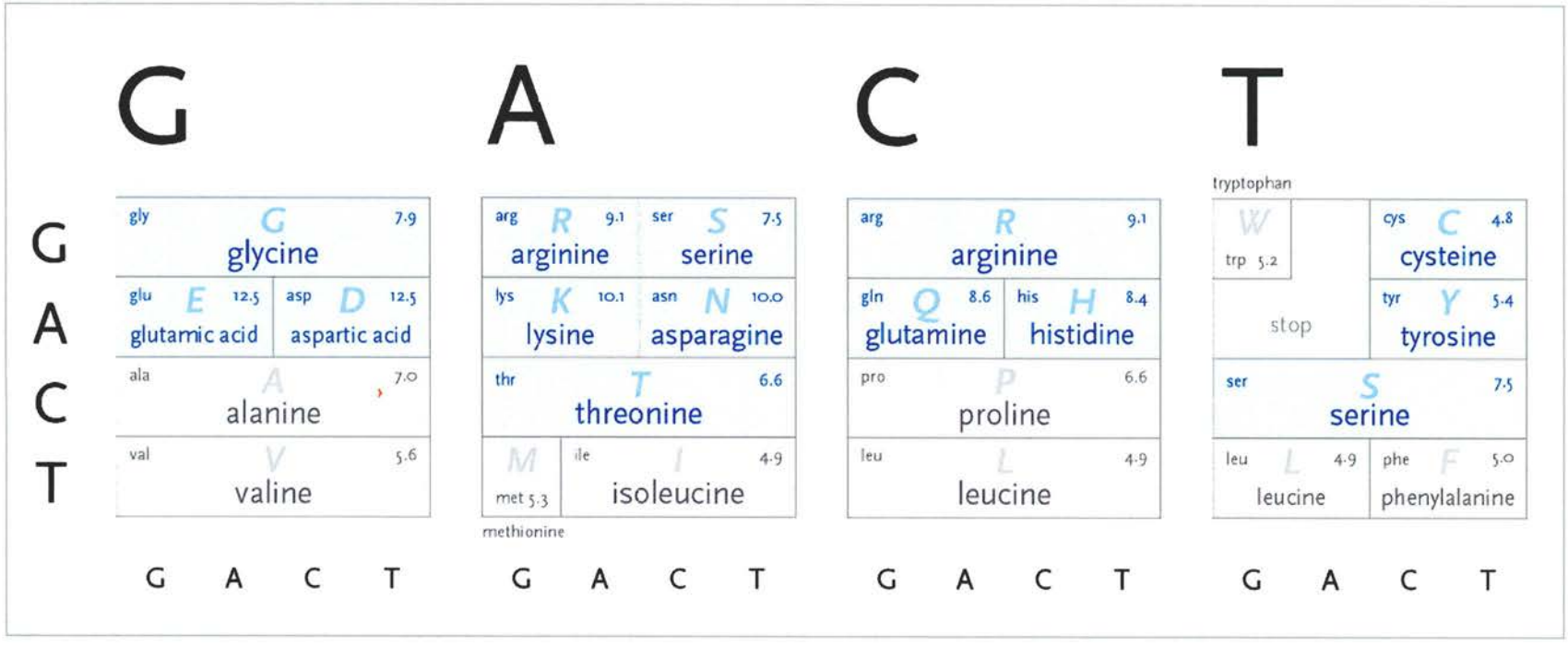
Πάνω απ'όλους, είμαι ευγνώμων στους γονείς μου Δημοσθένη και Βασιλική που έχουν κάνει τα πάντα δυνατά, υποστηρίζοντάς με με κάθε δυνατό τρόπο. Έχουν υπάρξει πάντοτε το κύριο σημείο αναφοράς μου και τους ευχαριστώ που είναι στοργικά μαζί μου όπου βρίσκομαι.

Genetic code

(Table obtained with permission from Benjamin Fry, MIT Media Laboratory/Aesthetics and Computation, Cambridge, USA; http://acg.media.mit.edu/people/fry)



Abbreviations

Units

% (v/v)	percentage concentration (volume per volume)
% (w/v)	percentage concentration (weight per volume)
°C	degrees Celcius
A	ampere
Å	angstrom (10^{-10})
bp	basepair
Ci	Curie ($3.7x10^{10}$ disintegrations/sec)
Da	Dalton
F	farad
g	gram
h	hour
kb	kilobase
l	litre
m	meter
M	molar concentration
min	minute
mol	mole
pH	$-\log_{10}[H^+]$
pfu	plaque-forming unit
RLU	relative light units
V	volt
Ω	ohm

Viruses

bPIV3	bovine Parainfluenza virus type 3
bRSV	bovine Respiratory Syncytial virus
CDV	Canine Distemper virus
DMV	Dolphin Morbillivirus
HeV	Hendra virus
hPIV1/2/3/4a/4b	human Parainfluenza virus types 1, 2, 3, 4a and 4b
hRSV	human Respiratory Syncytial virus
MeV	Measles virus
MuV	Mumps virus
NDV	Newcastle Disease virus
NiV	Nipah virus
PDV	Phocine Distemper virus
PPRV	Peste-des-petits ruminants virus
PVM	Pneumonia virus of mice
RPV	Rinderpest virus
SeV	Sendai virus
SV41	Simian virus type 41
SV5	Simian virus type 5

TPMV	Tupaia Paramyxovirus
TRTV	Avian pneumovirus
CPI	canine parainfluenza virus
mci-1/2	mouse cell isolate 1 and 2
rSV5N>D	recombinant rSV5-V/P N100D virus
W3	wild-type SV5
T4	bacteriophage 4
T7	bacteriophage 7

Nucleic acids

RNA	ribonucleic acid
dsRNA	double-stranded RNA
mRNA	messenger RNA
DNA	2' deoxyribonucleic acid
A	adenine
T	thymine
G	guanine
C	cytosine
cDNA	complementary DNA

Proteins and complexes

ADAR	adenosine deaminase
CPLA2	cytosolic phospholipase A2
DDB	damage-specific DNA binding protein
DDB1	127-kDa DDB subunit
eIF	eukaryotic translation initiation factor
GAF	gamma-activated factor
GFP	green fluorescence protein
GTPase	guanine triphosphatase
ICSBP	IFN-consensus sequence binding protein
IFN	interferon
Ig	immunoglobulin
IKKβ	β–subunit of the multicomponent IκB kinase
IRF	interferon-regulatory factor
ISGF3	interferon-stimulated gene factor 3
IκB	nuclear factor-κB inhibitor
MAP kinase	mitogen-activated protein kinase
NF-κB	nuclear factor-kappa B
PKR	protein kinase R
pRB	retinoblastoma protein
RNase L	endoribonuclease L
STAT	signal transducer and activator of transcription

C	C protein
F	fusion glycoprotein
HN	haemagglutinin-neuraminidase
L	large protein
M	matrix protein
NP	nucleocapsid protein
NS	nonstructural protein NS
P	phosphoprotein
SH	small hydrophobic protein
V	V protein

Chemicals and reagents

^{35}S	radioisotope sulphate-35
ATP	adenosine triphosphate
BSA	bovine serum albumin
DAPI	4,6-diamino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
EDTA	ethylenediaminetera-acetic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	isopropylthiogalactoside
LB	Luria-Bertani medium
ONPG	o-Nitrophenyl-β-D-Galactopyranoside
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulphate
TBE buffer	Tris-borate EDTA
TE	Tris-EDTA
Tris-HCl	tris-hydroxymethyl-aminomethane, pH adjusted with HCl SDS
Triton X-100	polyethylene glycol P-1,1,3,3-tetramethylbutylphenyl ether, octyl phenol ethoxylate, 4-octylphenol polyethoxylate, Mono 30
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Miscellaneous

2'5'A	2'-5' oligoadenylate
a.a.	amino acid
Ab	antibody
APC	antigen-presenting cells
BF	BALB/c fibroblast
C-	carboxy-terminal
C'	carboxy-terminus
CTL	cytotoxic T cell
E.coli	*Escherichia coli*
ECL	enhanced chemiluminescence

EPI	Expanded Programme on Immunization
ER	endoplasmic reticulum
GenBank	National Institutes of Health (USA) genetic sequence database
IRES	internal ribosome entry site
ISRE	IFN-stimulated response element
m.o.i.	multiplicity of infection
mAb	monoclonal antibody
MDCK	Madin-Darby canine kidney
N-	amino-terminal
N'	amino-terminus
OD	optical density
ORF	open reading frame
p.i.	post-infection
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PRD I	positive regulatory domain I
RT	reverse transcription
SCID	severe combined immunodeficiency
SSPE	subacute sclerosing panencephalitis
SWISS-PROT	European Bioinformatics Insititue protein database
Th1/2	T helper cell type 1 and 2
T_m	melting temperature
UV	ultraviolet
WHO	World Health Organisation

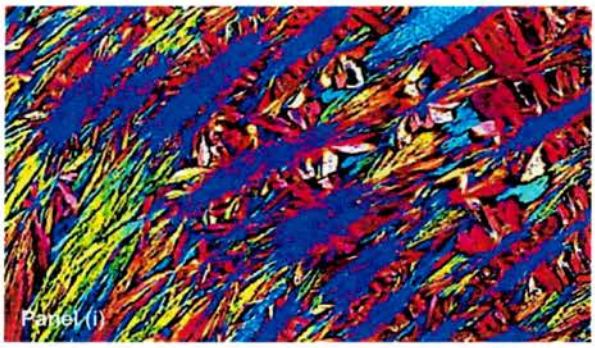
Contents

Chapter 1

Introduction

1.1 Interferons

1.1.1 Overview of the IFN system



The interferon (IFN) system is an essential component of the defence system of many organisms, comprising an important part of innate, nonspecific immunity. IFNs are soluble cytokines that are secreted by vertebrates and act as the first line of defence against virus infections, essentially buying time for the adaptive immune responses to be elicited. Being multifunctional proteins, IFNs play a key role in antiviral defence, as well as cell growth regulation and immune activation (reviewed in Goodbourn *et al.*, 2000). Like other cytokines, IFNs exert their effects following their binding to specific cellular receptors, which initiates intracellular signalling cascades that ultimately activate IFN-induced genes (Biron & Sen, 2001). IFNs are classified into two superfamilies, type I and type II IFNs.

Type I IFNs The type I IFN superfamily contains four subfamilies, namely IFN-α, -β, -ϖ and –τ. Of these subfamilies, IFN-α and IFN-β (commonly referred to as IFN–α/β or type I IFN) have been studied extensively and their essential role in antiviral immunity and mechanisms of action are now well-established. IFN-β is encoded by a single gene and is synthesised by most cell types, particularly by fibroblasts. In contrast, IFN–α is a product of a multigene family and is produced predominantly by leukocytes (Pfeffer *et al.*, 1998; Vilcek & Sen, 1996). The great importance of type I IFN as a defensive mechanism against viral infections has been demonstrated experimentally by a large number of researchers who have shown that mice defective for type I IFN-receptors are vulnerable to infection by many viruses (reviewed in Goodbourn *et al.*, 2000). Type I IFN induces an antiviral state in cells, thus preventing virus proliferation and spread. Furthermore, IFN–α/β is also able to slow down cell growth, promote apoptosis, as well

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Panel (i): Photomicrograph of interferon (printed with permission of Michael W. Davidson, National High Magnetic Field Laboratory, Tallahassee, USA; http://micro.magnet.fsu.edu/pharmaceuticals/pages/interferons.html).

as affect other components of the immune system (the antiviral mechanisms induced by IFNs are discussed in further detail below.)

Type II IFN

IFN-γ is the type II IFN, and, in contrast to type I IFNs, it is produced exclusively by cellular components of the immune system (Boehm *et al.*, 1997). IFN-γ is produced in innate immune responses, being secreted by natural killer (NK) cells, and it is also involved in adaptive immune mechanisms, as it can be synthesised by activated T lymphocytes (Vilcek & Sen, 1996). Although type I and type II IFN pathways overlap, thereby resulting in partial gene activation redundancy, IFN-γ has certain functions that are unique to its type, making it a very potent immune regulator. Thus, it is known that IFN-γ can directly stimulate cells of both the innate (activation of NK cell cytotoxicity) and adaptive immune system ($CD4^+$ T lymphocyte differentiation; immunoglobulin class switching in B cells), trigger the synthesis of antimicrobial enzymes (stimulation of mononuclear phagocyte-produced enzymes with antimicrobial activity; induction of nitric oxide synthase 2, NOS2 or iNOS, for the production of antiviral NO radicals), as well as enhance the expression of other chemokines or immune-related cell surface receptors, including MHC I and II, IL-12 and TNF receptors, which participate in inflammatory responses (reviewed in Pfeffer *et al.*, 1998).

Type I vs. type II

Both type I and II IFNs activate antiviral defence mechanisms by inducing genes that encode proteins with antiviral affects. Despite the lack of structural homology between the two types and the specificity that they have for distinct cell surface receptors (reviewed in Stark *et al.*, 1998), the signalling pathways and gene activation triggered by IFN–α/β and IFN-γ partially overlap. It is possible that this redundancy may have arisen due to virus countermeasures that target the IFN system, which may have evolutionarily favoured alternative pathways of antiviral gene activation. However, the two systems are not redundant in many cases (reviewed in Goodbourn *et al.*, 2000).

1.1.2 Cellular IFN response against viruses

1.1.2.1 Induction of IFN genes

IFN-β induction

The production of IFN-β by virus infection has been studied extensively and it is thought to occur mainly at a transcriptional initiation level. It is believed that the

causative agent that triggers the intracellular responses that ultimately lead to the induction of the IFN-β gene is double-stranded RNA (dsRNA), which is recognised as "foreign" in the intracellular environment. dsRNA can arise in the cell either as the viral genome itself (dsRNA viruses) or as an intermediate during the virus life cycle (Jacobs & Langland, 1996). In either case, the result is the activation of the NF-κB pathway, which induces not only IFN-β, but a plethora of immunomodulatory factors, such as other cytokines and MHC class I and II, thus promoting antigen presentation (reviewed in Baldwin, 1996).

NF-κB activation

NF-κB is a transcription factor critical for the induction of many immunomodulatory genes. In the absence of stimuli, NF-κB is present in the cytoplasm, associated with its inhibitor IκB. The critical event triggered by dsRNA is the translocation of NF-κB from the cytoplasm to the nucleus (Lenardo *et al.*, 1989; Visvanathan & Goodbourn, 1989), where it drives gene expression. For this translocation event to take place, it is necessary that NF-κB becomes free from its inhibitor, IκB, to which it is bound. This happens in response to stress signals, which result in the phosphorylation of IκB by a specific multicomponent IκB kinase. Following phosphorylation, IκB is ubiquitinated by an E3 ligase and thereby targeted for proteasomal degradation. IκB degradation renders NF-κB free to enter the nucleus, where it binds promoters of immunomodulatory genes, including the IFN-β promoter, and induces expression (reviewed in Israel, 2000). Amongst other stress signals (TNF, IL-1, lipopolysaccharide), dsRNA also activates NF-κB-dependent transcription, as it activates the dsRNA-dependent protein kinase R (PKR) which, in turn, activates the IKKβ subunit of the multicomponent IκB kinase (Chu *et al.*, 1999; Zamanian-Daryoush *et al.*, 2000).

The enhance-asome

Despite its critical role, NF-κB on its own is not sufficient to promote IFN-β expression but has to act in concert with other transcription factors to form a multicomponent protein complex that induces gene expression. This complex, referred to as the enhanceasome (reviewed in Thanos, 1996), also contains HMG-I/Y, ATF-2 homodimers or ATF-2/c-Jun heterodimers (Du *et al.*, 1993), as well as a factor that binds to the positive regulatory domain I (PRD I). The identity of this factor is yet ambiguous, as it is believed that it is either interferon-stimulated gene factor 3 (ISGF3;

see below) or a member of the interferon-regulatory factor (IRF) family. It is noteworthy that IRF proteins bind both to PRD I and to the IFN-stimulated response element (ISRE), thus providing redundancy which is thought to protect IFN-β induction signalling from inhibition by viruses (reviewed in Goodbourn *et al.*, 2000).

IFN-α induction

IFN-α synthesis is induced by virus infection and it is known to occur in fibroblastoid cells and leukocytes through distinct mechanisms, although the activation mechanism for the latter remains unclear. Extensive studies on IFN-α gene promoters (reviewed in Pitha & Au, 1995) have revealed that, in contrast to IFN-β promoters, they do not contain NF-κB-binding sequences. Nevertheless, apart from distinct elements, IFN-α promoters also contain PRD I and ATF-2-related binding sequences, and experimental evidence has indicated that fibroblasts require stimulation with IFN-β for IFN-α to be produced (Erlandsson *et al.*, 1998). Stimulation with IFN-β is believed to induce IRF-7 which, upon infection, activates IFN-α expression (Au *et al.*, 1998; Marie *et al.*, 1998; Sato *et al.*, 1998; Yeow *et al.*, 2000). Unlike fibroblastoid cells, the activation mechanism of IFN-α in leukocytes is not dependent on IFN-β stimulation (Erlandsson *et al.*, 1998).

IFN-γ induction

IFN-γ is produced by Th1 $CD4^+$ helper T cells, by nearly all $CD8^+$ cells, as well as by NK cells. The latter can produce IFN-γ irrespective of antigen presentation, whereas both Th1 $CD4^+$ helper T and $CD8^+$ cells rely heavily on exposure to antigen-presenting cells (APCs) for induction of IFN-γ production (Young, 1996). The regulatory sequences preceding the IFN-γ gene differ depending on cell type. Thus, in $CD4^+$ Th1 cells, the promoter contains two distinct regulatory elements, a proximal and a distal one (Aune *et al.*, 1997), which are activated by c-Jun- and ATF-2-containing transcription complexes, and GATA-3 and ATF-1, respectively (Penix *et al.*, 1996; Zhang *et al.*, 1998a). In $CD8^+$ cells, only the distal element is activated, and transcriptional activation of IFN-γ is therefore diminished in such cells. It is known that the signalling cascade that leads to the activation of IFN-γ promoters involves the p38 and JNK2 mitogen-activated protein kinase (MAP kinase) pathways (Rincon *et al.*, 1998; Yang *et al.*, 1998; Lu *et al.*, 1999), but the precise mechanism of signal transduction remains to be determined. Moreover, it is known that IL-12 and IL-18, cytokines produced by APCs (reviewed in Okamura *et al.*, 1998), also play a key role in

IFN-γ induction. When acting in concert, these cytokines can induce antigen-independent IFN-γ production (Tominaga *et al.*, 2000), although details of the mechanism behind this effect are yet to be identified (reviewed in Goodbourn *et al.*, 2000). Induction of IFN-γ production by NK cells, which occurs in an antigen-independent fashion, has also been shown to rely on APC-produced IL-12 and to be subject to stimulation by IL-18 (Singh *et al.*, 2000).

1.1.2.2 Intracellular IFN signalling cascade

IFNs exert their activities by inducing antiviral genes through distinct yet related signalling pathways. Thus, the signalling cascades involve protein components that are specific for each type of IFN (type I or type II IFN signalling). However, major components of the signalling machinery are common between the two types of pathways, namely the 'Janus' tyrosine kinase Jak1 and the signal transducer and activator of transcription (STAT) protein STAT1. The Jak/STAT pathways involved in the transduction of the IFN signal from the cell membrane receptors into the cell nucleus for activation of transcription is presented schematically in Figure 1.1 (reviewed in Stark *et al.*, 1998). The antiviral genes induced by IFNs contain upstream regulatory sequences that differ according to the type of pathway that induces them, as described below. Following stimulation with IFN and activation of the signalling cascades, transcription activation complexes form and bind to these regulatory sequences, resulting in expression of IFN-induced genes, which is otherwise minimal or null.

(i) IFN-α/β signalling

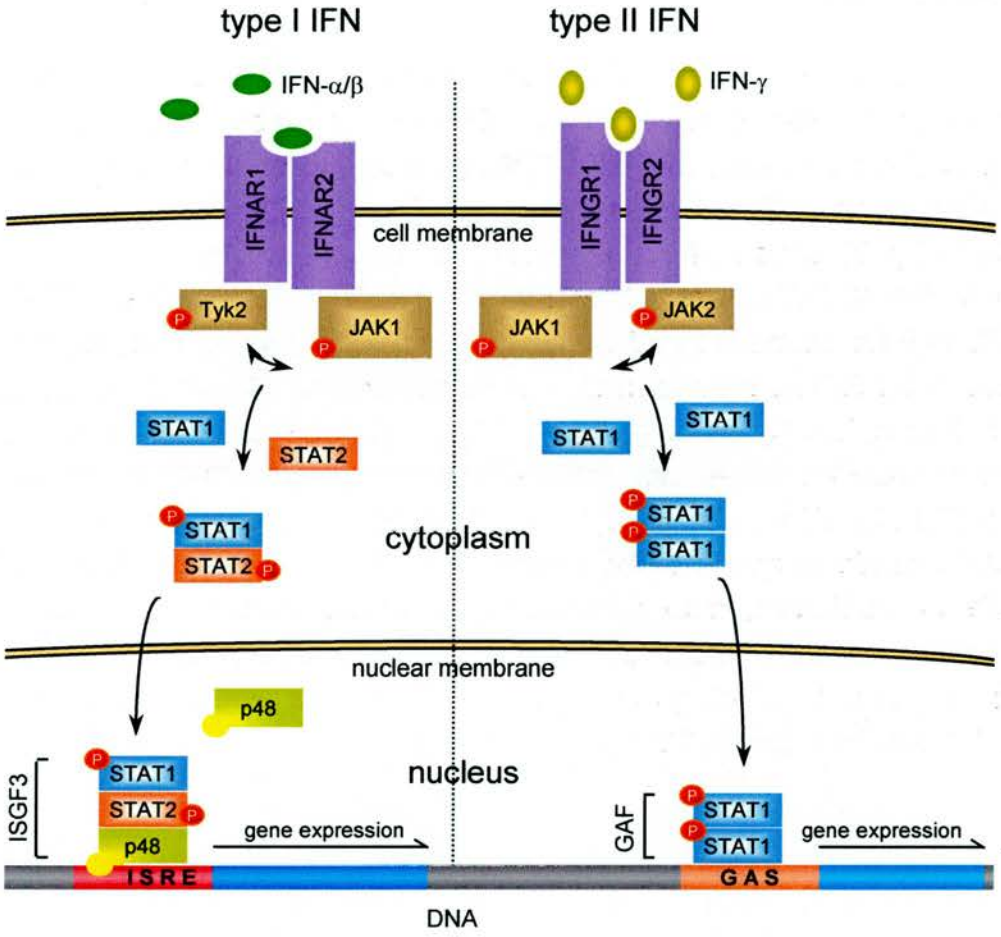
Receptor and associated proteins

The cell membrane receptor involved in type I IFN signalling consists of two major subunits, IFNAR1 and IFNAR2 (reviewed in Mogensen *et al.*, 1999). Normally, in the absence of any stimulatory signal, these proteins are associated with 'Janus' tyrosine kinases; the cytoplasmic domain of IFNAR1 is associated with $p135^{tyk2}$ (Tyk2) (Colamonici *et al.*, 1994), whilst that of IFNAR2 is associated with Jak1 (Novick *et al.*, 1994), as well as STAT2 (Li *et al.*, 1997). Furthermore, IFNAR1 is also known to be pre-associated with tyrosine phosphatases SHP1 and SHP2 (see below).

Figure 1.1: Schematic representation of the type I and type II interferon (IFN) signalling pathways.

Signal transduction is initiated by binding of the IFN-α/β and IFN-γ ligands to their cognate receptors, which triggers a cascade of downstream phosphorylation events. In type I IFN signalling, receptor activation leads to phosphorylation of the receptor-associated 'Janus' tyrosine kinases Jak1 and $p135^{tyk2}$ (Tyk2) which phosphorylate signal transducers and activators of transcription (STATs) 1 and 2 on tyrosine residues. This causes STAT1 and STAT2 to heterodimerise and translocate to the nucleus, where, together with protein p48 (IRF-9), they form the trimeric interferon-stimulated gene factor 3 (ISGF3) complex. By binding to the IFN-stimulated response element (ISRE) element, ISGF3 induces the expression of many IFN-α/β-inducible genes. In type II IFN signalling, phosphorylation of the receptor-associated kinases Jak1 and Jak2 leads to tyrosine-phosphorylation and homodimerisation of STAT1. STAT1 homodimers, also referred to as gamma activated factor (GAF), translocate to the nucleus and stimulate the transcription of many IFN-γ-inducible genes by binding to the gamma activation sequence (GAS) (adapted from Kolakofsky & Lamb, 2001).

Fig. 1.1


type I IFN
type II IFN
IFN-α/β
IFN-γ
IFNAR1
IFNAR2
IFNGR1
IFNGR2
cell membrane
Tyk2
JAK1
JAK1
JAK2
STAT1
STAT2
STAT1
STAT1
STAT1
STAT2
cytoplasm
STAT1
STAT1
nuclear membrane
p48
nucleus
ISGF3
STAT1
STAT2
p48
gene expression
GAF
STAT1
STAT1
gene expression
ISRE
GAS
DNA

Phosphorylation cascade

Binding of IFN-α/β molecules to the cell membrane receptor essentially initiates a downstream activation cascade, by leading to the association of the receptor domains and the transphosphorylation of their associated kinases, Tyk2 and Jak1 (Novick *et al.*, 1994). As a result of this transphosphorylation event, Tyk2 is activated and phosphorylates IFNAR1 at Tyr^{466} (Colamonici *et al.*, 1994), thereby giving rise to a new STAT2-binding site, via the SH2 domain of the phosphorylated receptor subunit (Yan *et al.*, 1996). Phosphorylation of STAT2 by Tyk2 at residue Tyr^{690} subsequently enables STAT1 to dock on STAT2 (Leung *et al.*, 1995; Qureshi *et al.*, 1996), via its SH2 domain, and to be phosphorylated at Tyr^{701} (Shuai *et al.*, 1993). STAT1 phosphorylation at Tyr^{701} is a prerequisite for its activation.

ISGF3 formation and ISRE stimulation

Phosphorylation of the STATs leads to their dissociation from the membrane receptor and their translocation into the nucleus, where they bind to the DNA-binding protein p48 (Veals *et al.*, 1992), which is a member of the IRF family (p48 is also known as IRF-9). The STAT1:STAT2:p48 heterotrimeric complex, referred to as interferon-stimulated gene factor 3 (ISFG3), then acts as a transcription factor, activating type I IFN-sensitive genes. Most genes that are activated by IFN-α/β are preceded by a variation of the IFN-stimulated response element (ISRE) sequence, which contains the consensus sequence GAAAN(N)GAAA, and it is the binding of the ISGF3 complex on this motif that drives their expression.

Several IRF proteins bind to ISRE

It is noteworthy that, in addition to p48, other IRF proteins also bind to the ISRE sequence, including IRF-1, IRF-2, and IRF-8 (also referred to as the IFN-consensus sequence binding protein, ICSBP). Strikingly, IRF-1 is induced by both IFN-α/β and IFN-γ, and, given its ISRE-binding property, it is believed that it gives rise to complex gene expression patterns and system activation redundancy, which is crucial to the host antiviral defence (reviewed in Goodbourn *et al.*, 2000). IRF-2 and IRF-8, on the other hand, also have a profound effect on IFN-induced gene expression system but these proteins are thought to be involved in signal attenuation, as described below.

Other proteins

Additional proteins that are involved in type I IFN signalling include tyrosine phosphatase SHP2, which participates in the signalling cascade by pre-associating with the IFNAR1 receptor subunit and becoming phosphorylated in response to stimulation

with IFN (David *et al.*, 1996). The cytosolic phospholipase A2 (CPLA2) is also phosphorylated and activated in response to IFN-α/β (via Jak1 and the p38 MAP kinase; Goh *et al.*, 1999) and experimental data have suggested that it is involved in the trans-activation of ISRE-regulated genes (Hannigan & Williams, 1991; Flati *et al.*, 1996).

(ii) IFN-γ signalling

Receptor and kinases

IFN-γ receptors comprise at least two major subunits, IFNGR1 and IFNGR2 (reviewed in Bach *et al.*, 1997), which are pre-associated weakly in naive cells (Bach *et al.*, 1996). Nevertheless, similar to IFN-α/β membrane receptors, the cytoplasmic domains of the receptor subunits are associated with 'Janus' kinases. Thus, IFNGR1 is known to be associated with Jak1, whilst IFNGR2 is associated with Jak2 (Kotenko *et al.*, 1995; Sakatsume *et al.*, 1995; Bach *et al.*, 1996; Kaplan *et al.*, 1996).

Phosphor-ylation cascade

Activation of the signalling pathway is initiated when dimeric IFN-γ binds the membrane receptor, which leads to receptor dimerisation, bringing the IFNGR1 and IFNGR2 subunits together. As a result, the associated kinases also come in close proximity (Greenlund *et al.*, 1994; Greenlund *et al.*, 1995; Igarashi *et al.*, 1994; Bach *et al.*, 1996), which triggers a phosphorylation cascade. Therefore, upon stimulation with IFN, Jak2 is activated and transphosphorylates Jak1, which is thus also activated (Briscoe *et al.*, 1996). In a situation similar to that of the IFN-α/β activation cascade, the activated kinases subsequently phosphorylate the cytoplasmic domain of the receptor, thereby creating a docking site for STATs. Specifically, a tyrosine-containing region (Tyr^{440}-Tyr^{444}) near the carboxy-terminal end of the IFNGR1 subunit is phosphorylated by the activated Jaks, which then serves as a binding site for STAT1 via the SH2 domains (Greenlund *et al.*, 1994; Greenlund *et al.*, 1995; Igarashi *et al.*, 1994). Recruitment of STAT1 to the receptor is followed by its phosphorylation at Tyr^{701}, which, as mentioned above, results in its activation (Shuai *et al.*, 1993; Shuai *et al.*, 1994; Greenlund *et al.*, 1994; Heim *et al.*, 1995).

GAF formation and GAS stimulation

Unlike the IFN-α/β signalling pathway, only STAT1 participates in IFN-γ signal transduction. Thus, following activation, phosphorylated STAT1 molecules dissociate from the IFN-γ receptor and dimerise through SH2 domain-tyrosine phosphate recognition. Active STAT1 homodimers, referred to as gamma-activated factor (GAF),

then translocate to the nucleus (Sekimoto *et al.*, 1996) where they drive the expression of type II IFN-inducible genes by binding to their unique gamma activation sequence (GAS) regulatory element (reviewed in Stark *et al.*, 1998), which contains the consensus sequence TTNCNNNAA. Intriguingly, STAT1 homodimers are known to be formed not only in response to IFN-γ, but also following stimulation with IFN-α/β, which, nevertheless, results in a far less efficient signal response (Haque & Williams, 1994), through a mechanism that requires further elucidation.

1.1.2.3 STAT1 in IFN signalling

Ser^{727} and protein interactions

Following activation of STAT1 by phosphorylation at Tyr^{701}, the protein translocates to the nucleus where another phosphorylation reaction occurs at serine position 727 (Ser^{727}). This phosphorylation event is a prerequisite for the transactivation function of the protein (Wen *et al.*, 1995), as it is known to facilitate the interaction of STAT1 not only with the basal transcription machinery, but also with the chromatin-associated protein MCM5 (Zhang *et al.*, 1998b) and a protein called Nmi. Nmi enhances binding of STAT1 to the CREB-binding protein (CBP)/p300 transcription factors (Zhu *et al.*, 1999), which occurs both through its carboxy- and amino-terminal domains (Zhang *et al.*, 1996); the role of CBP/p300 is reviewed in (Janknecht & Hunter, 1996). STAT2 is also known to bind to CBP/p300 and associate with the basal transcription machinery (Bhattacharya *et al.*, 1996).

Although Ser^{727} is known to be phosphorylated by a kinase with a MAP-like specificity, the precise candidate has been subject to controversy. However, it is believed that different kinases may be responsible for this reaction, according to cell type (reviewed in Goodbourn *et al.*, 2000). Moreover, Takaoka and colleagues (1999) have shown that Pyk2, a tyrosine kinase, is involved in Ser^{727} phosphorylation, although this is true for IFN-γ but not IFN-α/β signalling. PKR is also known to be involved in the process, but in a rather indirect fashion (Ramana *et al.*, 2000). Unlike STAT1, STAT2 does not have a MAP kinase consensus region and is not serine-phosphorylated in response to IFN stimulation (Bhattacharya *et al.*, 1996).

STAT1β form

As discussed above, STAT1 is a major component of both type I and type II IFN signalling. Nevertheless, STAT1, the predominant, active form of which is referred to as STAT1α, is additionally found in a second form, called STAT1β. STAT1β occurs by differential gene splicing and differs from STAT1α in that it lacks the carboxy-terminal 38 amino acid residues, including Ser^{727}, which disables its transcription activation function (Schindler *et al.*, 1992; Shuai *et al.*, 1993). However, when activated at the IFN receptor complex by the associated kinases, STAT1β is phosphorylated at Tyr^{701} and translocates to the nucleus to bind to DNA. Moreover, it can still participate in the formation of ISGF3 complexes which remain functional because of STAT2 activity (Muller *et al.*, 1993). Given the above, and while the function of STAT1β remains obscure, it has been suggested that the formation of STAT1α/STAT1β heterodimers might be involved in the downregulation of IFN-induced gene expression.

Signal attenuation

STAT1 dephosphorylation, which is catalysed by a tyrosine phosphatase, makes STAT1 activation transient, thus leading to downregulation of IFN signalling (Haque *et al.*, 1995). A possible candidate for the tyrosine phosphatase involved in this attenuation effect is SHP1, which is known to associate with the IFNAR1 receptor subunit following stimulation with IFN-α (David *et al.*, 1995). It is believed that it is this dephosphorylation process that mainly regulates STAT1 function and not its degradation by the proteasome (Kim & Maniatis, 1996). Although the mechanisms that attenuate IFN signals require further investigation, experimental evidence has suggested that IFN-induced proteins can act in a negative feedback loop and inhibit further expression of IFN-sensitive genes (Friedman *et al.*, 1984; Larner *et al.*, 1986). For example, IFN-γ (as well as other cytokines) are known to induce the SOCS/JAB/SSI protein family which bind and inhibit activated Jak kinases, thus negatively regulating IFN signalling (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997; Starr & Hilton, 1999). Finally, IRF proteins IRF-2 (Harada *et al.*, 1989) and IRF-8 (Nelson *et al.*, 1993) also downregulate IFN signalling, particularly type I IFN signalling, by binding to the ISRE gene regulatory element. Thus, in contrast to the ISGF3 complex and IRF-1, which bind to ISRE and induce gene expression, IRF-2 does not activate transcription, competing for binding to the ISRE sequence and thereby suppressing gene activation. This is thought to act not only as a signal attenuation mechanism (when signalling has been triggered by IFN-α/β) but also as a preventive mechanism that protects the cell

from the adverse effects of IFN-induced gene expression under normal conditions (no infection).

1.1.2.4 IFN antiviral action

Due to the major effects that IFN-activated mechanisms have on cell metabolism, growth and death, the enzymes involved in the processes against virus proliferation are only activated following viral infection. Since dsRNA is not a macromolecule that is found naturally in the intracellular environment, it is believed that its occurrence upon viral infections must act as an activation cofactor that triggers antiviral pathways (reviewed in Jacobs & Langland, 1996). Although the roles of PKR and the endoribonuclease L (RNase L) system are best established as IFN-induced antiviral mechanisms, undoubtedly, more cellular mechanisms are known to be involved in the IFN-induced antiviral response. As described below, these mechanisms appear to interfere directly with virus processes and RNA metabolism (Mx proteins and dsRNA-dependent adenosine deaminase, ADAR), the progression of the cell cycle and gene transcription (effect on p21, p202 and c-myc levels), as well as with programmed cell death (caspase and Fas activation).

(i) dsRNA-dependent protein kinase R (PKR)

PKR activation

PKR is a serine/threonine kinase with two domains, the amino-terminal regulatory domain and the carboxy-terminal catalytic domain, and is normally inactive in the cell, in a monomeric form. PKR is induced in response to IFN and is activated when dsRNA (or other polyanions) binds to its regulatory domain (Meurs *et al.*, 1990; Katze *et al.*, 1991; George *et al.*, 1996). Activation requires that dsRNA has a minimum length of 50 base pairs and, whilst RNA binding to PKR is not sequence-specific, some RNAs seem to be more powerful activators than others (reviewed in Robertson & Mathews, 1996). Upon activation, PKR undergoes a conformational change which essentially reveals the catalytic domain, and the protein is believed to dimerise, as one molecule of dsRNA binds and brings two PKR molecules together. This dimerisation event is followed by reciprocal transphosphorylation of the juxtaposed proteins on serine and threonine residues.

PKR function

PKR is a kinase with multiple roles in transcriptional and translational control. With regard to translation, it is known that PKR phosphorylates the eukaryotic translation initiation factor eIF2 α-subunit, thereby preventing the recycling of initiation factors (reviewed in Clemens & Elia, 1997) and inhibiting translational initiation. PKR is also involved in dsRNA-triggered signal transduction pathways (as well as cascades activated by other ligands; reviewed in Williams, 1999), such as dsRNA-dependent NF-kB-mediated IFN-β gene induction, which has been described above. In addition, PKR affects the activation of transcription factors involved in virus clearance, namely STAT1 (Wong *et al.*, 1997; Ramana *et al.*, 2000), IRF-1 (Kumar *et al.*, 1997) and p53 (Cuddihy *et al.*, 1999a; Cuddihy *et al.*, 1999b), through mechanisms that need to be resolved. Apoptosis is another cellular process that can be induced either directly by viral dsRNA (Der *et al.*, 1997; King & Goodbourn, 1998; Tanaka *et al.*, 1998) through a PKR-mediated mechanism (Takizawa *et al.*, 1996; Der *et al.*, 1997; Tan & Katze, 1999; reviewed in Jagus *et al.*, 1999), or indirectly via PKR-dependent induction of Fas ligand and receptor (Takizawa *et al.*, 1995; Balachandran *et al.*, 1998; Fujimoto *et al.*, 1998). Bcl-2- and caspase-dependent mechanisms have also been shown to be involved in PKR-mediated apoptosis. Nevertheless, certain viruses induce apoptotic processes via pathways that do not require PKR (Balachandran *et al.*, 2000), revealing that, despite the major role of PKR in antiviral mechanisms, there are cellular antiviral responses that depend on non-PKR-mediated processes.

(ii) The 2'-5' oligoadenylate synthetase/RNase L system

RNase L activity

In mammalian cells, IFNs induce a group of enzymes called the 2'-5' oligoadenylate synthetase system, which catalyses the synthesis of labile oligoadenosine molecules from ATP. These oligomers, termed 2'5'A, comprise three to five units linked by phosphodiester bonds in a 2'-5' conformation (Kerr & Brown, 1978) and bind and activate endoribonuclease L (RNase L) by mediating its dimerisation. The role of RNase L in the cellular antiviral response is of major importance, as it blocks protein synthesis by cleaving RNA (reviewed in Silverman, 1997). RNase L degrades single-stranded RNA, as well as 28S ribosomal RNA, thus targeting gene transcripts and disabling the ribosomal machinery required for their translation (Iordanov *et al.*, 2000). Despite the ability of the enzyme to degrade mRNA, which includes cellular mRNA, RNase L activity in the intracellular environment is primarily focussed on viral RNA, as

a result of 2'-5' oligoadenylate synthetase activation (and 2'5'A synthesis) near viral dsRNA (Nilsen & Baglioni, 1979).

RNase L and apoptosis

In addition to having ribonuclease activity against viral RNA, RNase L also mediates the apoptotic action of IFN in infected cells (Zhou *et al.*, 1997; Castelli *et al.*, 1998a; Castelli *et al.*, 1998b). Although the precise mechanism through which this is effected remains to be investigated, the link between RNase L and apoptosis has already been demonstrated *in vivo* with RNase $L^{-/-}$ mice that are defective for apoptosis (Zhou *et al.*, 1997), and *in vitro* by direct induction of apoptosis in animal cells through RNase L activation (Diaz-Guerra *et al.*, 1997). As described below, IFN-induced apoptosis is a key mechanism for the inhibition of virus proliferation and spread, which reveals yet another important role of RNase L in antiviral responses.

(iii) Alternative antiviral mechanisms

Mx proteins

Mx proteins are large, highly conserved GTPases homologous to dynamin, which have antiviral activity. Mx proteins are found in all vertebrates, including mammals, birds and fish (reviewed in Staeheli *et al.*, 1993; Arnheiter *et al.*, 1995), and are induced upon virus infections in response to IFN. It has been proposed that one of the ways that Mx proteins inhibit viral replication is by impeding virus polymerase trafficking or activity (Stranden *et al.*, 1993). A direct link between their GTPase activity and ability to inhibit virus proliferation has been demonstrated experimentally with mutant Mx proteins that cannot bind or hydrolyse GTP and thus lose their antiviral activity. Different Mx proteins have been shown to inhibit the proliferation of different RNA virus families (reviewed in Goodbourn *et al.*, 2000), and human cytoplasmic MxA has been shown to also be able to affect *Paramyxoviridae* (Schneider-Schaulies *et al.*, 1994; Zhao *et al.*, 1996).

Caspases and ADAR

In addition to PKR, RNase L, and Mx proteins, experimental findings from transgenic mice (Zhou *et al.*, 1999) have shown that other pathways must also be activated in response to IFNs. Thus, as discussed below, it is known that IFNs induce caspases, enzymes involved in apoptosis, as well as dsRNA-dependent adenosine deaminase (ADAR), which is known to have a direct mutagenic effect on dsRNA (reviewed in

(Goodbourn *et al.*, 2000) and contribute to the degradation of modified viral RNA (Scadden & Smith, 1997).

(iv) Antiproliferative action

Inhibition of cell cycle progression

The cytostatic action of IFNs is one of the mechanisms through which proliferation of certain viruses is inhibited, as, by slowing down cell growth, cellular processes that are necessary for virus replication are also arrested. This effect varies according to cell type and, because of its great clinical importance, it has been studied extensively. Experimental data have shown that PKR and RNase L are proteins that are involved in the antiproliferative action of IFN. Moreover, p21, a cyclin-dependent kinase inhibitor involved in cell cycle progression, is also subject to IFN stimulation; in response to IFN, p21 levels rise, causing the cell cycle to arrest through an increase in hypophosphorylated pRB and a consequent reduction in E2F transcription factor availability (reviewed in Goodbourn *et al.*, 2000). Given that E2F transcription factors are highly important for gene expression and transition through the G_1-S cell cycle phase, it is therefore obvious that IFN poses a key constraint on cell proliferation. However, p21 is not the only mediator of IFN-induced E2F inactivation; p202 is another IFN-induced protein which not only affects E2F function (Choubey *et al.*, 1996; Choubey & Gutterman, 1997), but inhibits gene transcription itself through its transcriptional repression domain (Johnstone *et al.*, 1998). Transcription of c-myc, which is required for cell growth, has also been also shown to be inhibited by IFNs, providing another direct link between IFNs and their antiproliferative properties.

(v) Apoptotic control

Links between IFN and apoptosis

Similar to other cytokines, IFNs are known to influence cellular apoptotic responses, by inducing or inhibiting apoptosis. Thus, as described above, IFNs can affect indirectly the apoptotic state of cells through PKR and the 2'-5'-oligoadenylate system. Nevertheless, the direct link between IFNs and apoptosis is also established, as IFNs are known to induce caspases (Chin *et al.*, 1997; Subramaniam *et al.*, 1998; Balachandran *et al.*, 2000), which are cellular enzymes with a central role in the apoptotic process. Given that a major role of IFN is to stimulate apoptosis in response to viral infection (Tanaka *et al.*, 1998), caspase induction seems to be a key IFN function. Moreover,

IFN-γ has been shown to be involved in apoptosis through induction of Fas ligand and Fas receptor (Xu *et al.*, 1998). It is noteworthy that, although the role of IFN in apoptosis is linked to viral infections, IFN action is not restricted to infected cells. Primary targets are uninfected cells which, in response to IFN secreted by neighbouring infected cells, enter a pro-apoptotic state (reviewed in Schindler, 1998), rendering the tissue in the vicinity of the infection nonpermissive for viral replication.

(vi) Immunomodulatory effects of IFN

IFNs, and particularly IFN-γ, are very potent immune regulators, with their action affecting both the innate and adaptive arms of the immune system. The effect of IFNs on adaptive immunity extends not only to the humoural and cellular branch of the system, but also to other critical immune processes, such as antigen processing and presentation, which, in turn, promote downstream immune responses.

Effects on innate immunity

Regarding innate immune responses, a key role of IFN-α/β is to stimulate NK cell proliferation (to some degree), but, mainly, to enhance NK cell cytotoxicity (reviewed in Reiter, 1993; Biron *et al.*, 1999), which is obviously of high significance for host antiviral measures. This is achieved through upregulation of perforin levels (Mori *et al.*, 1998; Kaser *et al.*, 1999), which is required for elimination of target cells. IFN-α/β is also known to induce production of IL-12 and IL-15, which seem to both stimulate and inhibit IFN-γ production via different mechanisms. IFN-γ also influences innate immune mechanisms, by affecting the activation of macrophages which, in turn, use IFN-γ-induced mechanisms to destroy their targets (reviewed in Goodbourn *et al.*, 2000).

Effects on adaptive immunity

All IFNs are known to be able to stimulate $CD8^+$ T cell responses, by upregulating MHC I protein expression (reviewed in Boehm *et al.*, 1997). However, MHC II-dependent antigen presentation is only boosted by IFN-γ which thus promotes $CD4^+$ T cell mechanisms. The humoural arm of the adaptive immune system is also affected by IFNs, as they influence the development of specific subsets of T helper (Th) cells and exert direct effects on the B cell population. This includes regulation of B cell development and proliferation, as well as regulation of immunoglobulin (Ig) secretion and heavy chain switching (Snapper & Paul, 1987). Moreover, IFN-α/β supports directly the survival of activated T cells (Marrack *et al.*, 1999), and, by inducing IL-15,

it also stimulates memory T cell division (reviewed in Tough *et al.*, 1999). IFN-γ is additionally known to regulate the balance between Th1 and Th2 cells (reviewed in Goodbourn *et al.*, 2000).

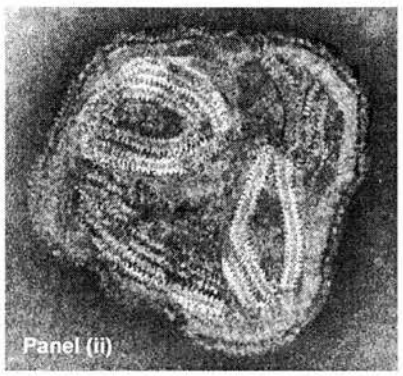
Antigen processing/ presentation

IFNs, and particularly IFN-γ, are intricately involved in the enhancement of immunogenicity, by broadening the antigen species range and increasing the amount of peptides presented to $CD8^+$ T cells. Thus, IFN-γ affects antigen processing by inducing proteasome subunits LMP2, LMP7 and MECL1, which favour the degradation of protein antigens to peptides that associate with MHC I (reviewed in York & Rock, 1996). Furthermore, IFN-γ promotes trafficking of processed peptides that will ultimately bind to MHC I for presentation to $CD8^+$ T cells, by upregulating expression of TAP1 and TAP2 (Trowsdale *et al.*, 1990; Epperson *et al.*, 1992), proteins that are crucial for intracellular antigen transportation to the cell surface. Since viral infections generate intracellular antigens that are subject to the above processing mechanisms, it is therefore obvious that IFNs favour strongly the ability of the cell to present viral antigens, thereby affecting virus survival.

1.1.3 Viral anti-IFN strategies

Viral immune evasion strategies have coevolved alongside cellular immune defences (Alcami & Koszinowski, 2000), giving rise to counter-IFN molecular mechanisms that enable viruses to circumvent the restrictions posed by IFN upon their replication cycle. Viral anti-IFN mechanisms focus at many levels, from inhibition of IFN induction to IFN signalling blocking and inhibition of IFN-induced enzymes. Thus, viruses are known to be able to inhibit IFN production by sequestering RNA (a factor critical to the activation of IFN-induced mechanisms), by targeting NF-κB (thereby disrupting NF-κB-dependent IFN production), or by inhibiting the activity of IRF proteins (which play a major role in the IFN expression system). Yet other viruses can inhibit host mRNA or protein synthesis, or employ strategies of molecular mimicry or upregulation of cytokines that are critical to the immune system, such as cytokine IL-10 (Bejarano & Masucci, 1998; Arena *et al.*, 1999). The IFN-induced PKR and 2'-5'oligoadenylate synthetase/RNase L systems are also direct targets of viral counter-IFN strategies (reviewed in Gale & Katze, 1998), which provides an obvious advantage to the

1.2 *Paramyxoviridae*: A virus family of worldwide importance



Paramyxoviruses infect vertebrates, causing human and animal diseases that have been known for a long time, with the description of parotitis and orchitis (mumps virus) by Hippocrates dating back to the 5th century BC. Today, there is still not any treatment for **mumps**, which can lead to sterility, deafness or encephalitis (WHO, 2002; Cann, 1999). **Measles** virus, a paramyxovirus that causes one of the most infectious diseases in children, and which has been targeted for eradication by the World Health Organisation (WHO), is still responsible for more deaths than any other Expanded Programme on Immunization (EPI) target disease. Every year, measles kills over one million children worldwide. **Parainfluenzaviruses** (hPIV) commonly cause a range of diseases to children, from mild influenza-like illness to bronchitis, pneumonia and croup disease. hPIV3 and **respiratory syncytial virus** account for one in four cases of pneumonia and one in two cases of bronchiolitis in hospitalised children, making them the main cause of acute respiratory disease of infancy and early childhood (WHO, 2002).

Amongst paramyxoviruses that infect animal host species, **Newcastle disease** and **rinderpest viruses** are of great economic importance, affecting poultry and animal farming. **Simian virus type 5**, also known as canine parainfluenza virus (CPI) in veterinary circles, causes respiratory illness to dogs (kennel cough) and has been suspect to other implications in humans, as discussed below.

New species

Over the last decade, an increasing number of novel paramyxoviruses has been discovered in a wide range of terrestrial (rodent, bat, rat, mouse, tree shrew, pig, horse, penguin, snake) and aquatic animals (porpoise, dolphin, seal; reviewed in Wang & Eaton, 2001). However, humans have also been reported to be infected with some of the new paramyxovirus species. Thus, illustrating the great significance of the ability of viruses to cross host species, the recently isolated paramyxoviruses **Hendra** (Australia,

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Panel (ii): Electron micrograph of a human paramyxovirus virion (obtained from Linda Stannard's Virus Ultrastructure web site, Department of Medical Microbiology, University of Cape Town; http://www.uct.ac.za/depts/mmi/stannard/paramyx.html).

1994) and **Nipah** (Malaysia, 1999), whose natural host has been shown to be the bat, have been directly linked to the death of over 67 human individuals by encephalitis (CDC, 1999a; CDC, 1999b; Wang & Eaton, 2001).

A synopsis of the human diseases caused by paramyxoviruses is presented below (Table 1.1).

Table 1.1: Diseases caused to humans by paramyxoviruses (information obtained from: Garry, 1995a; WHO, 2002; CDC, 1999a; CDC, 1999b).

Disease	Paramyxovirus
Bronchiolitis	RSV, Parainfluenza virus
Croup, infectious	Parainfluenza virus types 1-3
Measles (+SSPE)	Measles virus
Parotitis	Mumps virus
Pharyngitis	RSV, Parainfluenza virus
Pneumonia, viral	RSV
Encephalitis	Hendra, Nipah viruses

1.2.1 Classification of paramyxoviruses

The order

Paramyxoviridae are single-stranded, nonsegmented, negative-sense RNA viruses, with the length of their genome ranging from 15 to 19 kb (Garry, 1995b). The virus family itself belongs to the order of *Mononegavirales* that also includes the closely related *Filoviridae* (Marburg and Ebola viruses) and *Rhabdoviridae* (Rabies, Vesicular stomatitis, Bovine Ephemeral Fever viruses) families (Garry, 1995c).

The family

Paramyxoviridae have been classified into distinct subfamilies and genera (Table 1.2) by the International Committee on the Taxonomy of Viruses, on the basis of their morphological characteristics, the organisation of their genome, as well as the biological activities and the sequence relationship of the proteins that they encode (Kolakofsky & Lamb, 2001). The family is divided into two subfamilies, namely *Paramyxovirinae* and *Pneumovirinae*. The latter includes the genera Pneumovirus and Metapneumovirus, while the *Paramyxovirinae* subfamily contains the Respirovirus, Rubulavirus and

Table 1.2: Classification of paramyxovirus species into subfamilies and genera (adapted from Kolakofsky & Lamb, 2001).

Family ***Paramyxoviridae***

Subfamily ***Paramyxovirinae***

Genus **Respirovirus**

- Sendai virus (SeV)
- human Parainfluenza virus types 1 & 3 (hPIV1/3)
- bovine Parainfluenza virus type 3 (bPIV3)

Genus **Rubulavirus**

- Simian virus type 5 (SV5)
- Simian virus type 41 (SV41)
- Mumps virus (MuV)
- human Parainfluenza virus type 2 (hPIV2)
- human Parainfluenza virus type 4a & 4b (hPIV4a/4b)
- Newcastle Disease virus (NDV)

Genus **Morbillivirus**

- Measles virus (MeV)
- Dolphin Morbillivirus (DMV)
- Canine Distemper virus (CDV)
- Peste-des-petits ruminants virus (PPRV)
- Phocine Distemper virus (PDV)
- Rinderpest virus (RPV)

Unclassified Paramyxovirinae

- Hendra virus (HeV)
- Nipah virus (NiV)
- Tupaia Paramyxovirus (TPMV)

Subfamily ***Pneumovirinae***

Genus **Pneumovirus**

- human Respiratory Syncytial virus (hRSV)
- bovine Respiratory Syncytial virus (bRSV)
- Pneumonia virus of mice (PVM)

Genus **Metapneumovirus**

- Avian pneumovirus (TRTV)
  (formerly called Turkey Rhinotracheitis virus)

Morbillivirus genera. One of the criteria for the classification of virus species in the Rubulavirus genus, which includes Simian virus type 5 (SV5), is that these viruses do not express a set of gene products termed C proteins (explained below; please refer to Table 1.3 and Figure 1.5), as well as the fact that some of them (including SV5) contain an extra gene that codes for an additional protein, the small hydrophobic (SH) protein (Kolakofsky & Lamb, 2001).

Hendra and Nipah viruses, which have unusually long genomes (>18 kb; Chua *et al.*, 2000; Harcourt *et al.*, 2000), remain unclassified members of the *Paramyxovirinae* subfamily, together with the tree shrew Tupaia virus (Kolakofsky & Lamb, 2001).

1.2.2 Virion structure and life cycle

The virion

Paramyxoviruses are pleiomorphic, with typical spherical forms ranging from 150 to 350 nm in diameter. Embedded into the envelope of the particle, which is derived from the host cell membrane, are the attachment and fusion (F) glycoproteins, which extend approximately 8 to 12 nm from the surface of the membrane. Inserted into the lipid envelope are also copies of the SH (small hydrophobic) protein (only in some rubulaviruses, including SV5). The M (matrix) protein, which underlies the lipid membrane, is thought to hold the particle together, bridging the surface proteins with the nucleocapsid. The nucleocapsid, which is remarkably stable, is a helical protein:RNA structure in the core of the particle, comprising the nucleocapsid NP protein associated with the genome. Attached to the genome, which is single-stranded, nonsegmented RNA, are the P (phosphoprotein) and L (large) proteins (Kolakofsky & Lamb, 2001). In rubulaviruses, copies of the V protein are also associated with the nucleocapsid (Paterson *et al.*, 1995; Randall & Bermingham, 1996). A schematic representation of a paramyxovirus particle is shown in Figure 1.2.

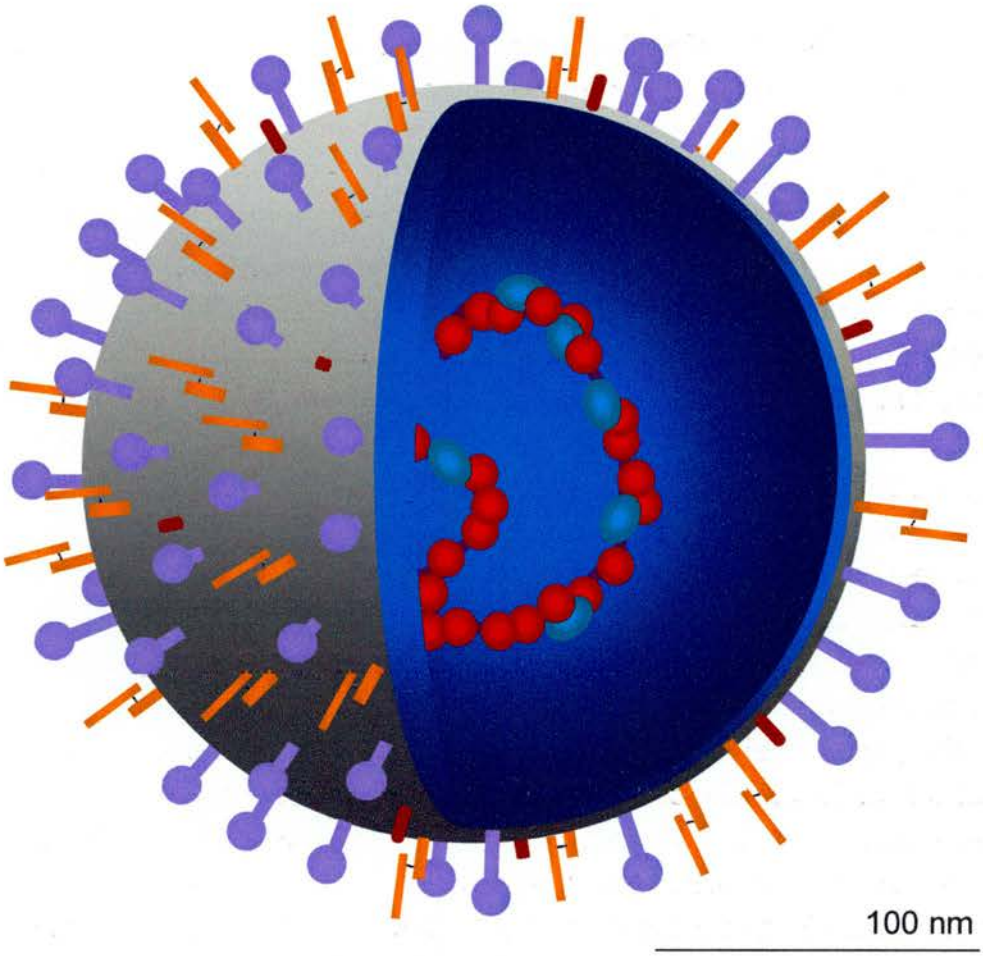
Life cycle

To establish an infection *in vivo*, upon entry, paramyxoviruses first have to infect the mucosal surfaces ('myxo-' meaning mucus in Greek) of the respiratory tract of the host. The replication cycle of a paramyxovirus, illustrated schematically in Figure 1.3, begins with the attachment of the virus particle to receptor proteins on the surface of the host cell, followed by fusion of the virion envelope with the cell membrane. These events are

Figure 1.2: Three-dimensional, schematic representation of the virion structure of simian virus type 5 (SV5). (not drawn to scale)

The virus is coated by a lipid envelope (outer layer shown in grey), underneath which the matrix (M) protein forms a coating structure that covers the inner membrane leaflet (drawn in blue colour). The matrix structure is believed to be essential for the architecture of the virion, which possibly acts as a bridge between different virus components. The surface glycoproteins haemagglutinin-neuraminidase (HN) and fusion (F) are embedded into the lipid bilayer and protrude from the surface of the virus particle. HN (drawn in purple) is believed to have a structure that consists of a stalk region and a globular head, whilst F (drawn in orange) comprises two subunits (F_1 and F_2) linked by a disulfide bond. HN is a tetramer, whereas F is a trimer (not shown). The small hydrophobic protein is also illustrated (short, brown-coloured stalks), whereas the orientation of all three membrane-anchored proteins is indicated in the key of the diagram. No attempt has been made to indicate a possible interaction of the cytoplasmic tails of the glycoproteins with M, as this issue remains subject to investigation. The helical nucleocapsid is found in the core of the particle and comprises the single-stranded, nonsegmented, negative-sense genomic RNA of the virus (presented as a thread-like structure), complexed with the nucleocapsid (NP), phosphoprotein (P) and large protein (L) (all three proteins are represented as red particles). P and L together form the RNA polymerase of the virion (RNA-dependent transcriptase activity). As in all rubulaviruses, the V protein of SV5 (depicted as green particles) is also associated with the nucleocapsid, whereas, for other paramyxovirus genera, this protein is only found in infected cells (adapted from Kolakofsky & Lamb, 2001).

Fig. 1.2


100 nm

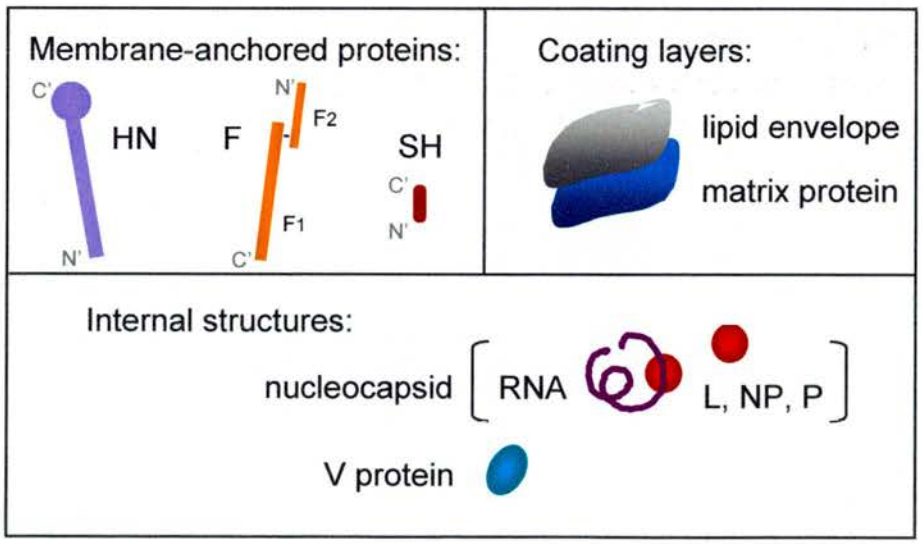
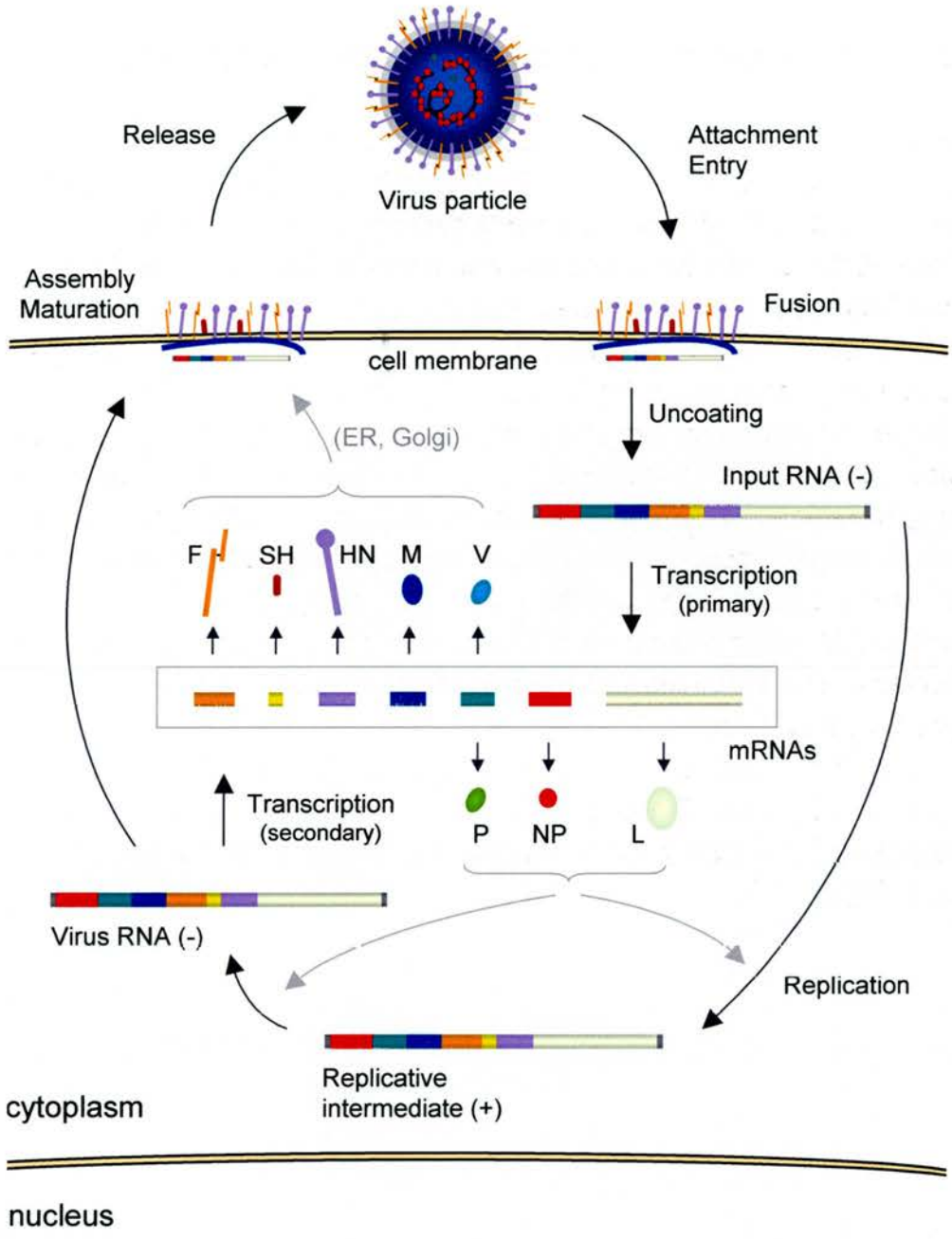

Membrane-anchored proteins:
C'
N'
HN
N'
F2
F
F1
C'
SH
C'
N'
Coating layers:
lipid envelope
matrix protein
Internal structures:
nucleocapsid
RNA
L, NP, P
V protein

Figure 1.3: Schematic representation of the SV5 replication cycle.

Virion attachment to the cell surface is the first step that is necessary for the virus to infect a cell. Haemagglutinin-neuraminidase (HN) has a central role in this process, as this viral protein binds protein receptors on the surface of the host cell. Entry of the virus into the cell involves fusion of the virus envelope with the host cell membrane, mediated primarily by the fusion (F) protein, and uncoating of the genome (negative-sense, input RNA) in the cytoplasm. Transcription is carried out by the nucleocapsid-associated phosphoprotein (P) and large (L) proteins, which recognise NP-associated genomic RNA as template. In addition to transcribing genes to mRNA for protein expression (primary transcription), the polymerase replicates the entire genome (negative-sense), through a replicative intermediate RNA (positive-sense). Genomic RNA copies are also used for gene expression (secondary transcription). During virus replication, V protein activity is known to counteract the innate immune responses of the host, whereas the role of SH remains unknown. HN, F and SH are produced and routed through the endoplasmic reticulum (ER) and Golgi apparatus to the cell membrane, where virus assembly and maturation are thought to take place. The M protein is believed to provide the driving force for the release of the newly formed virus particles (adapted from Kolakofsky & Lamb, 2001).

Fig. 1.3


Release
Virus particle
Attachment
Entry
Assembly
Maturation
Fusion
cell membrane
Uncoating
(ER, Golgi)
Input RNA (-)
F
SH
HN
M
V
Transcription
(primary)
mRNAs
Transcription
(secondary)
P
NP
L
Virus RNA (-)
Replication
Replicative
intermediate (+)
cytoplasm
nucleus

driven by the catalytic action of the attachment and F proteins, respectively. Following the entry of the virus into the cell, all subsequent intracellular phases of replication, i.e. transcription, translation, genome replication and assembly, take place in the cytoplasm. At least one homotetramer of P and a copy of L are required for the transcription of the NP-encapsidated genomic RNA into 5'-capped and 3'-polyadenylated mRNAs. The reaction is always initiated at the 3' end of the genome and is terminated and reinitiated at gene junctions, which, due to frequent reinitiation failure, leads to the accumulation of a gradient of gene transcripts. Thus, the number of mRNA transcripts is inversely proportional to the distance of the gene from the 3' end of the viral genome. Following the translation of the transcripts to viral proteins, and after the levels of NP have risen sufficiently, encapsidation of the positive-sense RNA becomes coupled to viral RNA synthesis. At this point, the virus replication machinery starts to ignore the gene junctions, producing complementary copies of the entire genome. The new nucleocapsids are assembled and, under the driving force of the M protein that lines the inner face of the cell membrane, new virus particles are released into the extracellular environment. The newly synthesised glycoproteins, which by that stage have also been embedded into the cell membrane, are believed to play a role in this final step, as the neuraminidase activity of the attachment glycoprotein (property found only in rubulaviruses and respiroviruses) prevents the aggregation of the newly formed virus particles (Kolakofsky & Lamb, 2001).

1.2.3 Genes and gene products

Genome organisation

The genome of paramyxoviruses is single-stranded, nonsegmented, negative-sense RNA and its length ranges from 15 kb, such as that of SV5 (the smallest genome in the *Paramyxovirinae* subfamily; 15246 bp; GenBank Accession no. AF052755), to over 18 kb (the recently identified Nipah species has the longest genome of all; 18246 bp; GenBank Accession no. AF212302). The complete genome sequence of nearly all known paramyxoviruses is currently available (NCBI, 2002) and the number of identified genes ranges from six to ten, depending on the species (SV5 contains seven genes). However, the number of proteins encoded by *Paramyxovirinae* is larger than the number of genes contained in their genome, as their V/P gene contains overlapping open reading frames (ORFs) that give rise to distinct gene products, as described below.

A schematic representation of the SV5 genome is illustrated in Figure 1.4. All genes are flanked by conserved transcriptional control sequences (that are transcribed to mRNA) and spaced apart by intergenic regions, the length of which varies for different viruses. In all paramyxoviruses, there are also extracistronic sequences at the beginning (3' end) and the end (5' end) of the genomic RNA, which are termed leader and trailer (or (-) leader) sequences, respectively. These sequences are necessary for both transcription and replication and also vary in length amongst different species.

Genes and proteins of SV5

As shown in Figure 1.4, SV5 features seven genes and eight gene products, the structural characteristics and function of which are discussed below. These include the membrane-anchored haemagglutinin-neuraminidase (HN), fusion (F) and small hydrophobic (SH) proteins, the matrix protein (M), the nucleocapsid protein (NP), as well as the V protein and phosphoprotein P, which are both encoded by the V/P gene.

1.2.3.1 Haemagglutinin-neuraminidase

SV5 HN

The nucleotide sequence of the HN genes of all *Paramyxoviridae* is currently available. The HN gene of SV5 (locus S76876, GenBank accession no. S76876) has a length of 1789 bp (Baty *et al.*, 1991) and codes for a protein (haemagglutinin-neuraminidase protein; accession no. AAB21114) of 565 amino acids, that has a molecular weight of 62204 Da (SWISS-PROT primary accession no. P04850; Hiebert *et al.*, 1985a).

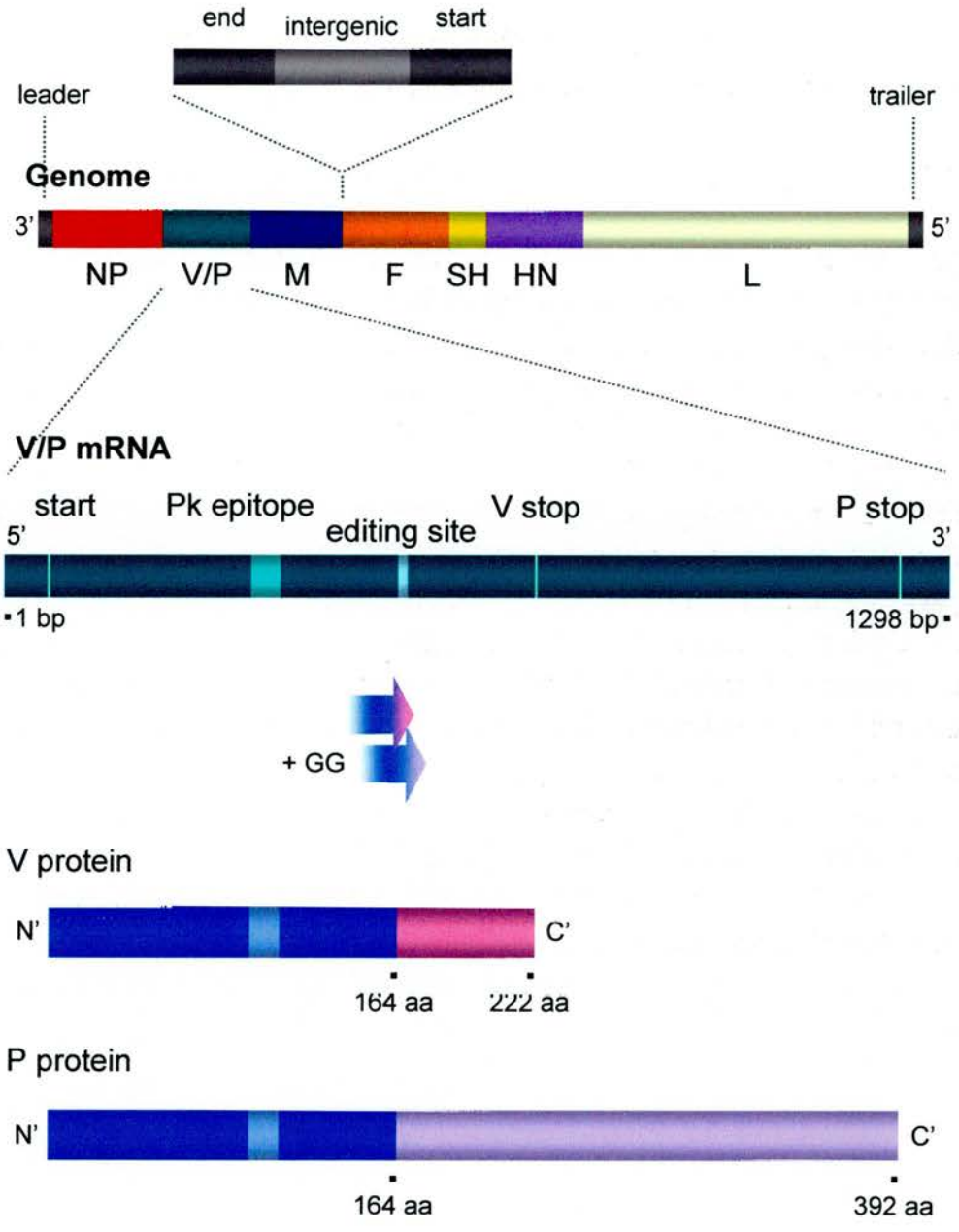
Structure

HN is a type II integral membrane protein, with the amino-terminal domain facing the cytoplasm and the carboxy-terminal domain protruding from the outer surface of the lipid bilayer (see Figure 1.2). The protein is anchored in the lipid membrane by a single hydrophobic domain near the amino-terminus. In paramyxoviruses, HN has four to six sites for the addition of N-linked carbohydrate chains (SV5 HN has four sites). It is anticipated that the protein structure of HN of different paramyxoviruses is similar, as conserved amino acid residues have been identified between related members of the family (Kolakofsky & Lamb, 2001). The atomic structure of the rubulavirus Newcastle disease virus (NDV) HN was recently solved by Crennell and colleagues (2000). The protein is known to form noncovalently-linked tetramers, which consist of disulfide-linked homodimers.

Figure 1.4: Schematic diagram showing the SV5 genome and V/P gene structure, as well as the encoded V and P proteins.

SV5 contains seven genes that code for eight distinct proteins, namely genes NP (that codes for the nucleocapsid protein), V/P (encodes the V protein and the phosphoprotein P), M (matrix protein), F (fusion protein), SH (small hydrophobic protein), HN (haemagglutinin-neuraminidase) and L (large protein). The genome, which is single-stranded, nonsegmented, negative-sense (3'-5') RNA, also contains two extragenic regions, the 3'-terminal leader sequence and the 5'-terminal trailer sequence. Each gene is flanked by transcriptional start and end sequences, which are separated from those of the adjacent genes by intergenic regions. For example, the coding regions of the M and F genes are spaced apart by the M transcriptional end sequence, the M-F intergenic region and the F transcriptional start sequence. The V/P gene codes for two distinct proteins, the V and P proteins, as a result of mRNA editing. Thus, whereas V mRNA is a faithful gene transcript, P mRNA arises by cotranscriptional modification (insertion of two nontemplated G residues), which alters the open reading frame (ORF). Consequently, the amino-terminal parts of V and P are identical (this region is referred to as the V/P common amino-terminal domain), whilst the carboxy-terminal domains are unique for each protein. The Pk epitope, which maps to the amino-terminal domain, is also shown in the diagram (adapted from Kolakofsky & Lamb, 2001).

Fig. 1.4


end
intergenic
start
leader
trailer
Genome
3'
5'
NP
V/P
M
F
SH
HN
L
V/P mRNA
start
Pk epitope
editing site
V stop
P stop
5'
3'
1 bp
1298 bp
+ GG
V protein
N'
C'
164 aa
222 aa
P protein
N'
C'
164 aa
392 aa

Function In respiroviruses and rubulaviruses, HN is the major antigenic determinant and is multifunctional, exhibiting both haemagglutinin and neuraminidase activity. Following the determination of the three-dimensional structure of NDV HN, it became clear that there is a single sialic acid-binding site on the molecule which is responsible for both functions. The haemagglutinin activity is associated with the ability of the protein to adsorb virus particles to sialic-acid-containing receptor molecules, glycoproteins or glycolipids, on the surface of the host cell upon infection. Moreover, HN has been assigned a fusion-promoting activity for most paramyxoviruses. The neuraminidase activity of HN enables the cleavage of sialic acid from the surface of virions, as well as from the surface of infected cells. Thus, HN also has an important role at the end of the replication cycle of the virus, by preventing the aggregation of newly produced virus particles at the cell membrane. In SV5, it is known that transportation of the synthesised copies of HN to the cell surface is followed by rapid internalisation by the clathrin-mediated endocytosis pathway, although the reason for this remains to be determined (Kolakofsky & Lamb, 2001).

1.2.3.2 Fusion glycoprotein

SV5 F The F gene of SV5 (locus SV5PFC, GenBank accession no. K02253) is 1873 bp long (Paterson *et al.*, 1984) and encodes a 529-amino acid, type I integral membrane protein (fusion protein; accession no. AAA47881), the unprocessed precursor of which has a molecular mass of 56597 Da (SWISS-PROT primary accession no. P04849). The protein is anchored to the membrane by a hydrophobic domain near the carboxy-terminal end, with the amino-terminus of the protein protruding from the outer surface of the lipid bilayer (Figure 1.2) and a remaining short stretch of amino acids (20-40 residues) at the carboxy-terminal end forming a cytoplasmic tail (Kolakofsky & Lamb, 2001). Studies on SV5 isolates with altered fusogenic properties have revealed that the length of the cytoplasmic tail of the protein can affect virus fusogenicity, as described later in this chapter.

Subunits The protein is synthesised as an inactive precursor (F_0; 510 amino acid residues), the cleavage of which to two disulfide-linked subunits, F_1 (427 residues) and F_2 (83 residues), is a prerequisite for its function. Thus, F becomes biologically active only

after host cell protease(s) have released the amino-terminus of the membrane-anchored F_1 subunit, which is thought to play a direct role in the fusogenic action of the protein. Cleavage-activation of F, which is a crucial determinant for viral infectivity, can take place either within the cell, with the Golgi-localised enzyme furin being the intracellular protease that current evidence points to, or at the surface of membranes (of infected cells or assembled virions) by secreted cellular proteases. In SV5, as in the rest of rubulaviruses and respiroviruses, the F_1 and F_2 subunits are glycosylated and there are a total of three to six potential sites for the addition of N-linked carbohydrate. In SV5, all four potential sites of the protein are glycosylated (Kolakofsky & Lamb, 2001).

Sequence comparison and structure

Sequence analysis of the nucleotide sequence of the F of paramyxoviruses has indicated that, despite the lack of major regions of sequence identity, all F proteins have similar structure, given their overall hydrophobicity and the similar placement of certain amino acid residues. Moreover, the hydrophobic amino-terminal region (25 amino acids) of the fusion peptide (F_1) is highly conserved among *Paramyxovirinae*, with an identity up to 90% (Kolakofsky & Lamb, 2001). The crystal structure of the SV5 F1 core trimer structure has been solved to a 1.4 Å resolution (Baker *et al.*, 1999).

Function

The F glycoprotein mediates the fusion of lipid membranes, thus playing a major role in virus penetration (virus envelope-cell membrane fusion) and syncytia formation (cell-cell membrane fusion; Kolakofsky & Lamb, 2001). Virus penetration, an event that takes place at neutral pH, occurs at the beginning of the virus replication cycle and results in the delivery of the virus nucleocapsid into the host cytoplasm. Syncytia formation is observed later in infection and involves the fusion of adjacent cells by the action of F that has been expressed on the membrane of infected cells, leading to the formation of syncytia (fused, multinucleated cells). This event provides a possible mechanism for virus spread and can lead to tissue necrosis *in vivo*. Furthermore, in addition to membrane fusion, F has been reported to have attachment activity in Sendai and RSV viruses.

Mechanism

The mechanism of action of F is currently under investigation. It is believed that the hydrophobic fusion peptide (F_1) of the protein initiates the fusion process by intercalating into the target membrane. Activation of F involves not only cleavage of the protein into its F_1 and F_2 subunits, but an overall conformation change of the protein.

Expression studies have shown that in SV5 (W3) (Paterson *et al.*, 1985), as in measles and RSV, F alone is sufficient to form syncytia. However, in other paramyxoviruses (NDV, hPIV2 and -3, bPIV3, mumps, CDV) HN co-expression has been shown to be a prerequisite for F-mediated fusion. Thus, it has been suggested that, following binding of HN to its receptor (Russell *et al.*, 2001), a homotypic (of the same virus) interaction between HN and F is required to trigger the putative conformational activation of F (Lamb, 1993; Russell *et al.*, 2001). This hypothesis was supported by coimmunoprecipitation assays which indicated that the F and HN proteins can exist together in a complex (Kolakofsky & Lamb, 2001). For viruses that do not require the presence of HN for F activation, it has been postulated that the conformation change in F is triggered by the contact event with the target membrane or by the binding of F to a yet unidentified receptor (Lamb, 1993).

1.2.3.3 Small hydrophobic protein

SV5 SH

The SH gene is present in SV5 and mumps (rubulaviruses), as well as RSV (pneumovirus). In SV5 (locus SV5SH, GenBank Accession no. M11785), the gene is 292 bp in length (Hiebert *et al.*, 1985b) and encodes a 44-amino acid residue protein (small hydrophobic protein; accession no. AAA47883) with a molecular mass of 5108 Da (SWISS-PROT primary accession no. P07577).

Characteristics

SH is a type II integral membrane protein, with its carboxy-terminus protruding from the surface of the lipid membrane of infected cells and virus particles (see Figure 1.2). The role of SH in virus growth remains unknown, being dispensable *in vitro* in SV5 (He *et al.*, 1998), whereas it does not seem to be required *in vitro* or *in vivo* for RSV. Nevertheless, experimental data from *in vitro* infection of Madin-Darby canine kidney (MDCK) cells with SV5 have shown that SH is required for apoptosis to be blocked (He *et al.*, 2001).

1.2.3.4 Matrix protein

SV5 M

The M gene of SV5 (locus PMSMP, GenBank accession no. M32248) is 1382 bp long (Sheshberadaran & Lamb, 1990) and codes for a protein of 377 amino acid residues

(matrix protein; accession no. AAA46901) that has a molecular weight of 42250 Da (SWISS-PROT primary accession no. P16629).

Association with other components

The protein is basic and hydrophobic to some extent (Kolakofsky & Lamb, 2001) and has been shown to be able to associate with membranes only peripherally, as it has domains that are insufficiently long to span the membrane. Therefore, it has been postulated that M has amphipathic α-helices that insert themselves into the inner leaflet of the membrane, thus coating the cytoplasmic face of the lipid bilayer (Figure 1.2). In addition, M is associated with the nucleocapsid and, at least in Sendai virus (SeV), evidence suggests that it also interacts with the cytoplasmic tails of the HN and F glycoproteins. M is the most abundant protein in the virion (Kolakofsky & Lamb, 2001).

Function

Given its multiple associations with other components of the virus (membrane, glycoproteins, nucleocapsid), it is reasonable to identify M as the virus protein that plays a central role in the architecture of the virion. Moreover, it is believed that, by associating with itself and nucleocapsids in infected cells, M provides the driving force for the assembly and release of the newly produced virus particles at the end of the replication cycle of the virus (Figure 1.3). The importance of M activity for virion assembly is demonstrated in cases of persistent paramyxovirus infections, in which virus budding fails to occur. Thus, in cases of subacute sclerosing panencephalitis (SSPE), a persistent measles infection of the brain, M is either absent or fails to associate with budding structures. Similarly, in persistent Sendai virus infections, M is unstable and budding structures are absent (Kolakofsky & Lamb, 2001).

1.2.3.5 Nucleocapsid protein

SV5 NP

The NP gene of SV5 (locus SV5NUCCAP, GenBank accession no. M81442) has a length of 1787 bp (Parks *et al.*, 1992) and encodes a protein (nucleocapsid protein; accession no. AAA47880) which contains 509 amino acids and has a molecular mass of 56535 Da (SWISS-PROT primary accession no. Q88435).

Structure NP is the protein that is tightly associated with genomic RNA, forming the helical nucleocapsid structure in the core of virus particles, and every copy of the protein is predicted to be associated with precisely six nucleotides (reviewed in Kolakofsky *et al.*, 1998). Nevertheless, the protein does not seem to be a classic RNA-binding protein, as it does not have any previously recognised RNA-binding motifs. Experimental data suggest that NP has two domains, a globular amino-terminal body that represents 80% of the protein, and a hypervariable tail-like carboxy-terminal domain (20% of the protein) that extends from the amino-terminal body. The amino-terminal domain is relatively conserved amongst related virus species and evidence suggests that it contains the RNA binding domains and the determinants for the helical nature of the nucleocapsid. In contrast, the carboxy-terminal domain of the protein, which contains invariably a highly charged, mostly negative region, is poorly conserved and contains most of the phosphorylation and antigenic sites of the protein (Kolakofsky & Lamb, 2001).

Function NP seems to be the protein that protects genomic RNA by forming an RNase-resistant nucleocapsid around it. In the replication cycle of the virus, NP also plays an important role in transcription and replication, as, not only does it associate with the P-L polymerase complex to participate in those processes (Figure 1.3), but it is also believed to regulate the relative rates of these reactions through the intracellular concentration of its free (unassembled) form. The interaction of NP with the phosphoprotein (P) is well-documented, as discussed below. Last but not least, at the end of the virus life cycle, and in concert with the M (matrix) protein, NP is thought to have a central role in virus assembly (Kolakofsky & Lamb, 2001).

1.2.3.6 Large protein (RNA polymerase β-subunit)

SV5 L The L gene of SV5 (locus SV5LPRO, GenBank Accession no. M81721) is 6859 bp in length (Parks *et al.*, 1992) and codes for a protein (Large protein; accession no. AAA47879) of 2255 amino acids and a molecular mass of 255923 Da (SWISS-PROT primary accession no. Q88434). As in all paramyxoviruses, the L gene localises at the far 5' end of the viral genome, which makes the relative concentration of its transcripts

lower than this of all other viral genes, and the protein is found in limited copy numbers in virions (approximately 50 copies per virus particle).

Structural features

The sequence of the L genes of most paramyxoviruses is currently available and comparison of the sequence data has shown that, whilst there is similarity in gene length, there is little homology outside the subfamily. Five highly homologous regions that are believed to represent structural features of an ancestral polymerase 'fold' have been identified near the centre of the large proteins of different paramyxoviruses, which are also present in RNA-dependent RNA polymerases of other virus families (Kolakofsky & Lamb, 2001).

Function

The large protein is part of the polymerase complex (RNA polymerase β-subunit), which also includes phosphoprotein P (RNA polymerase α-subunit; see below), and basically has all the polymerase catalytic activities. Thus, P-associated L is required for NP:RNA transcription and replication. Experimental data have also shown that capping at the 5' end of the newly synthesised strands is performed by L itself (guanylyl and methyl transferase activities), while polyadenylation at the 3' end of the RNA is thought to be the consequence of polymerase stuttering on a short sequence of U residues. Last, L has been identified as the kinase that is associated with the core of the virus (Kolakofsky & Lamb, 2001).

The structural features and function(s) of the V/P gene and products of SV5, with particular reference to the V protein, are described in the following section.

1.3 SV5 V/P gene and products

Pseudo-templated transcription

The V/P gene of paramyxoviruses is a striking example of how viruses have evolved to increase their coding capacity in a compact genome. Through a mechanism termed pseudotemplated transcription (Kolakofsky & Lamb, 2001), which is unique to the V/P genes of this virus family (Paterson *et al.*, 1995), paramyxoviruses encode more than one proteins in the V/P gene, by editing the mRNA gene transcript and inserting G residues at specific sites. As a result, the ORF is altered and translation of the edited mRNA gives rise to different gene products. This process, which seems to occur cotranscriptionally, has been attributed to the virus transcriptase which is believed to stutter at a sequence 3'-AAAAUUCU-5' upstream the insertion site (Vidal *et al.*, 1990).

Variation in paramyxo-viruses

Different paramyxoviruses require a different number of G residues to be inserted in the V/P gene transcript in order to give rise to distinct proteins. Thus, morbilliviruses, respiroviruses and NDV require a single G residue insertion, whilst this number can vary from one to six residues for bPIV3 and hPIV3. Moreover, depending on the paramyxovirus, the faithful transcript of the gene not always codes for the P protein as the default product. Thus, although in respiroviruses, morbilliviruses and NDV the P protein is translated from the unedited mRNA, in rubulaviruses, including SV5, translation of the unedited transcript gives rise to the V protein and insertion of two G residues is required for the expression of the P protein (Kolakofsky & Lamb, 2001). The V/P gene products of *Paramyxovirinae* are shown in Table 1.3, whereas a comparative diagram of the different gene products is presented in Figure 1.5.

The V/P gene of SV5

In SV5, the V/P gene (1298 bp; locus SV5PVA, GenBank accession no. J03142) codes for two proteins, the V and P proteins, which are amino-coterminal, having the first 164 amino acids in common. However, as the ORFs that encode the two proteins differ past the editing site (+2 G residues), the carboxy-terminal domains of these proteins are different, resulting in distinct proteins with different characteristics (Thomas *et al.*, 1988). The structure of the SV5 V/P gene and products is presented schematically in Figure 1.4; the properties of the encoded V and P proteins are discussed below.

Figure 1.5: Comparative presentation of the V/P gene products of different paramyxoviruses.

In rubulaviruses, the faithful transcript of the V/P gene encodes the V protein, whereas P mRNA results from the addition of two G nucleotides. Insertion of one G nucleotide gives rise to I mRNA (Panel A). The V/P gene structure of Newcastle disease virus (NDV), which is a rubulavirus, is more similar to that of respiroviruses and morbilliviruses. Thus, the faithful gene transcript of NDV V/P codes for the P protein and addition of one G nucleotide produces V mRNA (Panel B). While this is also true for respiroviruses and morbilliviruses, these genera produce an additional mRNA (insertion of two G nucleotides) which gives rise to W or D proteins (this varies according to virus). Moreover, the P transcripts of the respirovirus and morbillivirus V/P genes contain the C open reading frame (ORF) which maps to the 5' end of the mRNA (Panel C). The respirovirus C gene product is thought to be the functional counterpart of the V/P common amino-terminal domain of the rubulavirus V and P proteins (adapted from Kolakofsky & Lamb, 2001).

Fig. 1.5

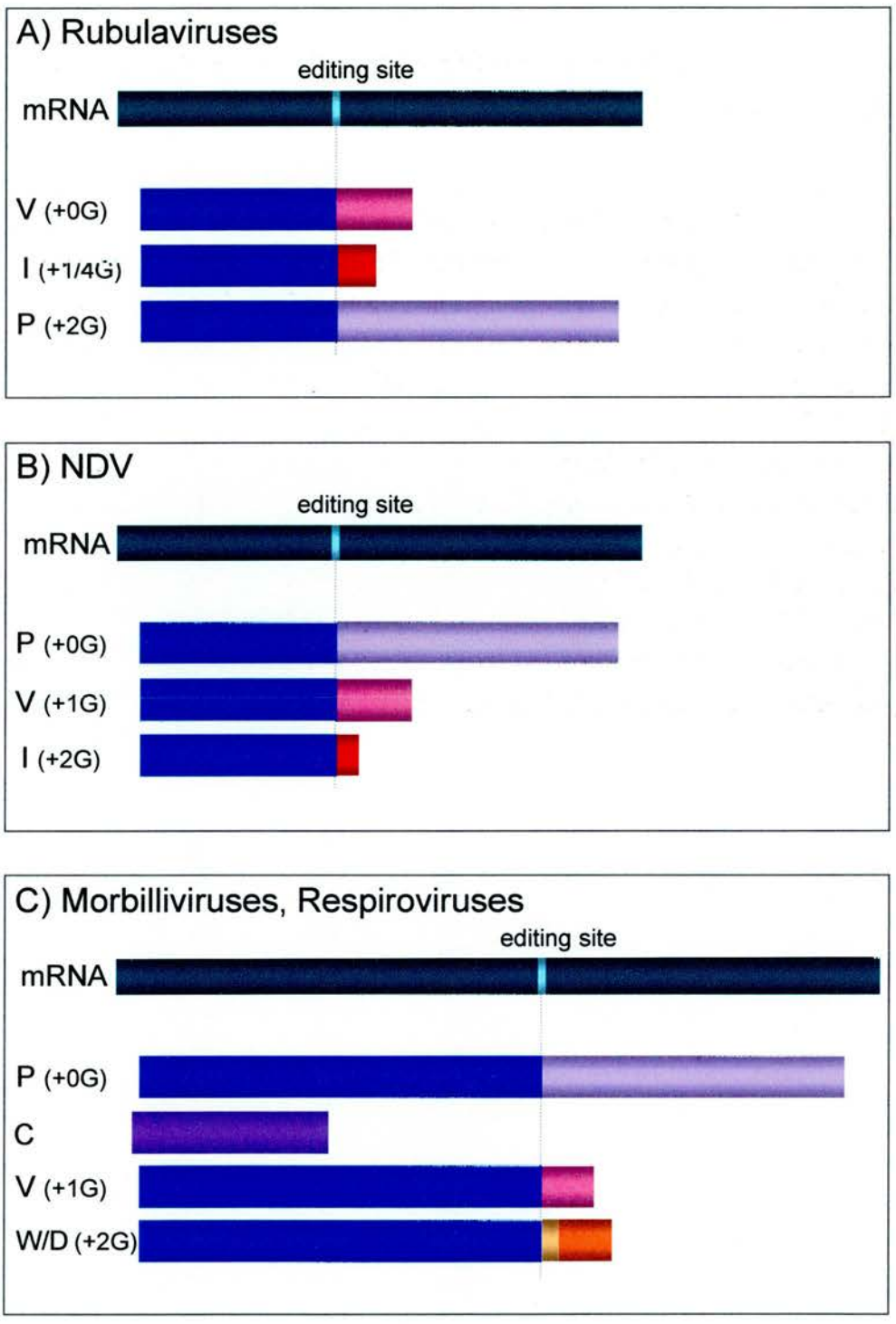

A) Rubulaviruses
editing site
mRNA
V (+0G)
I (+1/4G)
P (+2G)
B) NDV
editing site
mRNA
P (+0G)
V (+1G)
I (+2G)
C) Morbilliviruses, Respiroviruses
editing site
mRNA
P (+0G)
C
V (+1G)
W/D (+2G)

Table 1.3: V/P gene products of *Paramyxovirinae* (adapted from Gotoh *et al.*, 2001).

Genus	Species	RNA editing			Overlapping (C) ORF
		+0G	+1G (or +4)	+2G	
Rubulavirus					
	SV5	V	I	P	-
	hPIV2				
	MuV				
	SV41				
	NDV	P	V	I	
Respirovirus					
	SeV	P	V	W	C', C, Y1, Y2 (and X)
	bPIV3			D	C
	hPIV3*				
	hPIV1		-	-	C', C
Morbillivirus					
	MeV	P	V	W	C

* [There is no evidence for the presence of a V protein, but a V-like protein may be synthesised]

1.3.1 P protein (RNA polymerase α-subunit)

SV5 P

In SV5, phosphoprotein P is translated by the addition of two G residues at the editing site of the V/P gene transcript (see Figure 1.4). The expressed protein (GenBank accession no. AAC95513) contains 392 amino acids and has a molecular mass of 42106 Da (SWISS-PROT primary accession no. P11208; Thomas *et al.*, 1988). As indicated by its name, this protein is highly phosphorylated. Its carboxy-terminal portion is relatively well-conserved in predicted secondary structure in all *Paramyxovirinae* and it is found as an essential part of all P proteins. The structure of the carboxy-terminal domain is predicted to form an α-helical bundle and the entire protein is thought to be a tetramer (Kolakofsky & Lamb, 2001).

Function and association with other components

The P protein is essential for viral RNA synthesis, as it is a core component of the viral polymerase (RNA polymerase α-subunit), mediating the binding of the catalytic L protein (RNA polymerase β-subunit; see above) to the NP:RNA template. The part of the protein that is believed to be involved in this function is the conserved carboxy-terminal domain, which represents the polymerase cofactor module of the protein

(Kolakofsky & Lamb, 2001). P is known to bind soluble and polymeric NP and experiments have shown that, in contrast to V that has a single binding site for NP (see below), P has two binding sites, one mapping to the V/P common amino-terminal domain (the same binding site as in the V protein) and the other being in the P-unique carboxy-terminal domain. Of the two sites, the latter has been shown to be able to recognise NP in its polymeric form (Randall & Bermingham, 1996). Immunofluorescence experiments revealed that when NP alone is expressed in cells, it has a granular cytoplasmic distribution. However, Precious and colleagues (1995) showed that co-expression of P and NP leads to accumulation of large cytoplasmic aggregates, in contrast to V that seems to keep NP soluble in the cell (see below). The ability of P to form complexes with NP has been associated with NP function in genome replication (Curran *et al.*, 1995). P has also been found to bind to RNA (Lin *et al.*, 1997), whilst it is estimated that approximately 300 copies of the protein are associated with the nucleocapsid (Kolakofsky & Lamb, 2001).

1.3.2 V protein

1.3.2.1 V in SV5 and other paramyxoviruses

SV5 V

In contrast to the P protein, which requires editing of the V/P mRNA for the insertion of G residues, the V protein of SV5 is a direct product of the translation of the faithful V/P gene transcript (Figure 1.4). The protein (GenBank accession no. AAA47882) is 222 amino acid residues in length and has a molecular mass of 23935 Da (SWISS-PROT primary accession no. P11207; Thomas *et al.*, 1988).

V is a conserved protein

The V protein is evolutionarily conserved, as it appears in most paramyxovirus species, and there is high homology between the amino acid sequences of the V proteins of different paramyxoviruses (Thomas *et al.*, 1988). The protein is expressed not only by SV5 but by all rubulaviruses, as well as by all morbilliviruses and some respiroviruses (SeV and bPIV3). In contrast to SV5 and all rubulaviruses (except NDV), in which V is translated from unmodified mRNA, mRNA editing (insertion of one G residue) is required for its expression in the rest of paramyxoviruses (Table 1.3, Figure 1.5; Kolakofsky & Lamb, 2001; Gotoh *et al.*, 2001).

The carboxy-terminal domain

The part of the ORF that encodes the carboxy-terminal domain of V (67 amino acid residues) is highly conserved in all paramyxoviruses. Moreover, the carboxy-terminal domain of V is cysteine-rich, containing seven perfectly conserved, identically positioned cysteine residues (C189, C193, C205, C207, C210, C214, C217; Thomas *et al.*, 1988; Baron *et al.*, 1993).

1.3.2.2 Association of V with other SV5 components

V binds zinc

The identification of the cysteine residues in V led to the suggestion that it is a zinc-finger protein (Thomas *et al.*, 1988) or that it forms some type of zinc-coordinated structure (Borden & Freemont, 1996; Choo & Klug, 1997; O'Halloran, 1993). Indeed, several V proteins have been shown to bind zinc (Liston & Briedis, 1994; Steward *et al.*, 1995) and particularly the carboxy-terminal domain of the SV5 V protein has been shown to bind two atoms of Zn^{2+} (Paterson *et al.*, 1995). It has been postulated that this putative zinc-finger domain of SV5 V may be involved in a protein:protein interaction (Lin *et al.*, 1997; O'Halloran, 1993).

V is a structural protein.

In contrast to previous beliefs, it is known that V is a structural protein component of SV5 (approximately 350 copies of V per virion) and that it is associated with the nucleocapsid structure (Paterson *et al.*, 1995). However, experimental evidence has shown that V can bind to free NP, but not to polymeric NP that is organised in nucleocapsids (the importance of this is discussed below; Randall & Bermingham, 1996), thus indicating that V must interact directly with viral RNA. Experimental work by Lin and colleagues (1997) confirmed that V can interact with RNA and showed that the RNA-binding region of the protein maps to five basic residues (K74, K76, K77, R79 and K81) that are common for both V and P.

The V protein of SV5 is a multifunctional protein and its properties and mechanism(s) of action are currently subject to experimental investigation (see below). Distinct functions have been ascribed specifically to either of the two domains of the protein, the V/P common amino-terminal domain and the (V-unique) carboxy-terminal domain, which we distinguish on the basis of the mRNA editing site.

1.3.2.3 V keeps free NP soluble

The amino-terminal domain of V has been shown to be important in keeping NP soluble in the cell. NP encapsidates viral RNA into nucleocapsids as it is being replicated, but free NP also tends to self-assemble and form nucleocapsid structures. Immunofluorescence data has demonstrated that, when expressed alone, V has a diffuse cytoplasmic and nuclear distribution. In contrast, expression of NP alone results in a granular cytoplasmic distribution of the protein. However, when NP is co-expressed with V, the two proteins co-localise, changing the distribution pattern of NP to diffuse cytoplasmic and nuclear. Therefore, it has been suggested that one of the functions of V is to keep NP soluble in the cell prior to viral RNA encapsidation, by preventing it from polymerising (Precious *et al.*, 1995). If this is true, then V plays a significant role in RNA encapsidation and thus ensures viral transcription and replication, given that NP-encapsidation of RNA is an essential requirement for the viral polymerase to recognise the genome as template. It is also possible that V competes with P for binding to NP, which is thought to delay encapsidation as part of a transcription/replication regulatory mechanism (Randall & Bermingham, 1996).

1.3.2.4 V binds to DDB1 and slows down the cell cycle

V binds to DDB1

The replication cycle of paramyxoviruses is believed to take place in the cytoplasm (Figure 1.3; Kolakofsky & Lamb, 2001). However, immunofluorescence experiments (Paterson *et al.*, 1995; Precious *et al.*, 1995; Watanabe *et al.*, 1996) have shown that the V protein has a diffuse nuclear localisation. In inducible cell lines that express either V or P, only V co-immunoprecipitated a host cell protein of an estimated size of approximately 150 kDa (Precious *et al.*, 1995). The identification of a cell protein that interacts with V, and particularly with the V-unique carboxy-terminal domain (the identified host cell protein did not coimmunoprecipitate with P), verified the belief that the cysteine-rich region of V must be involved in protein:protein interaction(s) (Lin *et al.*, 1997; O'Halloran, 1993). Lin and colleagues (1998) showed that the protein that V binds to is the 127-kDa subunit (DDB1) of the damage-specific DNA binding protein (DDB) [also known as the UV-damaged DNA binding protein (UV-DDB), xeroderma pigmentosum group E binding factor (XPE-BF), or the hepatitis B virus X-associated protein 1 (XAP-1); SWISS-PROT primary accession no. Q16531]. Moreover, the

importance of the cysteine-rich carboxy-terminal domain of V in this interaction was demonstrated by mutational analyses which showed that deletion of the entire domain or substitution of any of the seven conserved cysteine residues that map to this domain (see above for residue position numbers) results in loss of the V:DDB1 interaction (Lin *et al.*, 1998). This finding was reinforced by Andrejeva and colleagues (in press) who also demonstrated that substitution of the conserved cysteine residues at positions 193, 207 and 214 abolishes binding of V to DDB1. Furthermore, it was reported that, in addition to the role of the carboxy-terminal domain, the amino-terminal domain also seems to be involved in the V:DDB1 interaction. Thus, although the first 20 amino-terminal amino acid residues of V were found to be dispensable for V binding to DDB1, deletion of the first 85 amino acids prevented this interaction. Interestingly, the V proteins of mumps, hPIV2 and measles were also shown to interact with DDB1 (Lin *et al.*, 1998).

DDB1 binds to other cellular proteins

DDB1 is the large subunit of the heterodimeric DDB protein complex, which was initially thought to be part of the nucleotide excision repair pathway (Chu & Chang, 1988). Subsequent investigation showed that DDB is not essential for this process and it is currently believed that it is a multifunctional protein participating in multiple transcription-related events. DDB is known to interact with the hepatitis B virus X protein (Lee *et al.*, 1995; Sitterlin *et al.*, 2000a; Sitterlin *et al.*, 2000b; Wentz *et al.*, 2000; Lin-Marq *et al.*, 2001), the cytoplasmic tail of the amyloid protein precursor (Watanabe *et al.*, 1999), Cul-4A, a cellular protein that belongs to a family of proteins involved in the ubiquitin-mediated degradation of cell cycle proteins (see section below; Yu *et al.*, 1998; Winston *et al.*, 1999; Shiyanov *et al.*, 1999; Nag *et al.*, 2001; Chen *et al.*, 2001), as well as with the transcription factor E2F1 (Hayes *et al.*, 1998).

V slows down the cell cycle

It has been postulated that the association of DDB with E2F1 may play a role in the delay of the cell cycle progression during the DNA repair process; when DDB binds to damaged DNA, it is not associated with E2F1, and, consequently, the latter remains associated with pRB thus not being able to activate transcription. In tissue culture, SV5-infected cells proliferate slower than mock-infected cells and this delay has been shown to occur through a p53-independent mechanism (Lin & Lamb, 2000). It is possible that in a SV5 infection DDB1 is prevented from binding E2F1, by becoming associated with the V protein. Experiments of co-expression of V and DDB1 showed that

overexpression of DDB1 partially restores normal progression through the cell cycle, suggesting that the V:DDB1 interaction is indeed likely to be involved in cell cycle changes. In addition, expression of truncated V that lacks the carboxy-terminal cysteine-rich domain has no effect on the speed of cell proliferation, indicating that this part of the protein mediates the delay of cell cycle progression (Lin & Lamb, 2000).

How the virus benefits

Cell division seems to be a process that does not favour virus replication as, during mitosis, the Golgi apparatus fragments and vesicle fusion is inhibited. Such inhibitory conditions would consequently block the intracellular transport of viral HN and F glycoproteins, thus having an obvious effect on virus metabolism. Downregulation of the cell cycle by the V protein may therefore be a benefit for SV5. By delaying cell proliferation, the action of V would essentially hold the intracellular environment in a virus replication-permissive state, ensuring virus assembly and progeny release. It is noteworthy that the V protein of Sendai virus (which replicates faster than SV5, mumps, hPIV2 and measles) does not bind to DDB1, in contrast to the V proteins of these viruses the association of which with DDB1 is documented (Lin & Lamb, 2000).

1.3.2.5 V induces the degradation of STAT1

SV5 inhibits IFN promoters

An insight into the properties of SV5 V was gained following the observation that SV5 can productively infect human but not murine cells. In mice whose adaptive immune system was disabled (severe combined immunodeficiency; SCID mice) SV5 remained restricted, indicating that it is an innate immune mechanism that prohibits virus proliferation. In addition, when murine cell cultures were supplemented with anti-IFN antibodies, SV5 was able to replicate efficiently and spread (Young *et al.*, 1997). Didcock and colleagues (1999a) showed that, in response to IFN, SV5 protein synthesis is rapidly switched off. Experiments of murine cells transfected with an IFN-α/β-responsive reporter revealed that SV5 induces strongly such promoters. In contrast, there was not any IFN-α/β-responsive promoter induction in SV5-infected cells of human origin, demonstrating that SV5 blocks activation of the IFN system in human cells.

SV5 V leads to STAT1 degradation

Further investigation of the ability of SV5 to block activation of the IFN system revealed that it is the structural V protein that is responsible for the ability of the virus to

circumvent the host IFN response (Didcock *et al.*, 1999b). Experimental data revealed that V allows SV5 to replicate in the presence of IFN by blocking the intracellular IFN signalling pathway that is normally activated upon infection. The ability of the V protein to block the IFN signalling cascade was shown to be a direct result of its ability to target STAT1 for degradation by the proteasome. Expression of the V protein alone in human cells led to the degradation of STAT1 and blocked IFN signalling in luciferase reporter assays, demonstrating clearly the anti-IFN effect of the protein. As described earlier in this chapter, STAT1 is a cellular transcription factor that plays a central role in signal transduction and IFN-induced gene expression. By 8 h post-infection (p.i.), infection of human cells with SV5 results in complete loss of STAT1. Strikingly, this effect has been shown to be prevented in cells treated with proteasome inhibitor MG132, in which STAT1 degradation is inhibited. Thus, these findings established that, by inducing STAT1 degradation, SV5 V blocks both type I and type II IFN signalling pathways (see Figure 1.1).

Strikingly, neither inhibition of transcription (treatment of cells with transcription inhibitor actinomycin D) nor inhibition of protein synthesis (UV-inactivation of virus or treatment of cells with cycloheximide, a protein synthesis inhibitor) changed this effect, as the virus rapidly eliminated STAT1 in all experiments, irrespective of treatment. Thus, it was revealed that the V protein carried in the virus particles is sufficient to induce STAT1 degradation and disable the IFN system in the initial stages of infection. Indeed, SV5 can proliferate in human cells that have been pretreated with IFN and entered an antiviral state, despite an initial delay in the onset of virus protein synthesis (24 to 48 h). This suggests that SV5 remains viable in such cells and is activated when the antiviral state cannot be sustained due to V-induced loss of STAT1 (24 h p.i.) (Didcock *et al.*, 1999b). Moreover, although treatment of human cells with IFN normally induces an antiviral state, a human cell line that expressed permanently SV5 V failed to respond to treatment with IFN, showing that V is directly involved in the inhibition of cellular antiviral mechanisms (Andrejeva *et al.*, 2002).

V:STAT1: STAT2 complex formation

Studies on V-induced STAT1 degradation revealed recently that both phosphorylated and nonphosphorylated forms of STAT1 are degraded by SV5 infection (Andrejeva *et al.*, 2002), as demonstrated by STAT1 immunoblots of extracts from infected cells treated with genistein (a general kinase inhibitor). Interestingly, Parisien and colleagues

(2002a) also reported that, whilst IFN signalling (JAK-mediated phosphorylation cascade) is not required for STAT1 degradation, both STAT1 and STAT2 need to be present in a functional V-dependent multisubunit complex for STAT1 to be targeted. The ability of V to associate with STAT1 and STAT2 was shown by co-immunoprecipitation assays, and complementation analysis further revealed that the amino-terminal domain of STAT2 is necessary for STAT1 degradation. Subsequent investigation showed that expression of human STAT2 in murine cells, in which SV5 does not eliminate STAT1, enables the virus to degrade STAT1, thus demonstrating the role of STAT2 as an important determinant for SV5 IFN antagonism (Parisien *et al.*, 2002b).

DDB1 involvement in STAT1 targeting

The requirement of STAT2 for V-mediated STAT1 targeting was also demonstrated recently by Andrejeva and colleagues (in press) who additionally showed that binding of V to DDB1 is another prerequisite for the proteolytic effect of V. Thus, mutational analysis of V revealed a direct correlation between the ability of the protein to bind to DDB1 and block IFN signalling, indicating that DDB1 must be a key factor in the proteasome-mediated degradation of STAT1. It therefore seems possible that a V:STAT1:STAT2:DDB1 multiprotein complex may have to form in order for STAT1 degradation to be effected, whilst the precise mechanism of targeting requires further elucidation. A possible involvement of Cul-4A in proteasome-mediated degradation of STAT1 was also proposed by the same researchers. Cul-4A (SWISS-PROT primary accession no. Q13619), which is known to interact with DDB1 (see previous section), is a protein of the cullin family of ubiquitin-protein E3 ligases. Whilst cullins are known to participate in protein targeting for degradation (reviewed in Pickart, 2001), the ability of Cul-4A to ubiquitinate but not target DDB1 is documented (Chen *et al.*, 2001), suggesting that V-recruited DDB1 could act as a chaperone for the ubiquitination and targeting of STAT1.

1.3.3 Blocking of IFN signalling by other paramyxoviruses

Other paramyxo-viruses also block IFN signalling

In addition to the V protein of SV5, other gene products of the V/P genes of *Paramyxovirinae* have also been shown to interfere with intracellular IFN signalling, thus disabling the host IFN defence mechanism and allowing virus proliferation

(reviewed in Gotoh *et al.*, 2001). For example, in mumps virus, a virus closely related to SV5, the V protein has the same function as in SV5, inducing the degradation of STAT1. Similarly, in hPIV2, another closely related rubulavirus species, V allows the virus to block IFN signalling by targeting STAT2. However, it is not only the V proteins that enable paramyxoviruses to evade the IFN response of the host. In Sendai virus, a representative member of the Respirovirus genus, the functional equivalent of V is the C protein which also blocks IFN signalling by targeting STAT1. Although paramyxoviruses are an established example of an RNA virus family that overcomes the IFN-induced antiviral defence mechanisms of the host by blocking IFN signalling, this feature has also been found in a Filovirus (Ebola virus VP35 protein blocks IFN-α/β and IFN-γ signalling; Harcourt *et al.*, 1998; Basler *et al.*, 2000), whilst a Bunyavirus is known to inhibit specifically the type I IFN system through a yet unknown mechanism (Rift valley virus NSs proteins; Haller *et al.*, 2000).

1.3.3.1 Other rubulavirus V proteins

hPIV2 vs. SV5

Similar to SV5, the V proteins of other rubulaviruses are also known to mediate the degradation of protein components of the intracellular IFN signalling pathway, thus disrupting the activation cascade of IFN-induced genes. Evolutionarily, hPIV2 is closely related to SV5, as shown by the sequence similarity in their HN proteins (43% identity; Precious *et al.*, 1990). Although hPIV2 is different from SV5 (Randall & Young, 1988), there is antigenic cross-reactivity between the two species, which has led to the assumption that hPIV2 is the human equivalent to SV5 (the latter was isolated originally from simian cell cultures; see below) (Pringle, 1987; Tsurudome *et al.*, 1989). The V/P gene of hPIV2 is identical to that of SV5 in that the faithful transcript of the gene codes for the V protein, whereas the addition of two nontemplated G residues to the gene transcript is required for P mRNA to arise. Given its high degree of conservation within the virus family, it is not surprising that the V-unique cysteine-rich carboxy-terminal region of hPIV2 V is highly homologous to that of SV5 V. However, there is less homology between the P ORFs of the two viruses, although the overall identity between the encoded proteins is relatively high (44% amino acid homology; Southern *et al.*, 1990).

hPIV2 blocks IFN signalling by inducing STAT2 degradation

Young and colleagues (2000) first reported that hPIV2 leads to the degradation of STAT2, as demonstrated by immunoblot analysis, whilst luciferase reporter assays confirmed that hPIV2 blocks type I but not type II IFN signalling (STAT2 participates exclusively in type I IFN signalling; see Figure 1.1). Consistent with this result, ISGF3 transcription complexes (type I-specific) were also inhibited in cells infected with hPIV2, whereas GAF complexes (type II-specific) were not affected by the virus. Parisien and colleagues (2001) later demonstrated that it is the V protein of hPIV2 that is responsible for the degradation of STAT2. Thus, while leaving STAT2 mRNA levels unaffected, expression of V was shown to induce degradation of STAT2. It was also shown that proteasome-mediated degradation must be involved in the activity of V, as treatment of hPIV2 V-transfected cells with proteasome inhibitor MG132 resulted in at least partial rescue of STAT2 from proteolysis. This suggested that other cellular mechanisms that result in the elimination of STAT2 must be involved in the proteolytic effect of the virus protein (Parisien *et al.*, 2001).

hPIV2:cell protein interactions

Similar to STAT1 degradation by SV5 V, it is known that the presence of both STAT1 and STAT2 is a prerequisite for STAT2 degradation by hPIV2 V, with the amino-terminal domain of STAT2 playing a critical role in this event (Parisien *et al.*, 2002a). Evidence for the association of hPIV2 V with STAT proteins has also suggested that a V:STAT1:STAT2 complex may be involved in the proteolytic degradation of STAT2. Furthermore, since hPIV2 V has been previously shown to bind DDB1 (Lin *et al.*, 1998) and STAT1 degradation by SV5 V is known to be DDB1-dependent, it has been postulated that hPIV2 is likely to employ a similar proteolytic mechanism for STAT2 targeting (Andrejeva *et al.*, in press).

hPIV2 V C' is essential

Highlighting the importance of hPIV2 V in virus replication, Nishio and colleagues (2001) demonstrated that cells infected with hPIV2, as well as cells that permanently express the V protein of this virus, show high resistance to type I IFNs, as revealed by challenges with Vesicular stomatitis or Sindbis viruses. The same researchers also emphasised on the importance of the carboxy-terminal domain of the protein, demonstrating that it is a prerequisite for STAT2 degradation, whilst Kawano and colleagues (Kawano *et al.*, 2001) showed that a recombinant hPIV2 virus lacking the V-specific carboxy-terminal domain exhibits impaired growth due to an IFN-sensitive phenotype.

MuV and SV41

Mumps virus (MuV) is also a rubulavirus that is closely related to SV5 (and hPIV2). Sequence analysis has revealed that all three viruses (MuV, hPIV2 and SV5) share 30% amino acid identity in their HN proteins, with MuV and hPIV2 having even higher homology (40% identity; Precious *et al.*, 1990). However, in terms of their anti-IFN strategies, MuV seems to be identical to SV5. In contrast to hPIV2, MuV blocks IFN signalling, and thereby prevents the activation of IFN-stimulated genes, by targeting STAT1 for degradation (Yokosawa *et al.*, 1998). Not surprisingly, this strategy is also known to be employed by Simian virus type 41 (SV41), another member of the Rubulavirus genus. Nevertheless, it has been suggested that, in contrast to SV5, the proteasome-mediated degradation pathway may play only a partial role in the proteolytic effect of MuV and SV41 V, this issue yet requiring further investigation (Gotoh *et al.*, 2001; Nishio *et al.*, 2001). The importance of the carboxy-terminal domain of MuV V in the ability of the virus to target STAT1 is documented (Kubota *et al.*, 2001).

1.3.3.2 Respirovirus C proteins and IFN signalling

SeV blocks IFN signalling

Sendai virus (SeV) is a representative of the Respirovirus genus, all members of which express the C proteins, as do Morbilliviruses (Table 1.3). The V/P gene of SeV expresses eight proteins (proteins P, V, W, C', C, Y1, Y2 and X), and extensive studies have been performed with regard to the pathogenicity and ability of this virus to circumvent the IFN response. SeV has been known to be a classic IFN inducer, which, nevertheless, remains unaffected by the antiviral IFN-induced effect. Although the V protein has been shown to be important for the pathogenicity of the virus *in vivo* (Kato *et al.*, 1997a; Delenda *et al.*, 1998), with the determinant mapping to the carboxy-terminal of the protein (Kato *et al.*, 1997b), an increasing number of reports has been focussing on the C proteins. With a single point mutation in the C proteins being sufficient to attenuate the phenotype of the virus *in vivo* (Garcin *et al.*, 1997), subsequent experiments revealed that it is this set of proteins that counteracts the cellular IFN response, enabling SeV to proliferate in an environment that would otherwise inhibit virus replication (Garcin *et al.*, 1999). Luciferase reporter experiments further elucidated the anti-IFN strategy of SeV by demonstrating that, in its natural host,

SeV blocks the activation cascade of IFN-responsive gene promoters (Didcock *et al.*, 1999a).

SeV C and STAT1

This observation was soon reinforced by studies on C gene-knockout SeV, which showed that the C proteins are essential for the ability of the virus to circumvent the type I IFN response (Gotoh *et al.*, 1999). However, unlike SV5, which blocks IFN signalling by inducing STAT1 degradation, Young and colleagues (2000) demonstrated that SeV does not affect STAT1 levels, yet inhibiting the formation of both type I and type II IFN transcription complexes (ISGF3 and GAF, respectively). In contrast, experimental evidence later showed that SeV blocks IFN signalling by inhibiting the phosphorylation of STAT1, thus providing an explanation of how the virus prevents the activation of the signalling cascade (Young *et al.*, 2000; Komatsu *et al.*, 2000). The same was found to be true for respirovirus hPIV3, which interdicts the IFN signalling pathway by inhibiting STAT1 phosphorylation (Young *et al.*, 2000). The ability of the C proteins to block IFN signalling has also been shown in luciferase reporter experiments, in which C expression inhibited the activation of both type I and type II IFN-responsive promoters (Garcin *et al.*, 2000). These data suggested that the C protein must interact directly with a component of the IFN signalling pathway, an assumption that was proved to be correct by Takeuchi and colleagues (2001) who provided evidence for the direct interaction of SeV C with STAT1.

1.3.3.3 Rubulavirus V vs. respirovirus C – an overview

Rubulavirus V and Respirovirus C are functional counterparts

The ability of paramyxoviruses to interdict IFN signalling is established and this function has been attributed to different viral proteins, depending on genus. Although the V protein is encoded by all *Paramyxovirinae*, it is found packaged in virions as a structural protein only in rubulaviruses, such as SV5 (Curran *et al.*, 1991). In contrast, the respirovirus SeV C protein is present in virions and, similar to the rubulavirus hPIV2 V protein, it is known to have an important role in virus assembly and particle morphogenesis. Rubulavirus V and respirovirus C proteins also share the anti-IFN function, as both proteins block IFN signalling, which is the reason why respirovirus C is considered to be the counterpart of rubulavirus V (Gotoh *et al.*, 2001). Thus, although the conserved carboxy-terminal domain of rubulavirus V has been shown to be

important in counteracting the IFN response, in SeV it is the C (and not the V protein) that has this function. Strikingly, pneumovirus RSV also exhibits an anti-IFN function that has yet been attributed to the NS proteins, which are known to substantially support virus replication (Jin *et al.*, 2000; Schlender *et al.*, 2000). Nevertheless, it is noteworthy that RSV does not block IFN signalling, which indicates that a different anti-IFN strategy is employed by this virus (Young *et al.*, 2000).

The amino-terminal region of V is important for function

Apart from the functional similarities between rubulavirus V and respirovirus C, experimental data have also revealed that there is amino acid sequence homology between these proteins, with regions of the respirovirus C protein showing sequence identity with the amino-terminal domain of rubulavirus V. Whilst reinforcing the current belief that respirovirus C is the counterpart of rubulavirus V, this finding has shed light on the rubulavirus V protein, drawing attention to the fact that, although its carboxy-terminal region is essential for function, the amino-terminal domain must also be necessary for its anti-IFN action (Kolakofsky & Lamb, 2001).

An overview of the ability of SV5, hPIV2, SeV, hPIV3 and RSV to (i) interfere with protein components of the IFN activation cascades, (ii) inhibit the formation of transcription complexes necessary for the activation of IFN-responsive genes, and thus (iii) block IFN signalling, is presented below (Table 1.4).

Table 1.4: Comparative overview of the ability of different paramyxoviruses to interfere with intracellular IFN signalling and relevant protein components in human cells (data adapted from Young *et al.*, 2000).

Effect	SV5	hPIV2	SeV	hPIV3	RSV
Blocking of IFN signalling					
type I	+	+	+	+	-
type II	+	-	+	+	-
Inhibition of complex formation					
ISGF3	+	+	+	+	-
GAF	+	-	+	-	-
Protein degradation					
STAT1	+	-	(*)	(*)	-
STAT2	-	+	-	-	-

(*) [reduction in (S727)-phosphorylated forms of STAT1α, indicating interference with STAT1 processing]

1.4 Recombinant RNA virus technology and paramyxoviruses

Method principle

Recent advances in virus reverse genetics have opened a new field of investigation in virus studies, providing answers to questions that conventional virology could not address. It is now possible to recover an infectious virus entirely from cDNA, using a method that is well-established for RNA viruses. In principle, intracellular transcription of genomic RNA from cDNA suffices for the initiation of a replication cycle, meaning that a virus can be recovered from genetically engineered full-length cDNA that encodes the entire viral genome. This method has proved tremendously powerful, allowing researchers to introduce specific mutations or other changes to viral genes and produce recombinant viruses with altered properties.

In the case of negative-sense RNA viruses, this technique took years to become available, as simple RNA expression is not sufficient for their recovery, adding a level of complication to the strategy. Negative-sense RNA viruses require not only intracellular transcription of the RNA genome from cDNA, but also expression of viral proteins that bind to RNA (naked RNA cannot be recognised as template unless associated with the viral RNA-binding protein NP), together with expression of the polymerase that will initiate the replication cycle (reviewed in Nagai, 1999; Nagai & Kato, 1999).

Application on paramyxoviruses

In paramyxoviruses, this method, which requires intracellular co-expression of proteins NP, P and L for the recombinant RNA genome to be expressed, has been applied with success to a growing number of family members. Today, at least one member of each paramyxovirus genus has been manipulated using reverse genetics, including respiroviruses **SeV** (Garcin *et al.*, 1995; Kato *et al.*, 1996) and **hPIV3** (Hoffman & Banerjee, 1997; Durbin *et al.*, 1997; Durbin *et al.*, 1999; Bailly *et al.*, 2000), morbilliviruses **MeV** (Radecke *et al.*, 1995) and **Rinderpest** virus (Baron & Barrett, 1997; Baron & Barrett, 2000), pneumovirus **RSV** (Collins *et al.*, 1995; Buchholz *et al.*, 1999; Jin *et al.*, 2000), as well as rubulaviruses **NDV** (Peeters *et al.*, 1999), **hPIV2** (Kawano *et al.*, 2001) and **SV5** (He *et al.*, 1997).

SV5 and hPIV2

In SV5, the use of reverse genetics not only proved the applicability of this method on this virus species, but demonstrated that it is possible to express foreign genes in recombinant paramyxoviruses. Thus, in a T7 polymerase-driven system, He and colleagues (1997) were able to recover a recombinant SV5 virus that expressed green fluorescence protein (GFP) under the control of SV5 transcriptional start-stop signals. More recent work on SV5 involved the introduction of a single point mutation in the SV5 genome (V/P gene), which demonstrated clearly the importance of this amino acid substitution in the ability of the virus to block IFN signalling in a new host species (Young *et al.*, 2001). The experimental findings from this case are presented in Chapter 3. With regard to rubulaviruses and IFN sensitivity, Kawano and colleagues (2001) reported that a recombinant hPIV2 virus with a defective V protein lacking the V-unique domain was susceptible to IFN. This virus was shown to be able to replicate only in non-IFN-producing cells (Vero), whereas virus replication in IFN-producing cell lines (CV-1 and FL cells) was largely inhibited and partially enabled in the presence of anti-IFN antibodies, showing that the cysteine-rich region of hPIV2 V is indispensable for function.

1.5 SV5 Isolates and Persistence

1.5.1 SV5 Host Species and Isolates

1.5.1.1 Simian origin and human isolates of SV5

Antibodies raised against SV5

Paramyxoviruses show marked differences in host range and SV5 has been isolated from several host species. Studies based on monoclonal antibodies have demonstrated that SV5 isolates of different host species origin exhibit antigenic differences (Randall *et al.*, 1987). Strikingly, a specific epitope on the V and P proteins (mapping to the V/P amino-terminal domain and commonly referred to as the Pk epitope; see Figure 1.4; (Dunn *et al.*, 1999) has proved extremely useful not only by distinguishing between closely related isolates (Southern *et al.*, 1991; see below) but, even further, by highlighting the role of the amino-terminal domain of V in its function. Thus, given that V enables SV5 to circumvent the IFN response, any changes in the ability of the Pk epitope to bind to the monoclonal anti-Pk antibody SV5-P-k (Randall *et al.*, 1987) can serve as an indicator of potential changes in the ability of the virus to block IFN signalling. Indeed, as presented in Chapter 3, this proved to be true in the cases of two SV5 isolates (termed mci-2 and CPI-) which have alterations in their Pk regions and exhibit altered anti-IFN properties. The Pk epitope and the anti-Pk mAb have given rise to a versatile antibody-tag system which is currently commercially available for a variety of applications in biochemical research.

Origin and human isolates

SV5 was originally isolated from rhesus monkey kidney cell cultures (the wild-type strain being referred to as W3A or, commonly, W3) (Hull *et al.*, 1956), which gave rise to the belief that the monkey is its natural host. Nevertheless, experimental evidence subsequently showed that SV5 can also infect humans (Hsiung, 1972; Goswami *et al.*, 1984; Robbins *et al.*, 1981), leading to the suggestion that the virus is probably transferred to monkeys via human contact (Tribe, 1966). Furthermore, work by Goswami and colleagues (1984) on several SV5 strains isolated from bone marrow cells of human patients with multiple sclerosis (human isolates LN, RQ, DEN, MEL and MIL) suggested strongly that there might be a link between SV5 and this disease. This hypothesis was further supported by serological studies which revealed that anti-SV5 antibodies could be identified in the cerebrospinal fluid of multiple sclerosis patients (Goswami *et al.*, 1987). However, this controversial issue was later resolved by further

investigation which eventually dissociated SV5 from multiple sclerosis (Vandvik & Norrby, 1989; Russell *et al.*, 1989).

1.5.1.2 Canine isolates

Strains CPI+ and CPI-

The neurotropic character of SV5 has been demonstrated in dogs. SV5, also referred to as canine parainfuenza (CPI) virus in veterinary medicine, is known to cause tracheobronchitis in dogs and is often associated with kennel cough (Cornwell *et al.*, 1976; McCandlish *et al.*, 1978). It has been suggested previously that the virus may be able to establish persistent infections in canine hosts. A SV5 strain, termed CPI+ (formerly referred to as CPI-78-238), was isolated from the cerebrospinal fluid of a dog with temporary posterior paralysis and has been further studied as a neurotropic isolate (Evermann *et al.*, 1980; Evermann *et al.*, 1981; Baumgartner *et al.*, 1981). Intracerebral infection of gnotobiotic dogs with CPI+ resulted in acute encephalitis and enabled the re-isolation of another SV5 isolate, termed CPI-, from the brain tissue, 12 days post-infection (p.i.) (Baumgartner *et al.*, 1982). Characterisation of CPI- showed that it differed significantly from parental CPI+ in that it established persistent infections in tissue culture more readily (Baumgartner *et al.*, 1987a; Baumgartner *et al.*, 1987b). In addition, CPI- was found to be attenuated regarding both its *in vitro* cytopathogenicity, causing only a mild cytopathic effect (compared to CPI+ which induces syncytial giant cell formation and cytolysis), and its *in vivo* virulence, as demonstrated by experimental infections of ferrets in which it caused only mild inflammation (CPI+ exhibited severe virulence in the same experiment; Baumgartner *et al.*, 1991). Interestingly, of a panel of 50 mAbs, only one could distinguish between these two closely related isolates, the anti-Pk mAb (SV5-P-k; see above). Sequence analysis of the CPI- V/P gene revealed that the V (and P) protein of CPI- has several amino acid differences to that of CPI+ (Southern *et al.*, 1991), one of which maps to the Pk epitope. Furthermore, findings from the characterisation of CPI-, in terms of its ability to block IFN signalling, reinforced the notion that changes in this epitope region may be indicative of changes in V function, as discussed in Chapters 3 and 4.

Other canine strains

Additional canine variants of SV5 include strains H221 and 78524 (obtained from Prof. O. Jarrett, Faculty of Veterinary Medicine, University of Glasgow, Glasgow, UK), as

well as the T1 variant which was isolated from a dog with kennel cough complex (Azetaka & Konishi, 1988). Characterisation of T1, in terms of its fusogenicity, showed that, unlike W3, this strain was not able to induce cell fusion. Furthermore, fusion was not induced when the F protein from this isolate was expressed in cells, not even when co-expressed with the HN glycoprotein (the activity of which is thought to promote the fusion process; see above). Interestingly, sequence analysis of the F gene of T1 revealed a short extension (22 amino acid residues) in the cytoplasmic tail of the protein which, when reduced in length or replaced with a functional counterpart, the fusogenic properties of the virus were restored (Ito *et al.*, 2000).

1.5.1.3 A porcine isolate

Similar to T1, a virus isolate of porcine origin, termed SER, was also found to have an altered F protein (locus PPA278916, GenBank accession no. AJ278916; Klenk & Klenk, 2000), which featured a longer cytoplasmic tail (with an additional 22 amino acids) and failed to induce cell fusion *in vitro*. Truncation studies on the protein revealed that syncytia formation was rescued completely when the entire extension was deleted, demonstrating again that the 22-amino acid cytoplasmic extension in F interferes with membrane fusion. Moreover, the fusogenic activity of several mutant proteins was enhanced when HN was co-expressed, whilst transferring of the cytoplasmic tail to SV5 F was shown to affect partially the function of the wild-type protein (Seth *et al.*, 2002).

1.5.2 Persistence and SV5 replication in murine cells

SV5 isolated from a murine cell line

In addition to simian, human and canine hosts in which it naturally proliferates, SV5 can also infect mice, nevertheless causing only self-limiting infections (Randall *et al.*, 1988; Young *et al.*, 1990) due to the IFN constraints posed on the virus by this host species (Young *et al.*, 1997). Although it is difficult for the virus to establish infections in murine cells *in vitro*, Young and colleagues (1997) reported the isolation of a new SV5 strain from persistently infected murine cells, following prolonged passaging (over 40 passages). This isolate, termed W3-f (currently also referred to as mouse cell isolate 1, mci-1), has a markedly greater fusogenic ability, which gives rise to massive syncytia formation in murine cell monolayers. However, this property was shown to be evident

only in the absence of IFN, as, similar to its parental W3, W3-f is sensitive to IFN in murine cells. Strikingly, despite the ability of W3-f to spread more rapidly than W3, no amino acid differences were found between the F proteins of these strains.

SV5 and replicative quiescence

The belief that SV5 can readily establish persistent infections was initially based on experimental evidence of SV5 recovery from simian kidney cell cultures long after infection (Tribe, 1966; Atoynatan & Hsiung, 1969; Hsiung, 1972). Since then, studies of *in vitro* SV5 infections have further shown that murine cells infected with SV5 show a nonpermissive pattern of infection which, upon prolonged passaging, can give rise to a persistent infection (Young *et al.*, 1997). However, this is not true in human and simian cell cultures, in which cells become easily infected, resulting in productive infections. For example, African green monkey kidney (Vero) cells are fully permissive for virus replication and are typically used for virus growing and stock preparation. Immunofluorescence analysis of passaged murine cells that had been infected at high multiplicity showed that the virus was transcriptionally inactive in those cells, whilst tight aggregates of viral origin, referred to as inclusion bodies, were readily detected in the cytoplasm. Strikingly, co-cultivation of these cells with Vero cells resulted in recovery of infectious SV5, which suggested that the virus genome can remain quiescent in the cytoplasm, while retaining its ability to be fully reactivated under permissive conditions (Fearns *et al.*, 1994). The formation of inclusion bodies in the cytoplasm of infected cells has been confirmed for certain strains under certain conditions (see Chapter 3) and it is possible that this phenotypic feature may be directly linked to virus repression and persistence, as a consequence of sensitivity to IFN (see Chapter 4 for proposed model of SV5 persistence).

Paramyxovirus persistence

The ability of paramyxoviruses to establish persistent infections both *in vivo* and *in vitro* is well documented and it is believed that, following infection, they can remain quiescent in the host without causing any symptoms of harmful pathology. This property also influences the epidemiology of the family, and persistent paramyxovirus infections have been linked to paramyxovirus-induced chronic diseases, such as subacute sclerosing panencephalitis (SSPE), Paget's bone disease and autoimmune chronic active hepatitis (reviewed in Randall & Russell, 1991).

Today, SV5 is used as an experimental paramyxovirus model in studies that aim to resolve the relationship between paramyxoviruses and host immune defences, particularly with regard to the molecular mechanisms that underlie the virus:host interactions. This thesis focuses primarily on SV5 V, its interaction with the IFN system, as well as its effect on virus replication. Characterisation of the murine isolate mci-2 and the canine strains CPI+ and CPI- aimed at the determination of their ability to overcome the IFN response as an outcome of V protein function. Similarly, examination of SV5 isolates of different host species origin was performed to determine their sensitivity to IFN and obtain an insight into virus:host interactions and how viral infections are established.

Chapter 2

Materials and Methods

2.1 Cells and Viruses

2.1.1 Mammalian cell culture and transfections

2.1.1.1 Basic cell lines

In this work, cells of human, simian, canine and murine species origin were used:

Human cells

- 2fTGH (Pellegrini *et al.*, 1989; McKendry *et al.*, 1991); diploid fibroblasts. Cell line provided by Dr. S. Goodbourn (St. George's Hospital Medical School, London, UK).

Simian cells

- Vero cell line; fibroblast-like cell line originating from kidney cells of the African Green monkey. Cell line obtained from ICN Pharmaceuticals Ltd., UK.

Canine cells

- Dog134 cell line; cells of canine species origin.

- Madin-Darby canine kidney (MDCK) (ECCC, 2002).

Murine cells

- BF; fibroblast cells cloned from a primary BALB/c mouse embryo cell culture. The gene encoding Mx in BALB/c mice has a large deletion and therefore any product made in these cells is inactivated (Staeheli *et al.*, 1988). Consequently, any effect of IFN on virus protein synthesis in these cells is not mediated through the induction of Mx proteins.

2.1.1.2 Cell lines with altered properties

In addition to the basic cells lines listed above, cell lines that have altered properties were also used:

Persistently infected cells

- BF-E7; BF cells persistently infected with SV5. This cell line arose by infection of BF cells with SV5 (strain W3) and continuous passaging (cell line established by Dan Young; Young *et al.*, 1997). Mci-2 was isolated from these cells after passage 80 (Young *et al.*, 2001).

Permanent cell lines

- 2fTGH.SV5-V; 2fTGH cells that express W3 V constitutively (produced by Dr. L. Andrejeva; Andrejeva *et al.*, 2002).

- 2fTGH.SV5-V/CPI+/-; 2fTGH cells that express constitutively the V protein of CPI+ or CPI-, respectively.

The preparation method of these cell lines is described below.

2.1.1.3 Cell culture

Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Ltd., UK) supplemented with 10% foetal serum in 25-cm^2 flasks (Greiner, UK). Cells were routinely passaged, trypsinised (trypsin, EDTA; Becton Dickinson UK Limited) and diluted every three to five days, depending on the growth rate of the cell line. When required, media were supplemented with recombinant human interferon-α_/D (rHuIFN-α_/D) (Rehberg *et al.*, 1982; PBL Biomedical Labs, New Brunswick, catalog no. 11200). For stimulation of IFN-responsive promoters, cells were incubated with IFN at a concentration of 10^4 units/ml for 4 hours (h) prior to harvesting, whereas an IFN concentration of 10^3 units/ml was used for longer incubations (IFN pretreatment or post-infection treatment).

2.1.1.4 Transfection of mammalian cells with plasmid DNA

Lipid-based method

In this method, cells were transfected with DNA using FuGENE™ 6 Transfection Reagent (Roche Diagnostics Co., UK), according to the manufacturer's instructions.

Cells were incubated with the lipid:DNA mix overnight and lysed the following day for further sample processing.

Electroporation

The culture medium of confluent cell monolayers was replaced with fresh growth medium approximately 1 h prior to electroporation. Cells were trypsinised, centrifuged at 1200 rpm for 5 min and resuspended in growth medium containing 15 mM HEPES pH7.5. The cell suspension ($5x10^6$ cells resuspended in 200 µl HEPES-containing medium) was transferred into a 4-mm electroporation cuvette (Flowgen Instruments Ltd., UK) to which 50 µl of the appropriate DNA suspension was added. The DNA suspension contained a total of 10 µg of reporter and test plasmid DNA, 30 µg salmon sperm carrier DNA, and 200 mM NaCl. Cells were given an electric pulse for 35 to 45 msec at 240 V, 1200 µF, 99 Ω, using an Easyject Plus electroporator (Flowgen Instruments Ltd., UK). Immediately after electroporation, cells were washed in fresh growth medium (resuspended in 5 ml medium, then centrifuged at 1200 rpm for 5 min), resuspended in 4 ml medium and plated out in two wells of a 6-well plate (or an equivalent surface area).

2.1.1.5 Preparation of stable mammalian cell lines

To produce a mammalian cell line that would express permanently a protein of interest, monolayers of 2fTGH cells were grown in a 25-cm^2 flask to 50 to 70% confluence and transfected with 3 µg DNA that encodes this protein, using the lipid-based transfection method described in the previous section. The plasmid DNA constructs were based on the pEF.IRES.neo plasmid, which facilitates the selection of transfected cells, as explained below. At 24 h post-transfection, the cells were diluted and plated out into petri dishes, in culture media supplemented with geneticin (400 µg/ml) so as to select cell clones that stably expressed the transgene of interest. Cell colonies were picked and transferred (pipetted) into a separate well of a six-well plate and grown as a monolayer. The prepared cells were subsequently passed and tested by immunofluorescence to confirm that they expressed the protein of interest. The cell clones that showed the highest protein expression levels were selected and stored at -70°C for future reference.

2.1.2 Viruses and virus infection of mammalian cells

2.1.2.1 Virus isolates and infections

In this study, the viruses that were used to infect mammalian cells or clone genes from were different isolates of Simian virus type 5 (SV5).

- W3 (Choppin, 1964; subfamily *Paramyxovirinae*, genus Rubulavirus. This is the standard laboratory strain of SV5 (wild-type virus).

- mci-2 (mouse cell isolate 2; Young *et al.*, 2001); strain isolated from the persistently infected BF-E7 cell line (see above).

- $rSV5_{N>D}$ (complete term: rSV5-V/P $N_{100}D$; Young *et al.*, 2001); recombinant SV5 strain, featuring a single amino acid substitution ($N_{100}D$) in the V/P gene. Produced by Prof. R.A. Lamb's research group (Molecular Biology & Cell Biology, Northwestern University, Evanston, USA).

- CPI+ (formerly called 78-238) and CPI- (Baumgartner *et al.*, 1981; Baumgartner *et al.* 1982; Baumgartner *et al.*, 1987a; Baumgartner *et al.*, 1991); closely related canine isolates. CPI+ was isolated from the cerebrospinal fluid of a dog with temporary posterior paralysis; CPI- was isolated from the brain of a gnotobiotic dog that had been infected experimentally with CPI+.

- MIL, DEN, LN, MEL and RQ (Goswami *et al.*, 1984); human isolates from bone marrow cells of multiple sclerosis patients.

- H221 and 78524 (obtained from Prof. O. Jarrett, Faculty of Veterinary Medicine, University of Glasgow, Glasgow, UK); canine virus isolates.

- SER (obtained from Dr. Klenk H.D., Institut fuer Virologie, Philipps-Universitaet Marburg, Germany); a porcine virus isolate.

Mammalian cells were infected either as monolayers or in suspension, depending on infection efficiency in different cell types. Human, simian and canine cells were typically infected at a multiplicity of infection (m.o.i.) of 10 pfu/cell and murine cells

were infected at 100 pfu/cell, unless a lower m.o.i. was required (<0.1 pfu/cell). Cells were incubated with the virus inoculum (or plain growth medium, where mock infections were required as control treatments) for 1-2 h on a rocking platform at 37°C and the inoculum was subsequently replaced with fresh growth medium.

2.1.2.2 Preparation of virus stocks

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

2.1.2.3 Titration of virus preparations

Vero cells were grown in monolayers in 6-well plates (Greiner, UK). When the cells were approximately 80% confluent, they were incubated with the virus preparation (1ml/well) at different dilutions (duplicate wells were set up for each dilution). The virus was adsorbed for 2 h (37°C, 5% CO_2) on a rocking platform and a solution of 0.5% Methocel (Methocel MC; Sigma-Aldrich Co Ltd., UK) and 2% NCS in DMEM was subsequently added on top of the inoculum (1ml/well). The plates were incubated (37°C, 5% CO_2) for 8 to 10 days without any mechanical agitation. When plaques were distinctly formed, the medium was aspirated off and the monolayers were fixed (5% formaldehyde and 2% sucrose in PBS) for 30 min, stained with Coomassie blue (for 10 min on a rocking platform) and washed briefly with water. Plaques were counted and

the titre was estimated (plaque forming units per ml of virus preparation; pfu/ml), taking into account the dilutions made.

2.1.2.4 Construction of the recombinant virus

The recombinant virus used in this study (termed rSV5-V/P $N_{100}D$ and abbreviated to $rSV5_{N>D}$) was produced by reverse genetics (recovery of the virus from an infectious SV5 DNA clone). The recombinant virus was produced by Prof. R.A. Lamb's research group (Molecular Biology & Cell Biology, Northwestern University, Evanston, USA) who have provided information about the materials and method (Young *et al.*, 2001).

2.2 Protein analysis

2.2.1 Antibodies

The antibodies used in this study are shown below (Table 2.1). Antibodies were used in immunofluorescence, immunoblotting or immune precipitation, as described in the following sections.

Table 2.1: Antibodies and target proteins.

Antibody	Target Protein	Source
SV5-NP-a (mAb)	SV5 NP	Randall *et al.*, 1987
SV5-P-e (mAb)	SV5 P (unique C')	
SV5-P-k (mAb)	SV5 V and P (common N' domain)	
14E8 (mAb)	SV5 V (unique C' domain)	Lamb, R.A. *
11C6, 31F6 (mAbs)	SV5 V (unique C' domain)	
SV5-HN-4a (mAb)	SV5 HN	Randall *et al.*, 1987
SV5-F-1a (mAb)	SV5 F	
SV5-M-h (mAb)	SV5 M	
hPIV2-P-a (mAb)	hPIV2 V and P (common N' domain)	Randall and Young, 1988
a-STAT1 (pAb)	STAT1 (N' 194 amino acids)	commercial source **

* Provided by Prof. R.A. Lamb (Northwestern University, Evanston, USA)
** Transduction Laboratories (Becton Dickinson UK Limited) (cat. no. G16930)

2.2.2 Immunofluorescence

For immunofluorescence analysis, cells were grown on multispot slides or 10mm-diameter coverslips (General Scientific Co. Ltd., UK). A detailed account of the staining procedure can be found in (Randall & Dinwoodie, 1986). Cells were first treated with fixing solution (5% formaldehyde, 2% sucrose in PBS) for 10 min, then permeabilised (0.5% Nonidet-P40, 10% sucrose in PBS) for 5 min and washed repeatedly in 1% calf serum PBS. To detect the proteins of interest, cell monolayers were incubated with 10 to 15µl of appropriately diluted antibody for 1 h (primary antibody). Cells were subsequently washed (1% calf serum PBS) several times and incubated for 1 h with a cocktail solution that contained secondary goat anti-mouse Ig Texas Red-conjugated antibody (Seralab, UK; catalog number SBA 1010-02) and DAPI (0.5 µg/ml; Sigma-Aldrich Co Ltd., UK) for nuclear staining. All reactions were performed at room temperature and antibody treatments were allowed to progress in a humidified chamber in order to prevent desiccation of the monolayers. Following staining, samples were washed with PBS, mounted with coverslips using Citifluor AF-1 mounting solution (Citifluor Ltd., UK) and examined under a Nikon Microphot-FXA immunofluorescence microscope.

2.2.3 Metabolic labelling with [^{35}S]methionine

Cells were grown and infected as described above. At various times p.i. cells were washed in PBS and incubated with L-[^{35}S]methionine (500 Ci/mmol; Amersham International Ltd., UK) in methionine-free tissue culture medium for 2 h at 37°C. At the end of the labelling interval, cells were washed twice in ice-cold PBS, lysed and samples were further processed for immune precipitation (see below).

2.2.4 Immune precipitation

Following metabolic labelling, cells were washed twice in ice-cold PBS and lysed into immune precipitation buffer (10 mM Tris-HCl pH7.8, 5 mM EDTA, 0.5% Nonidet P-40, and 0.65 M NaCl; 4 x 10^6 to 6 x 10^6 cells per ml buffer) by sonication with an ultrasonic probe. Soluble antigen extracts were obtained after pelleting the particulate

matter from the total cell antigen extracts by centrifugation at 400,000 *g* for 30 min. Immune complexes were formed by incubating (for 2 h at 4°C) 0.2- to 1-ml samples of the soluble antigen extracts with an excess of anti-SV5 mAbs to the HN, F, P, M, and NP proteins (1 µl of concentrated tissue culture fluid of relevant mAbs; see Table 2.1). The immune complexes were isolated using an excess of fixed *Staphylococcus aureus* strain Cowan A suspension (20 ml of a 10% (w/v) suspension per ml of concentrated tissue culture fluid or ascitic fluid for 1 h at 4°C). The proteins in the immune complexes were dissociated by heating (100°C for 5 min) in gel electrophoresis sample buffer (0.05 M Tris-HCl pH7.0, 0.2% SDS, 5% 2-mercaptoethanol, and 5% glycerol) and subjected to SDS-PAGE, a described below. Analysed proteins were subsequently fixed and stained, electrophoresis gels were dried, and resolved polypeptide bands were visualised by phosphorimage analysis.

2.2.5 SDS-PAGE

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

2.2.6 Immunoblotting

Cells were washed twice in PBS prior to harvesting and subsequently lysed in gel electrophoresis sample buffer. Cell lysates were then sonicated and heated at 100°C for 5 min. Samples were analysed by SDS-PAGE as described above, polypeptides were transferred to nitrocellulose membranes using a Trans-Blot Cell (Bio-Rad, UK), assembled according to the manufacturer's instructions. The transfer apparatus was submerged in transfer buffer (0.025 M Tris, 0.19 M glycine, 20% (v/v) methanol) in a tank containing an ice cooling unit and run at 200 mA for 1 h (or 30 mA overnight). Following electroblotting, the membrane was briefly rinsed with distilled water and incubated in blocking buffer (10% (w/v) skimmed milk powder, 0.2% (v/v) Tween 20

in PBS) for 1 h to block nonspecific protein binding sites. STAT1 was subsequently detected with polyclonal anti-STAT1 antibody (see Table 2.1) by incubating the membrane in antibody suspension (1/100 to 1/1000 dilution in blocking buffer) for 1 h. In order to detect bound antibodies, the membrane was then washed (three washes in 0.2% (v/v) Tween 20 PBS, incubation in blocking buffer for 10 min on a rocking platform, then another three washes in 0.2% (v/v) Tween 20 PBS) and incubated in a horse-radish peroxidase-conjugated donkey anti-rabbit IgG (Amersham International Ltd., UK) suspension (1/2000 dilution in blocking buffer) for a maximum of 1 h on a rocking platform. The membrane was then washed as above and STAT1 bands were visualised by enhanced chemiluminescence (ECL) according to the manufacturer's recommendations (Amersham Biosciences UK Ltd.).

2.2.7 Reporter gene assays and determination of relative protein expression levels

Luciferase assays

Transfected cell monolayers in 6-well or 24-well plates were washed once in PBS and lysed in 200 or 300 µl (per well of a 24- or 6-well plate, respectively) luciferase buffer A (25 mM Tris phosphate pH7.8, 8 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1% Triton X-100) for 2 min. Following lysis, 200 µl or 300 µl (per well of a 24- or 6-well plate, respectively) luciferase buffer B (30% glycerol, 0.8 mM ATP, 2% BSA in luciferase buffer A) were added to the samples. A portion of the soluble cell extract (300 µl) was subsequently transferred to a luciferase cuvette into which 100 µl of luciferase substrate (1.5 mM luciferin in TE) were injected. Substrate injection and measurement of the light emitted over the first 10 sec from substrate addition (measured in relative light units; RLU) was performed by a Lumat LB9501 luminometer (Bertold, Wildbad, Germany).

β-galactosidase assays

Following the luciferase assay, the same cell extract samples were further assayed for β-galactosidase activity, in order to measure the transfection efficiency of the β-galactosidase reporter plasmid (see plasmid list above) and thus normalise the luciferase assay results. To every sample, 1 ml of β-galactosidase assay mix was added, which was

made of 4 volumes of lacZ buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_20$, 10 mM KCl, 1 mM $MgSO_4.7H_2O$, 2.7 ml/l β-mercaptoethanol) and 1 volume of chromogenic β-galactosidase substrate (4 mg/ml o-Nitrophenyl-β-D-Galactopyranoside; ONPG). Samples were incubated at 37°C until their colour became lemon-yellow (typically 8 to 12 h). Sample OD_{420nm} was then measured using a spectrophotometer (UVIKON 923, Bio-Tek Kontron Instruments Ltd., UK).

Relative protein expression levels

Relative expression levels were calculated by dividing the luciferase assay data values (RLU measurements) by the β-galactosidase assay OD_{420nm} values. Experiments presented have been repeated several times with equivalent results.

2.3 Nucleic acid processing and analysis

2.3.1 Plasmid DNA

2.3.1.1 Plasmid vectors and constructs

Mammalian cells were transfected with *reporter plasmids*, in order to perform reporter assay experiments (see above) and thus determine the effect of different test proteins on IFN signalling. *Backbone plasmids* were the basic plasmids that were used for the construction of the test plasmids. *Plasmids encoding the V proteins of viruses* were constructed to test the effect of the V proteins of different viruses on IFN signalling. *Plasmids encoding single- and double-mutant CPI+ V proteins* were produced by PCR-based mutagenesis (see below), in order to investigate the effect of the relevant mutations on the ability of V to block IFN signalling. *Selectable plasmids encoding the V proteins of CPI+ and CPI-* enabled the production of stable cell lines that produce the relevant proteins. *Plasmids encoding SV5-hPIV2 domain hybrid V proteins* were constructed to determine the role of the respective domains in STAT protein degradation.

All the V/P constructs described below contain the full-length V/P gene (complete V and P ORFs) but express the V ORF (V is the default product that arises from the faithful SV5 V/P gene transcript). If the P protein was to be expressed from these

plasmids, the editing site would need to be modified by the insertion of two guanine (G) residues, in order to shift the reading frame to the P ORF (see Chapter 1, Figure 1.4).

Reporter plasmids

- IFN-α/β-responsive plasmid; full term: p(9-27)4tkΔ(-39)lucter (King & Goodbourn, 1998); contains four tandem repeat sequences of the ISRE from the IFN-inducible gene, 9-27, fused to the firefly luciferase gene.

- IFN-γ-responsive plasmid; full name: p(GAS)2tkΔ(-39)lucter; contains a minimal tk promoter and two tandem repeat sequences of the IRF-1 GAS site fused to the luciferase gene (King & Goodbourn, 1998).

- β-gal plasmid; termed: pJATlacz; used as a transfection standard, to correct for any differences in transfection efficiency between different experimental treatments. It contains a β-galactosidase gene under the control of the rat β-actin promoter (Masson *et al.*, 1992). This reporter plasmid has been previously used as a transfection strandard in experiments involving stimulation of cells with IFN, as its activity is known not to be affected by IFN.

All reporter plasmids were provided by Dr. S. Goodbourn (St. George's Hospital Medical School, London, UK).

Plasmid vectors

- pEF-plink2; the plasmid vector used for the construction of the test plasmids described below. This plasmid DNA was also used as a negative control treatment (blank DNA) in transfection experiments. Expression of transgenes inserted in pEF-plink2 is driven by the mammalian elongation factor 1α (EF1α) promoter and have a human β-globin 5' untranslated region for optimal translation (a kind gift from R.H. Treisman, Imperial Cancer Research Fund). The map of this plasmid vector is presented in Appendix 1.

- pEF.IRES.neo; the plasmid vector used for the production of stable cell lines (see above). pEF.IRES.neo is similar to the pEF-plink2 vector, but it additionally enables the expression of the transgene and the G418 resistance gene from a single transcript (the G418 resistance gene is translated from an internal ribosome entry site (IRES) derived

from the encephalomyocarditis virus; Andrejeva *et al.*, 2002). The map of this plasmid vector is presented in Appendix 2.

- pGEM® T-Easy; standard commercial vector used for subcloning of DNA fragments (Promega UK Ltd.).

Plasmids encoding the V proteins of viruses

- pEF.SV5-V; encodes the V protein of W3 (wild-type SV5 strain). Its construction has been reported by Didcock and colleagues (1999b).

- pEF.hPIV2-V; encodes the V protein of hPIV2. This construct was made by PCR amplification of the hPIV2 (Colindale "prototype" strain) V/P gene from a bacteriophage-based hPIV2 gene library (produced by Bernie Precious), followed by insertion of the amplified gene into the pEF-plink2 vector (restriction enzymes *Nco*I and *EcoR*I; Promega UK Ltd.).

- pEF.SV5-V/mci-2; encodes the V protein of mci-2 (Young *et al.*, 2001). This construct was produced by RT-PCR amplification of the mci-2 V/P gene from infected cells, digestion of the obtained product with restriction enzyme *Bam*HI and insertion of the *Bam*HI site-flanked gene region into similarly digested pEF.SV5-V. A schematic diagram of the cloning strategy for this plasmid construct is presented in Chapter 3, Figure 3.5. In all plasmid constructs obtained from single-enzyme (*Bam*HI) cloning strategies, the orientation of the inserted fragment was confirmed by DNA sequence analysis (see below).

- pEF.W3/CPI+/V and pEF.W3/CPI-/V; code for the V protein of CPI+ and CPI-, respectively. These plasmids were constructed using the same cloning strategy as the one described above for pEF.SV5-V/mci-2 (*Bam*HI digestion and fragment replacement; see Figure 3.5; reported in Chatziandreou *et al.*, 2002).

Plasmids encoding single-mutant CPI+ V proteins

- pEF.W3/CPI+/V-($Y_{26}H$); similar to the pEF.W3/CPI+/V construct, but contains the first (nucleotide position 136; amino acid substitution $Y_{26}H$) of the three mutations present exclusively in the V ORF of CPI-.

- pEF.W3/CPI+/V-($L_{50}P$); similar to the pEF.W3/CPI+/V construct, but contains the second (nucleotide position 209; a.a. substitution $L_{50}P$) of the three mutations that were identified exclusively in the CPI- V ORF.

- pEF.W3/CPI+/V-($L_{102}P$); similar to the pEF.W3/CPI+/V construct, but contains the third (nucleotide position 365; a.a. substitution $L_{102}P$) of the three unique mutations found in the CPI- V ORF.

The cloning strategy for these plasmids was similar to the one described above for pEF.SV5-V/mci-2 (*Bam*HI fragment digestion and insertion; see Figure 3.5; Chatziandreou *et al.*, 2002).

Plasmids encoding double-mutant CPI+ V proteins

- pEF.W3/CPI+/V-($Y_{26}H/L_{50}P$); similar to the pEF.W3/CPI+/V construct, but contains the first and second (nucleotide positions 136 [a.a substitution $Y_{26}H$] and 209 [a.a. substitution $L_{50}P$], respectively) of the three point mutations present exclusively in the CPI- V ORF .

- pEF.W3/CPI+/V-($L_{50}P/L_{102}P$); similar to the pEF.W3/CPI+/V construct, but contains the second and third (nucleotide positions 209 [a.a. substitution $L_{50}P$] and 365 [a.a. substitution $L_{102}P$], respectively) of the three mutations that were identified exclusively in the CPI- V ORF.

- pEF.W3/CPI+/V-($Y_{26}H/L_{102}P$); similar to the pEF.W3/CPI+/V construct, but contains the first and third (nucleotide positions 136 [a.a. substitution $Y_{26}H$] and 365 [a.a. substitution $L_{102}P$], respectively) of the three mutations unique in the CPI- V ORF.

These plasmids were constructed using the same cloning strategy as the one described for pEF.SV5-V/mci-2 (*Bam*HI digestion and fragment replacement in pEF.SV5-V; see Figure 3.5; Chatziandreou *et al.*, 2002).

Selectable plasmids encoding the V proteins of CPI+ and CPI-

- pEF.W3/CPI+/V.IRES.neo and pEF.W3/CPI-/V.IRES.neo; encode the V protein of CPI+ and CPI-, respectively. The plasmid backbone of this construct facilitates the selection of permanent cell lines (pIRES.neo plasmid vector; see above).

Plasmids encoding SV5-hPIV2 domain hybrid V proteins

- pEF.SV5/hPIV2-V; contains a hybrid V/P gene, consisting (5'-3') of the portion of the SV5 (W3) V/P gene that encodes the amino-terminal domain of the SV5 V protein (i.e. the portion from the start of the gene to the SV5 V/P editing site), followed by the portion of the hPIV2 V/P gene that codes for the carboxy-terminal domain of the hPIV2 V protein (i.e. the portion from the hPIV2 V/P editing site to the end of the gene).

- pEF.hPIV2/SV5-V; contains a hybrid V/P gene, which consists (5'-3') of the portion of the hPIV2 V/P gene that codes for the amino-terminal domain of the hPIV2 V protein (i.e. the portion from the start of the gene to the hPIV2 V/P editing site), followed by the portion of the SV5 (W3) V/P gene that encodes the carboxy-terminal domain of the SV5 V protein (i.e. the portion from the SV5 V/P editing site to the end of the gene).

The construction strategy for the hybrid V-encoding genes is presented in Chapter 3, Figure 3.23.

2.3.1.2 Preparation of plasmid DNA

For small scale preparations, bacterial cell cultures of 4 ml were grown overnight at 37°C in a shaking incubator. DNA was extracted from cells using the Qiagen DNA mini-prep kit (Thermo Hybaid Limited, UK), according to the manufacturer's instructions. This method is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt.

Alternatively, for large numbers of small preparations, DNA was extracted by alkaline lysis, followed by ethanol precipitation. In this method, the cell suspension was transferred into Eppendorf (microfuge) tubes. Cells were then pelleted by centrifugation for 5 min at 6.5K rpm (this step was repeated twice to load a total of 3 ml of culture into the 1.5 ml Eppendorf tubes), resuspended in 200 µl resuspension buffer (50 mM Tris pH8.0, 10 mM EDTA, 100 µg/ml RNase A), lysed in 200 µl lysis buffer (200 mM NaOH, 1% SDS), neutralised in 250 µl neutralisation buffer (3.0 M K-acetate pH5.5) and centrifuged for 10 min at 14K rpm to pellet cell debris. All solutions were provided by (Thermo Hybaid Limited, UK). DNA was ethanol precipitated from the supernatant (addition of 750 µl cold ethanol 100%, centrifugation for 15 min at 14K rpm) and

washed with ethanol 70%. The pellet was finally air-dried and resuspended in 50 to 100 µl de-ionised water.

For large scale preparations, bacterial cultures of 300 to 500 ml were grown overnight at 37°C in a shaking incubator. Similarly to small scale preparations, DNA was extracted from cells and purified on silica gel membrane columns (on the principle described above), using the Qiagen DNA maxi-prep kit (Thermo Hybaid Limited, UK) according to the manufacturer's instructions.

Using a UV spectrophotometer (UVIKON 923, Bio-Tek Kontron Instruments Ltd., UK), prepared DNA was quantified by measuring sample OD_{260nm} (50 ng/µl for OD_{260nm}=1) and the purity of the preparation (in terms of protein carryover) was estimated by calculating the OD_{260nm}/OD_{260nm} ratio (ratios above 1.8 were considered to be acceptable for downstream applications). Prepared DNA was also visualised by agarose gel electrophoresis, as described below.

2.3.2 DNA processing, RNA purification and gene cloning

2.3.2.1 Restriction enzyme digestion of DNA

In analytical restriction enzyme digestion reactions, DNA (typically 3 to 5 µg) was mixed with 5 units of (each of) the desired enzyme(s), enzyme buffer and acetylated BSA, in a reaction made up to a total volume of 10 to 20 µl in de-ionised water. All restriction reagents were provided by Promega UK Ltd. or New England Biolabs (UK) Ltd. The reactions were incubated for 2 to 4 h at the appropriate temperature, which varied depending on the enzyme(s) used (37°C, in the majority of cases). In preparative restriction digests, >5 µg DNA were digested in similar reactions scaled up to a maximum of 50 µl. Where a single enzyme was used to create cohesive ends at both ends of the fragment, following digestion, DNA was dephosphorylated with CIAP (0.5 units) in the appropriate buffer (Promega UK Ltd.). The dephosphorylation reaction was carried out for 15 min at 37°C, and then DNA was treated again (addition of another 0.5 units CIAP and incubation for another 15 min at 37°C), in order to ensure effective dephosphorylation. Prior to use in ligation reactions, digested and dephosphorylated

DNA was purified (cleaned of enzymes and buffers), using Qiagen silica membrane gel columns (Thermo Hybaid Limited, UK) according to the manufacturer's instructions. DNA purification was done either directly or following separation of the digested fragments by agarose gel electrophoresis.

2.3.2.2 Agarose gel electrophoresis

DNA was analysed by gel electrophoresis in horizontal mini-gels of 1% (w/v) agarose (Sigma-Aldrich Co Ltd., UK) in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA). Prior to electrophoresis, DNA samples were mixed with the appropriate volume of DNA loading buffer (Promega UK Ltd.). Samples were run at 45 to 80 V in TBE buffer containing 1 μg/ml ethidium bromide, until bands were clearly resolved. In most cases, samples were run alongside known DNA molecular weight markers (1kb ladder; Promega UK Ltd.). Electrophoresed DNA was exposed to UV light and photographed (Gel Doc 2000 UV transilluminator/photography system; Bio-Rad, UK). When preparative gels were run to separate fragments or plasmids for cloning, exposure of DNA to the transilluminator was kept to a minimum, in order to prevent damage by UV radiation.

2.3.2.3 Purification of DNA fragments from agarose gels

DNA was analysed by agarose gel electrophoresis, as described above, and the DNA fragments of interest were subsequently purified by excision of the resolved bands from the gel and recovery on Qiaquick gel extraction columns (Thermo Hybaid Limited, UK) according to the manufacturer's instructions. A fraction of the purified samples (typically 1/10 of the total volume of the eluate) was usually re-run on agarose gels, in order to estimate the purification efficiency.

2.3.2.4 Ligation of DNA fragments

Prior to the addition of any ligation reaction component, relevant DNA fragments were pooled together (aiming typically at 5-10 : 1 molar ratio of insert to vector), heated for 5 min at 45°C to melt any cohesive ends that might have annealed, and chilled at 0°C to

prevent reannealling. Following this step, T4 DNA ligase (New England Biolabs (UK) Ltd. or Promega UK Ltd.) was added to the mixture, together with the appropriate buffer (supplied by the company). The reaction mix was made up to a total volume of 10 to 15 µl with de-ionised water, and incubated at 14 to 16°C or 4°C overnight, depending on the manufacturer's recommendations.

Ligation reaction products were used for transformation of competent *Escherichia coli* (*E.coli*) cells either directly (in heat-shock transformation) or following purification by ethanol precipitation (recommended for transformation by electroporation).

2.3.2.5 Preparation of RNA from virus-infected cells for Reverse Transcription

mRNA

Vero cells (or BF cells, in some cases) were typically used for viral RNA isolation for Reverse Transcription. Cells were grown as monolayers (25- or 75-cm^2 flasks; Greiner, UK) and infected with the virus of interest at a high m.o.i. (typically 10 pfu/cells) to ensure high efficiency of infection. The monolayers were washed in PBS twice and harvested at 22 h post-infection (p.i.). Total RNA purification was performed using the Qiagen RNeasy™ RNA extraction kit (Thermo Hybaid Limited, UK), according to the manufacturer's instructions. RNA was eluted from the silica gel membrane in 50µl RNase free water, and the suspension was aliquoted and stored immediately at –70°C.

Genomic RNA

The appropriate number of cells was infected in suspension, as described above, and plated out in 75-cm^2 flasks in fresh medium supplemented with 10% serum. At 21 h p.i. the medium was replaced with serum-free medium (of a volume that matched the volume capacity of the ultracentrifuge tube, in this case approximately 11 ml medium per 75-cm^2 flask), in order to prevent precipitation of serum in subsequent ultra-centrifugation steps. At 30 h p.i. the medium was harvested (this limit was never exceeded), transferred to an ultracentrifuge tube and centrifuged at 30000 rpm for 90 min. The pellet, which was usually visible at the bottom of the tube (translucent, granular appearance), was subsequently lysed in a maximum volume of 500 µl lysis

buffer (Qiagen RNeasy ™ RNA extraction kit; Thermo Hybaid Limited, UK). At this point, lysed samples could be stored at –70°C. Lysates were processed using the Qiagen RNeasy ™ RNA extraction kit (Thermo Hybaid Limited, UK), according to the manufacturer's instructions. Following elution of RNA from the silica gel membrane column (50 µl RNase free water), the purified suspension was aliquoted and stored immediately at -70°C.

2.3.2.6 cDNA synthesis by Reverse Transcription

For cDNA synthesis, virus RNA (an estimated amount of 2 µg) was mixed with the appropriate reverse orientation primer (0.5 µg). The mix was heated at 70 °C for 5 min to melt any secondary structure within the template. Samples were then cooled immediately on ice to prevent secondary structure from reforming, and centrifuged briefly at 13K rpm to collect condensation. Following primer annealing to RNA, RNasin (25 units; Promega UK Ltd.), dNTPs (0.2 mM of A, T, G and C; Amersham International Ltd., UK) M-MLV (200 units) and supplied M-MLV buffer (Promega UK Ltd.) were added to the mix, to a final volume of 25 µl in RNase free water. The reaction was incubated for 60 to 75 min at 42° C and the cDNA product was subsequently used in polymerase chain reactions, as described below.

2.3.2.7 Polymerase Chain Reaction (PCR)

Being a very powerful and versatile method for DNA amplification and engineering, polymerase chain reaction (PCR) was used for three main purposes in this study: *DNA fragment amplification*, *DNA mutagenesis* and *hybrid DNA engineering*.

DNA fragment amplification

The 100-µl PCR reaction mixture (made up in sterile water) consisted typically of PCR enzyme buffer (at an appropriate dilution), 0.2 mM of each dNTP, 50 to 200 ng DNA (in cDNA-templated reactions, the whole 25-µl RT reaction mixture was added to the PCR microtube; see previous section), 0.1 to 0.5 µM of each primer (0.5 µM usually yielded optimal results), and 3 units DNA polymerase. PCR condition parameters were typically as follows: melting at 95°C for 45 sec, annealing at 55°C (or other) for 45 sec,

and strand extension at 72°C for 1min/1kb. The PCR set (typically 30 cycles) was preceded by an initial step of melting at 95°C for 2 min, followed by a final step of strand extension at 72°C for 10 min. Annealing temperature varied in different PCRs, depending on oligonucleotide composition, and was calculated on the principle of {(lowest primer T_m)-8°C}.

The DNA polymerase typically used was cloned *Taq* DNA polymerase (DyNAzyme™, *DyNA II*) (Finnzymes Oy, Finland). In some cases, *rTth* DNA polymerase (Perkin-Elmer Life Sciences Ltd., UK) was the preferred enzyme, as this polymerase has higher fidelity than *Taq*. Nevertheless, although *Taq* DyNAzyme™ lacks proofreading activity, its error rate is still relatively low (max. 3.6×10^{-5}), especially when used for amplification of relatively short DNA fragments.

DNA mutagenesis

PCR-based site-directed mutagenesis was carried out using the method described in the instruction manual for a commercial mutagenesis kit (Stratagene Europe, UK; manual for QuickChange Multi Site-Directed Mutagenesis Kit product no. 200514; Stratagene, 2000).

Initially, copies of the mutant plasmid were produced in a PCR reaction. The 100 µl PCR reaction mixture (made up in sterile water) consisted of PCR enzyme buffer (at an appropriate dilution) and 1.5 mM $Mg(OAc)_2$ (Perkin-Elmer Life Sciences Ltd., UK), 0.2 mM of each dNTP (Amersham International Ltd., UK), 20 ng DNA (plasmid DNA to be mutagenised), 0.05 µM of each mutagenic primer (see below), and 2 units *rTth* DNA polymerase (Perkin-Elmer Life Sciences Ltd., UK). In brief, the PCR condition parameters were typically: an initial step at 95°C for 5 min, [95°C for 30 sec, typically 55°C (calculated on the principle of {lowest primer T_m}-8°C) for 45 sec, 72°C for 1min/1kb] x 20 cycles, and a final step at 72°C for 10 min. In this reaction, the entire plasmid was copied and the mutagenic primers were incorporated into newly synthesised plasmid DNA. At the end of the reaction, the final mixture contained copies of both methylated, bacterial plasmid DNA (template) and nonmethylated, PCR-synthesised plasmid DNA (mutated product).

In the second step of this method, restriction enzyme *DpnI* was added to the reaction mixture and DNA digestion was allowed to proceed at 37°C for 3 h. *DpnI* recognises only methylated DNA as substrate, so, following incubation with this enzyme, methylated, template DNA (extracted from bacteria) was digested, in contrast to nonmethylated, PCR-synthesised DNA which remained unaffected. A portion of the final reaction mixture was then used to transform bacterial cells and prepare mutant plasmid DNA in large quantities.

A schematic diagram of the mutations introduced in the CPI+ V/P gene for the production of the CPI+/- mutants is presented in Chapter 3, Figure 3.5.

Hybrid DNA engineering

PCR-based SV5-hPIV2 hybrid gene construction was carried out in two phases; details about the construction strategy are explained in Chapter 3, Figure 3.23. Initially, hybrid gene fragments were produced in individual PCRs (PCR I and II) using internal oligonucleotide primers that had overlapping regions. The fragments obtained from these reactions were subsequently combined in another PCR reaction (PCR III), in which they annealed to each other thus reciprocally priming their extension. Briefly, the condition parameters for PCR I and II were: an initial step at 95°C for 5 min, [95°C for 45 sec, 52°C for 45 sec, 72°C for 1 min] x 30 cycles, and a final step at 72°C for 10 min. For the final reaction, PCR III, the cycling parameters were the following: an initial step at 95°C for 5 min, [95°C for 45 sec, 48°C for 45 sec, 72°C for 2 min] x 30 cycles, and a final step at 72°C for 10 min.

2.3.2.8 Oligonucleotide PCR primers

Oligonucleotide primers were designed taking a number of parameters into consideration, including melting temperature (T_m), GC content, GC frequency at the beginning and end of the selected region, sequence variation and length, as well as restriction enzyme site usage. Oligonucleotide primers used in PCR-based DNA mutagenesis (see above) were additionally designed according to the mutagenic primer design guidelines available for this method (Stratagene Europe, UK; manual for QuickChange Multi Site-Directed Mutagenesis Kit product no. 200514; Stratagene,

2000). In the case of hybrid SV5/PIV2 V construction (see above), SV5/PIV2 hybrid primers were designed by fusing portions of the V/P genes from the two viruses (see Figure 3.23 for details). Cloning of the F gene from the different SV5 isolates also required new primers. Thus, in order to enable amplification of the complete gene and allow any variation in the length of F ORF to be detected, oligonucleotide primers were designed to anneal on either extragenic (genomic RNA) regions that flank the F gene (see Figure 1.4). Information about all oligonucleotide PCR primers used in the present study (obtained from Oswel Research Products Ltd., UK) is presented in Appendix 3.

2.3.2.9 Nucleotide sequence analysis

Nucleotide sequence analysis was typically performed on DNA samples (0.5 μg) that had been extracted from bacterial cells, and purified on silica gel membrane columns (Thermo Hybaid Limited, UK) or by ethanol precipitation, as described above. Samples were analysed by the fluorescent dideoxynucleotide method, using an ABI PRISM™ 377 DNA Sequencer (Perkin-Elmer Life Sciences Ltd., UK). Sequence analysis was carried out at the DNA Sequencing Unit of the University of St.Andrews by Alex Houston.

2.3.2.10 Computer software for nucleotide sequence data analysis

Nucleotide sequence data were processed using a variety of computer software tools. To identify the obtained sequences, data were compared to the National Institutes of Health (NIH; USA) genetic sequence database (GenBank) using the online BLAST sequence similarity search tool (http://www.ncbi.nlm.nih.gov/BLAST/) of the National Centre for Biotechnology Information (Bethesda, USA). For further processing, different programs were used, including Gene Jockey II Sequence Processor (Biosoft, UK) and BioEdit Sequence Alignment Editor (Tom Hall, USA) for conversion and multiple sequence alignments, DNA Strider™ 1.2 (C. Marck, France) for the identification of ORFs and restriction enzyme sites, and ABI PRISM AutoAssembler™ (Perkin-Elmer Life Sciences Ltd., UK) for the assembly of full-length gene sequences from shorter nucleotide sequences.

2.3.3 Bacterial transformations

2.3.3.1 Bacterial strains

Escherichia coli strains DH5a [*supE44* D*lac*U169 (*f*80*lac*ZDM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1*relA*1] and XL-1 Blue [*rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 (*rk*[+] *mk*[+]) *sup*E44 *rel*A1 [[lambda]][-] *lac* [F' *pro*AB *lac*IqZ[[Delta]]M15 Tn10 (*tet*[R])] were used in gene cloning. Bacterial cells were grown in liquid Luria-Bertani (LB) medium containing 10 g/l bacto-tryptone (Becton Dickinson UK Limited), 5 g/l yeast extract (Becton Dickinson UK Limited) and 10 mM NaCl pH 7.5, or plated on solid LB medium supplemented with 1.5% (w/v) agar (Becton Dickinson UK Limited) and 10M $MgSO_4$ in 90 mm-diameter petri dishes (Scientific Laboratory Supplies Ltd., UK). To make use of antibiotic resistance markers, when required, media were supplemented with ampicillin (100 μg/ml). For blue-white selection of colonies that contained the plasmid of interest (option available with strain XL1-blue), solid LB-agar medium was also supplemented with the appropriate chromogenic βgalactosidase substrate (40 μg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside; X-gal) and lacZ inducer (0.1 mM isopropylthiogalactoside; IPTG).

2.3.3.2 Preparation of competent bacterial cells

Heat-competent cells

Fresh bacterial mini-cultures (10 ml LB medium) were diluted 1:100 in fresh LB medium (1-litre flasks) and incubated at 37°C in a shaking incubator, until their OD_{600nm} reached 0.500 to 0.700 units. Cultures were then incubated on ice for at least 15 min and pelleted by centrifugation at 3500 rpm for 10 min. Cells were resuspended in 15 ml buffer TFB1 (30 mM KOAc, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15% glycerol, pH5.8 with 1M acetic acid; filter sterilised), incubated on ice for 30 min, re-centrifuged and finally resuspended in 2 to 4 ml buffer TFB2 (10 mM MOPS pH6.5, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol, pH 6.5 with 1M KOH; filter sterilised), depending on cell density. The cell suspension was aliquoted (10% glycerol) in appropriate volumes (multiple of 100 μl), frozen immediately in a dry ice/ethanol (100%) bath and stored at -70°C.

Electrocompetent cells

Freshly prepared 10-ml bacterial cultures in LB medium were diluted 1:50 (4 ml pre-culture in 200 ml fresh LB medium) and grown at 37°C until OD_{600nm} reached 0.500 to 0.700 units. Cultures were then chilled on ice, centrifuged in 50-ml tubes for 5 min at 3000 rpm, and pellets were resuspended in 25 ml sterile water at ice temperature. Cells were washed twice (centrifuged for 5 min at 3000 rpm and resuspended in ice-cold sterile water) and finally pooled and resuspended in 400 µl ice-cold sterile water. Bacterial stocks (10% glycerol) were prepared in aliquots of 100 µl, frozen immediately in a dry ice/ethanol (100%) bath and stored at -70°C.

2.3.3.3 Transformation of competent bacterial cells

Electroporation

Prior to electroporation, plasmid DNA was purified by ethanol precipitation, as described above, in order to remove salts (e.g. from the ligation reaction buffer). Precipitated DNA was resuspended in 10 µl sterile de-ionised water, of which half (or all of the suspension, in some cases) was added to 40 µl electrocompetent *E.coli* cells (cell and DNA suspensions must be kept on ice during the process). The mix was transferred to a pre-chilled 2-mm electroporation cuvette (Flowgen Instruments Ltd., UK) and given an electric pulse for 5 msec at 2500 V, 201 Ω, 25 mF, using an Easyject Plus electroporator (Flowgen Instruments Ltd., UK).

Heat-shock transformation

In contrast to electroporation, this method does not require purification of the prepared DNA by ethanol precipitation and, therefore, after completion of the ligation reaction, the reaction mix (10 to 20 µl) was added directly to heat-shock competent *E.coli* cells (100 µl). The mix was incubated on ice for at least 30 min and cells were then given a heat shock by incubation at 42°C for precisely 60 sec. Following this treatment, the suspension was transferred swiftly back on ice and incubated for 2 min.

In both methods, immediately after transformation, cells were resuspended in 1 ml fresh LB medium, transferred into an Eppendorf tube and incubated for 1 h at 37°C in a

shaking incubator, in order to assist cell recovery and allow the expression of the plasmid-contained antibiotic resistance gene. Following incubation, cells were pelleted by brief centrifugation at 13K rpm, resuspended in 100 μl LB medium and finally plated in a 90 mm-diameter petri dish (Scientific Laboratory Supplies Ltd., UK) containing solid LB-agar medium supplemented with the appropriate antibiotic and, if required, chromogenic substrate (see above). Plates were incubated at 37°C overnight and bacterial mini-cultures were prepared the next day from selected colonies.

Chapter 3

Results

3.1 SV5 mci-2: A single amino acid substitution in the V protein enables the virus to proliferate in cells of a different host species origin

3.1.1 Identification of mci-2

W3 in murine cells

Previous work has shown that simian virus type 5 (SV5) is IFN-sensitive in murine cells (Didcock *et al.*, 1999a). Thus, although the virus can proliferate productively in human cells (a natural host), in murine cells it is restricted by the IFN response of the cell. In murine cells, following infection, the virus enters an initial phase of high protein synthesis which is subsequently switched off by the antiviral action of IFN-induced cellular proteins. The sensitivity of SV5 to IFN in murine cells has also been linked to its ability to establish persistent infections in cells of this host species (Young *et al.*, 1997). Thus, upon prolonged passaging, SV5-infected cells become persistently infected, with the virus fluxing between an active and inactive state, depending on localised levels of IFN.

Isolation of mci-2

In that study, Young and colleagues (1997) isolated SV5 variants from persistently infected murine (Balb/c fibroblast; BF) cells (BF-E7 cell line). These variants arose following regular passaging (over 90 times) and they were selected for their ability to replicate better in the cell population. The first variant that was isolated (passage 40) was more fusogenic than W3 (albeit still IFN-sensitive in murine cells) and hence termed SV5 W3-f or mci-1 (mouse-cell isolate 1). After passage 80, however, a second variant predominated in the population, which was IFN-resistant (Young *et al.*, 2001). This isolate was named mci-2 (mouse-cell isolate 2) and, intriguingly, it could not be detected with the monoclonal anti-Pk antibody SV5-P-k that recognises the Pk epitope in the SV5 V and P (common) amino-terminal domain.

mci-2 does not bind the anti-Pk Ab

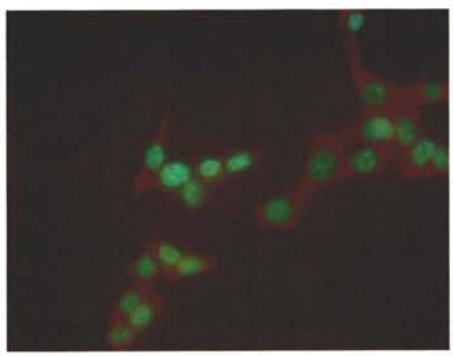
An example of the inability of the anti-Pk mAb to bind the Pk epitope of mci-2 is illustrated in Figure 3.1. Persistently infected murine BF-E7 cells (after passage 80, when mci-2 arose) were fixed and stained with two different monoclonal antibodies that recognise different epitopes on SV5 V and P. The results showed that staining with the anti-Pk mAb (Panel C) failed to detect the viral proteins. However, when stained with a different anti-P antibody, which binds to a different epitope (mAb SV5-P-e), cells

Figure 3.1: Immunofluorescence analysis reveals that there are changes in the Pk epitope of the V and P protein of mci-2.

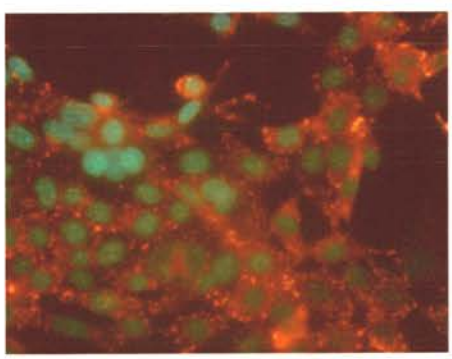
Murine cells persistently infected with SV5 (BF-E7 cell line) stained positive for P when treated with the monoclonal anti-P antibody which recognises an epitope other than the Pk epitope (mAb SV5-P-e; Panel B). When cells of the same cell line were immunostained with the anti-Pk antibody (mAb SV5-P-k), which binds to the Pk epitope in the amino-terminal domain of V and P, there was not any fluorescence, indicating that this antibody fails to recognise these proteins (Panel C). Panel A shows mock-infected cells stained with mAb SV5-P-e. DAPI staining was included in all treatments.

Fig. 3.1

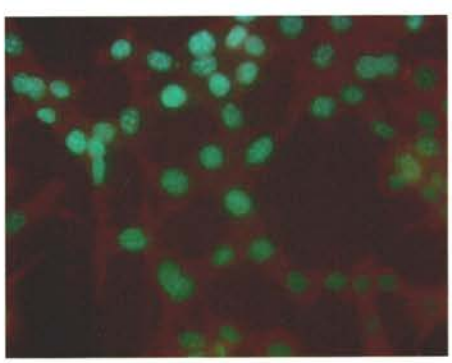
A)



B)



C)



stained positive for mci-2 (Panel B). Thus, this immunofluorescence analysis suggested the presence of mutation(s) in the Pk epitope of V and P of the IFN-resistant mci-2.

3.1.2 Characterisation of mci-2

3.1.2.1 Mci-2 can degrade STAT1 in murine cells

Mci-2 was selected as a variant that had a greater ability to replicate in murine cells, compared to the wild-type virus (W3 strain) which is IFN-sensitive in the same cell line. Given that SV5 V is the protein responsible for STAT1 degradation in infected cells (thereby determining IFN sensitivity), it was therefore of interest to determine whether the mutation(s) identified in the Pk epitope of mci-2 conferred IFN resistance on this isolate. To address this question, STAT1 levels were examined in murine BF cells infected with mci-2, as well as in the persistently infected BF-E7 cells (p80), which mci-2 was originally isolated from.

mci-2 degrades STAT1 in murine cells

Cells were infected at a high m.o.i., to ensure complete infection so that any STAT1 degradation effect would become evident, and treated with exogenous IFN for 24 hours (h) prior to harvesting, so as to induce STAT1 to detectable levels. Cell lysates were analysed by SDS-PAGE and immunoblotted with the appropriate antibody for STAT1 detection. The results from this analysis are shown in Figure 3.2. Clearly, infection with mci-2 resulted in loss of STAT1, as shown in lane 7. The same effect is apparent in lane 1 where STAT1 was not detectable in the persistently infected BF-E7 cell line. However, STAT1 remained unaffected in BF cells infected with W3 (lane 5), in confirmation of previous findings (Didcock *et al.*, 1999b).

mci-2 prevents STAT1 up-regulation

Cells respond to virus infection by producing IFN, which results in upregulation of STAT1 levels. Consequently, STAT1-based signalling is enhanced and IFN production increases in a positive feedback loop that essentially accelerates virus clearance. STAT1 induction is evident in murine cells infected with W3 (lane 6), as wild-type virus does not degrade STAT1 in murine cells and therefore post-infection STAT1 induction is not disrupted. However, STAT1 upregulation was not manifested in the BF-E7 or mci-2-infected BF cells (lanes 2 and 8, respectively), presumably due to the prevention of IFN signalling by STAT1 degradation. Lane 4 shows that, in murine cells, STAT1 levels are

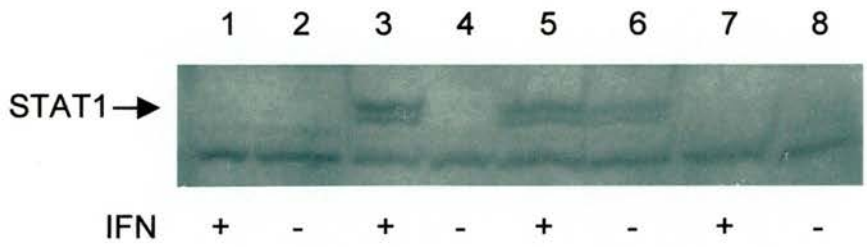


Figure 3.2: Mci-2 leads to the degradation of STAT1 in murine cells.

Murine (BF) cells were inoculated with SV5, treated with exogenous IFN (or not) for 24 h prior to harvest and immunoblotted with polyclonal anti-STAT1 antibody. Treatment with exogenous IFN induced STAT1 to detectable levels, as shown in mock-infected cells (compare lanes 3 and 4). In cells that were persistenty infected with SV5 (BF-E7 cell line) STAT1 was absent (lanes 1 and 2). STAT1 was also degraded in cells infected with mci-2 (lanes 7 and 8). Infection with W3 not only left STAT1 levels unaffected (lane 5), but induced it to detectable levels in cells that had not been treated with IFN (lane 6). The STAT1 double-band represents STAT1α (upper band) and STAT1β (lower band).

normally below detection level, whilst STAT1 induction by treatment with exogenous IFN is confirmed in lane 3.

3.1.2.2 Sequence analysis of the V/P, HN and F genes of mci-2

Sequence analysis of the V/P gene

In order to identify the amino acid change(s) in the V protein of mci-2, the nucleotide sequence of the V/P gene of this isolate was analysed. The gene was cloned by RT-PCR from Vero cells that had been infected with mci-2. The cDNA preparation was directly sequenced by the dideoxy-nucleotide method (at the DNA sequencing unit of the University of St.Andrews), using oligonucleotide primers that flanked the entire coding region of the gene (from the V/P start to the P stop codon). Consistent with the immunofluorescence observations, sequence analysis results revealed a single amino acid substitution in the V/P common amino-terminal domain, which mapped to the Pk epitope [asparagine (N) to aspartic acid (D) substitution at a.a. position 100; hereinafter referred to as $N_{100}D$]. A second amino acid change was also identified, which uniquely affected the carboxy-terminal domain of the P protein. The relative position of these two mutations in the V/P mRNA is presented schematically in Figure 3.3 and information about their precise location is provided in Table 3.1.

Sequence analysis of the HN and F genes

To characterise the mci-2 isolate further, particularly in relation to the more fusogenic mci-1 that had preceded its occurrence, the nucleotide sequence of mci-2 HN and F genes was determined as described previously. Analysis of the sequence data showed that, compared to W3, mci-2 has two amino acid substitutions in the HN protein and only one silent nucleotide mutation in the F gene. Further comparison of the HN and F sequences of mci-2 to those of mci-1 (Young *et al.*, 1997; Didcock, 1999) revealed that there is hardly any relationship between the two isolates. Of the nine amino acid substitutions identified in the mci-1 HN gene, only one was found in mci-2. In addition, mci-2 HN has a second amino acid substitution which is not present in mci-1. Similarly, only one (silent) mutation is common between the mci-1 and mci-2 F genes, as mci-1 has an additional mutation that is not present in mci-2. Thus, sequence analysis of the mci-2 and mci-1 HN and F gene sequences made clear that mci-2 did not originate from mci-1 but was derived directly from W3. An overview of these results is illustrated in

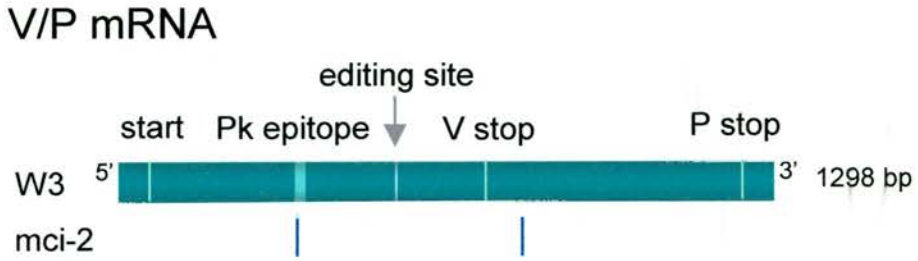


Figure 3.3: Map of the mutations identified in the V/P gene of mci-2.

The entire mRNA is represented schematically by the green band and the amino acid substitutions identified in mci-2 are shown below with blue lines. Annotation on the mRNA reveals the relative position of the mutations with regard to the V and P open reading frames (ORFs), and the Pk epitope.

Table 3.1: Nucleotide and amino acid differences between the W3 and mci-2 V/P genes and proteins.

Nucleotide and amino acid changes are shown in bold. Position numbers refer to the nucleotide residue information available for the V/P gene of SV5 W3 (SV5PVA locus, GenBank Accession no. J03142).

nucleotides			amino acids		
position	W3	mci-2	position	W3	mci-2
358	A	**G**	100	N	**D**
798	A	**G**	247	Q	**R**

Figure 3.4, while full nucleotide and amino acid residue information about the mci-2 and mci-1 mutations in HN and F is provided in Table 3.2.

3.1.2.3 The $N_{100}D$ amino acid substitution in the V protein of mci-2 enables the virus to block IFN signalling in murine cells

Mci-2 was isolated from a population of cells that produce and respond to IFN, in which it predominated as a result of its ability to degrade STAT1. It was thus found that this isolate has an IFN-resistant phenotype, in contrast to W3 which fails to reduce STAT1 levels and is IFN-sensitive in the same cells. Nevertheless, despite this dramatic phenotypic difference, sequence analysis showed that there is only one amino acid difference between the mci-2 and W3 V proteins ($N_{100}D$). To establish the role of this mutation further and confirm its importance in the prevention of IFN action, the ability of mci-2 V to block IFN signalling in murine cells was assessed in a reporter assay experiment.

transfection experiment

To perform this analysis, the portion of the gene that contained the $N_{100}D$ mutation was introduced into the W3 V/P gene, which had been previously engineered into a constitutive eukaryotic expression vector (pEF-plink2 plasmid vector; see Chapter 2). The cloning strategy for this genetic manipulation is presented graphically in Figure 3.5. The construct (pEF.SV5-V/mci-2) was co-transfected into human (2fTGH), canine (Dog134) and murine (BF) cells together with a type I IFN-responsive reporter plasmid (pISRE) and the ability of the reporter (luciferase) gene to respond to IFN was measured.

mci-2 V blocks IFN signalling in murine cells

The results from this experiment are plotted in Figure 3.6. As shown in Panel B, it became clear that the V protein of mci-2 blocks type I IFN signalling efficiently in murine cells. In agreement with previous findings (Didcock *et al.*, 1999b), W3 V failed to block signalling in the same cells; however, it reproducibly inhibited approximately 30% of the activity of the IFN-responsive promoter, suggesting that it may still be weakly active. Furthermore, very low levels of IFN signalling were reproducibly observed in murine cells transfected with the mci-2 V gene. In human and canine cells, mci-2 V behaved like the wild-type (W3) protein, blocking IFN signalling very efficiently, as shown in Panels A and C, respectively. This experiment thus

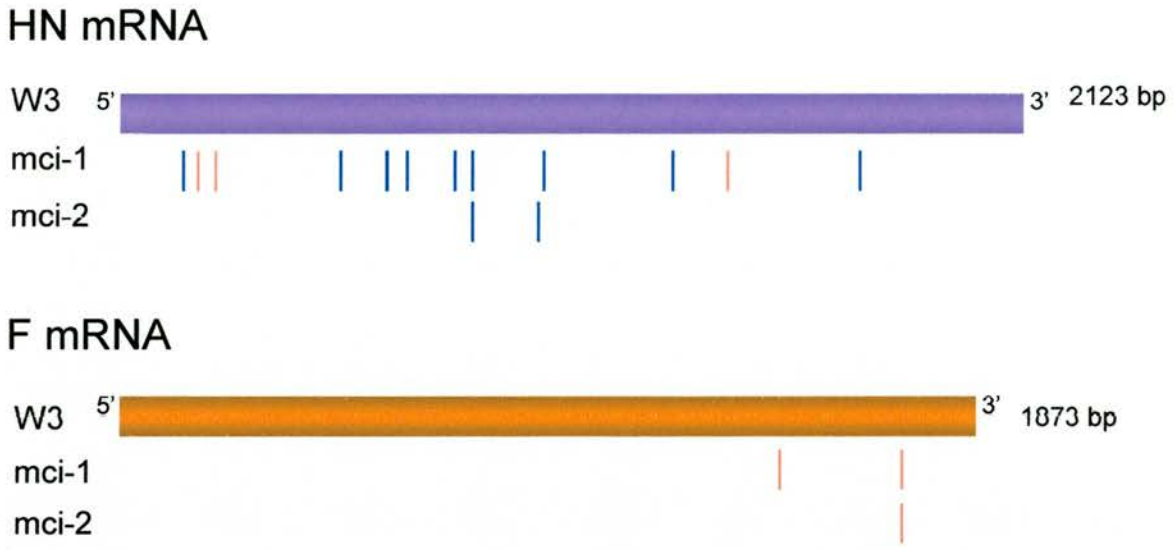


Figure 3.4: Gene map showing the mutations identified in the HN and F genes of mci-2, in comparison to those of mci-1.

In each panel, the length of the entire mRNA is represented schematically by a band. Mutations are shown below each mRNA, with blue lines indicating amino acid substitutions and orange lines showing silent mutations.

Table 3.2: Nucleotide and amino acid differences in the HN and F genes and proteins of W3, mci-1 and mci-2.

Nucleotide and amino acid changes are shown in bold. Position numbers refer to the nucleotide sequence information available for the SV5HNP and SV5PFC loci (GenBank Accession no.s K02870 and K02253, respectively).

gene	nucleotides				amino acids			
	position	W3	mci-1	mci-2	position	W3	mci-1	mci-2
HN	149	T	**C**	T	28	L	**P**	L
	183	A	**G**	A	39	L	L	L
	225	A	**G**	A	53	G	G	G
	530	C	**T**	C	155	A	**V**	A
	628	C	**A**	C	188	H	**S**	H
	629	A	**G**	A	188	H	**S**	H
	676	T	**C**	T	204	S	**P**	S
	788	T	**C**	T	241	F	**S**	F
	829	G	**A**	**A**	255	A	**T**	**T**
	994	A	A	**C**	310	I	I	**L**
	1007	G	**A**	G	314	S	**N**	S
	1300	A	**G**	A	412	M	**V**	M
	1428	T	**C**	T	454	C	C	C
	1739	T	**C**	T	558	F	**S**	F
F	1300	C	**T**	C	369	F	F	F
	1546	A	**G**	**G**	451	Q	Q	Q

Figure 3.5: Schematic presentation of the cloning strategy for the introduction of the mci-2, CPI+ and CPI- V ORF mutations in the wild-type (W3) V/P gene.

In all three cases, the V/P gene was cloned by RT-PCR from infected cells and digested with *Bam*HI, in order to release the portion of the gene that contains mutations affecting the V ORF (grey region). The resulting *Bam*HI-*Bam*HI fragment (370 bp in length) was then inserted in the W3 V/P gene, which had been digested similarly to release the equivalent (wild-type) *Bam*HI-*Bam*HI fragment.

In the diagram, silent mutations are shown with orange lines, whereas blue lines indicate mutations that lead to amino acid substitutions. To produce the CPI+/CPI- V single-mutant constructs, each of the three mutations that were identified exclusively in CPI- was introduced into the CPI+ V/P construct, using site-directed mutagenesis. Three single-mutants were thus produced, containing the

a) nt136 [a.a.26],
b) nt209 [a.a.50], or
c) nt365 [a.a.102] mutation.

In a second round of mutagenesis, double-mutants were similarly produced, by introducing an additional (of the three) mutations into the constructs obtained from the first mutagenesis step. Thus, three double-mutant CPI+/CPI- constructs were produced, which contained the

d) nt136/209 [a.a.26/50],
e) nt209/365 [a.a.50/102], or
f) nt136/365 [a.a.26/102] combination of mutations.

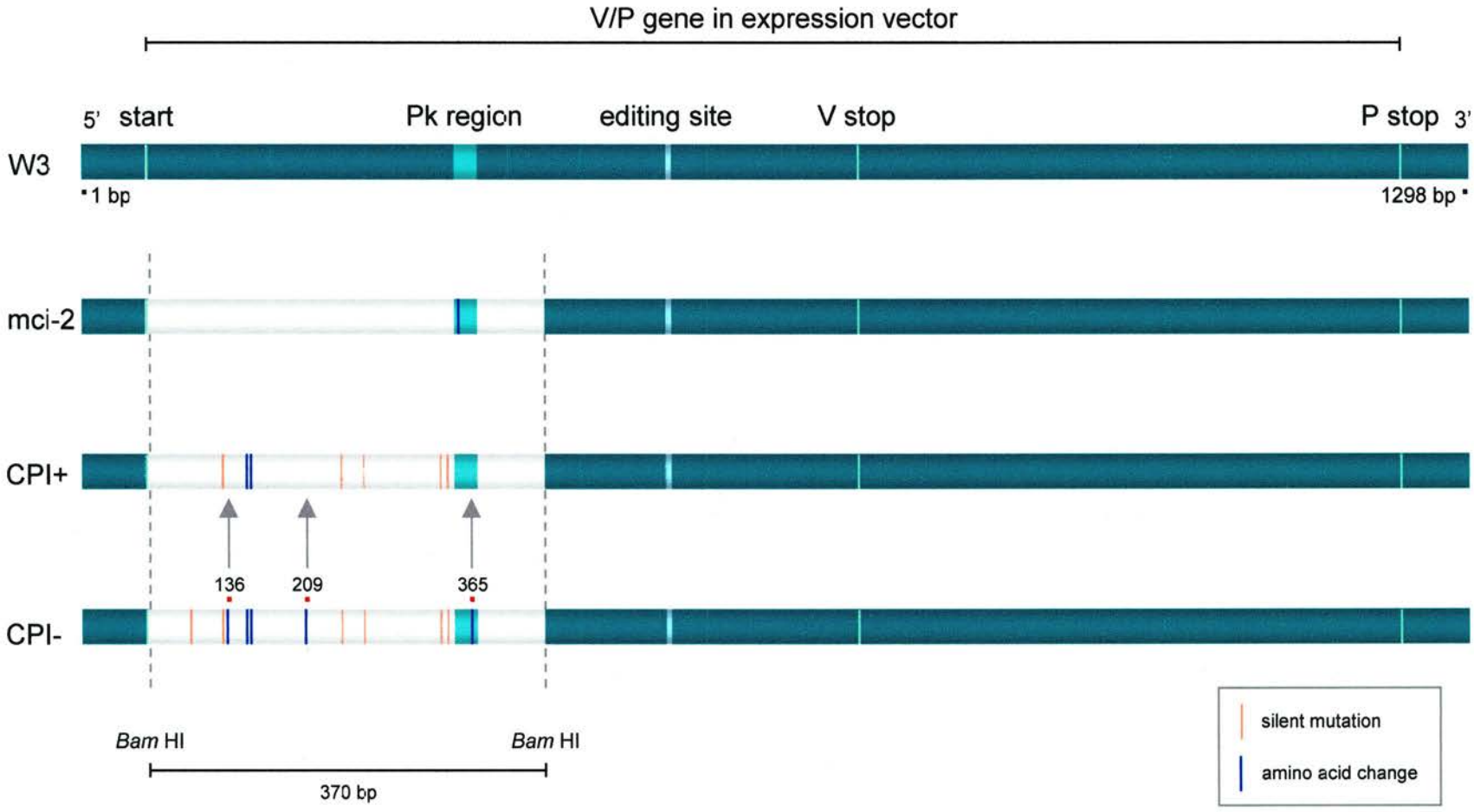

V/P gene in expression vector
5' start
Pk region
editing site
V stop
P stop 3'
W3
1 bp
1298 bp
mci-2
CPI+
136
209
365
CPI-
Bam HI
Bam HI
370 bp
silent mutation
amino acid change

Fig. 3.5

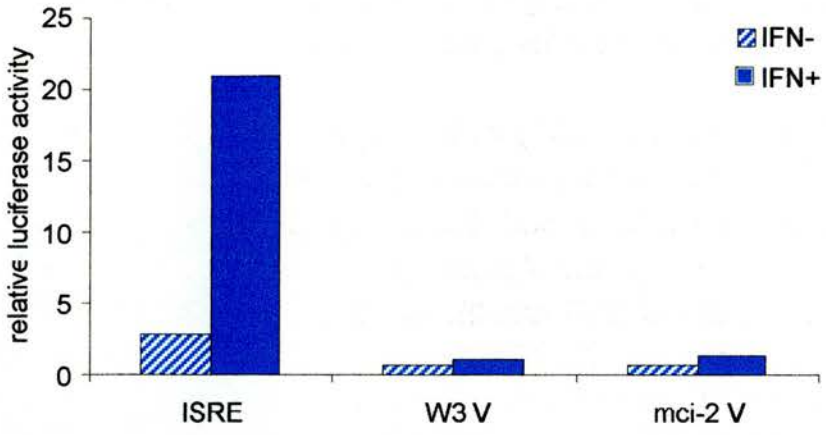
Figure 3.6: Mci-2 V blocks IFN signalling in murine cells, in addition to blocking IFN signalling in human and canine cells as W3 V.

Human (2fTGH) and murine (BF) cells (Panels A and B, respectively) were transfected (lipid-based method) with 0.1 μg of pJATlacz, 0.1 μg of the IFN-α/β-responsive plasmid (pISRE) and 0.3 μg of pEFplink2 (control plasmid), pEF.SV5-V/wt (that encodes the V protein of SV5 W3) or pEF.SV5-V/mci-2 (that encodes the V protein of SV5 mci-2). At 40 h post-transfection, the culture medium was supplemented with IFN (IFN+) or left untreated (IFN-). Four hours later, the cells were lysed and luciferase activity was measured (relative light units, RLU) and normalised to β-galactosidase activity ($OD_{420\ nm}$ units).

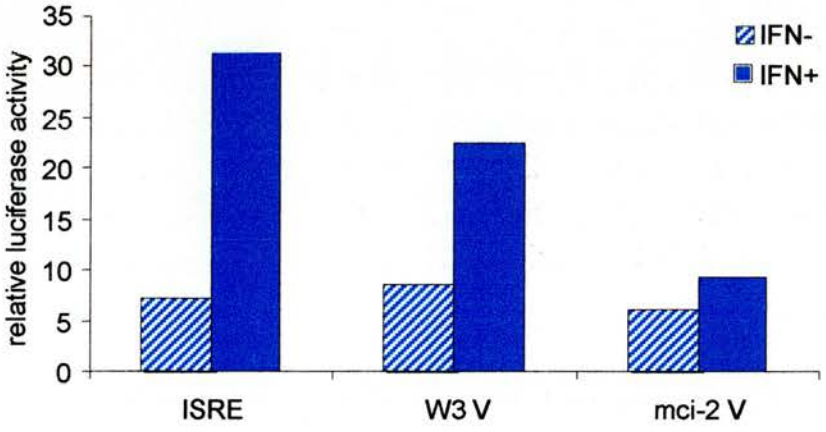
Canine (Dog134) cells (Panel C) were transfected (electroporation) with 2 μg of pJATlacz, 2 μg of the IFN-α/β-responsive plasmid (pISRE) and 6 μg of the pEFplink2 (control plasmid), pEF.SV5-V/wt (that encodes the V protein of SV5 W3) or pEF.SV5-V/mci-2 (that encodes the V protein of SV5 mci-2). At 16 h post-transfection, the culture medium was supplemented with IFN (IFN+) or left untreated (IFN-). Four hours later, the cells were lysed and luciferase activity was measured (relative light units, RLU) and normalised to β-galactosidase activity ($OD_{420\ nm}$ units).

These are representative results from a set of independently repeated experiments with equivalent findings.

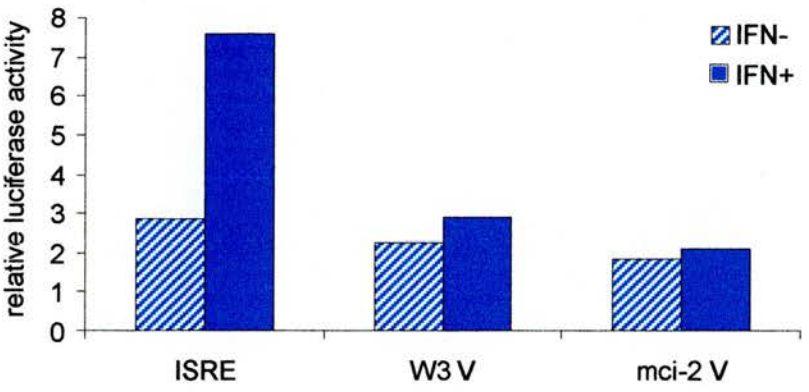
A) Human cells



B) Murine cells



C) Canine cells



demonstrated that mci-2 V blocks IFN signalling in murine cells, while retaining its efficiency of function in human and canine cells.

3.1.2.4 A recombinant SV5 virus that has only the $N_{100}D$ mutation in the V/P gene can also overcome the IFN response and proliferate in murine cells without losing its ability to block IFN signalling in human cells

The recombinant virus

Having confirmed that the $N_{100}D$ mutation enables the V protein of mci-2 to block signalling in murine cells, it was of interest to study the effect of this mutation alone on the biological properties of the wild-type virus (mci-2 was also found to have mutations in the examined HN and F genes, which could possibly contribute to its murine cell-adapted phenotype). For this reason, a plasmid DNA construct containing the $N_{100}D$ substitution in a W3 V/P backbone was sent to Prof. R.A. Lamb's research group (Young *et al.*, 2001) who, using reverse genetics, introduced it into wild-type SV5 (W3), thus producing a recombinant virus termed rSV5-V/P $N_{100}D$ (abbreviated to $rSV5_{N>D}$).

rSV5 blocks IFN signalling in murine cells

In order to test the ability of this recombinant virus to block IFN signalling in murine cells, BF cells were transfected with the type I IFN-responsive plasmid (pISRE) and subsequently (24 h post-transfection) infected with $rSV5_{N>D}$, mci-2 or W3. At 68 h post-transfection the culture medium was supplemented with exogenous IFN or left untreated, and 4 hours later the relative activation of the type I IFN reporter (luciferase) gene was estimated by measuring the luciferase activity in the cell lysates. Evidently, as presented in Figure 3.7, the results from this reporter assay experiment showed that, in murine cells, $rSV5_{N>D}$ blocks IFN signalling as efficiently as mci-2. Thus, a single amino acid substitution was shown to confer on wild-type SV5 the ability to block IFN signalling in a new species. In contrast, no prevention of ISRE promoter activation was observed in cells infected with W3, which again agreed with previous results (Didcock *et al.*, 1999b).

rSV5 still blocks IFN signalling in human cells

Since the $N_{100}D$ mutation enabled SV5 to block signalling in murine cells, another reporter assay experiment was performed, in order to determine whether the mutation affected the ability of the virus to block signalling in human cells. Human (2fTGH) and murine (BF) cells were transfected with the type I IFN-responsive reporter plasmid and

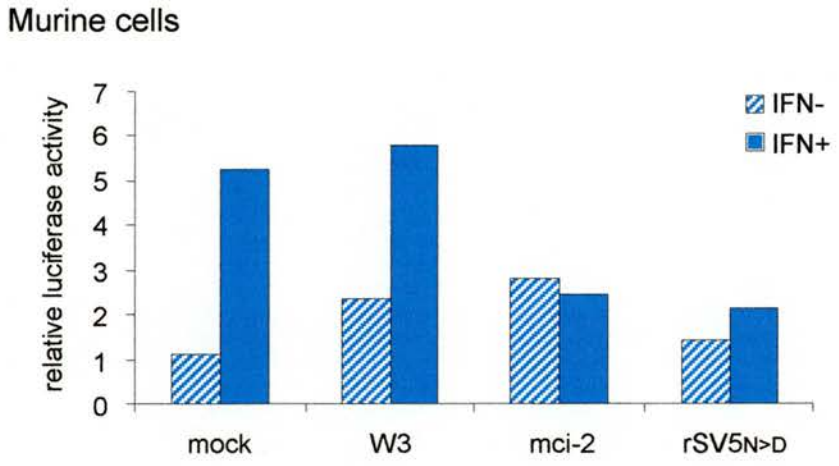


Figure 3.7: A single amino acid substitution ($N_{100}D$) in the V protein of SV5 enables the virus to block IFN signalling in murine cells.

Murine (BF) cells were transfected (lipid-based method) with control plasmids (pJATlacz, 0.02 μg; pUC13, 0.06 μg) and the IFN-α/β-responsive plasmid (pISRE, 0.02 μg). At 24 h post-transfection, the cells were infected with W3, mci-2, $rSV5_{N>D}$ (the recombinant virus containing the $N_{100}D$ mutation), or mock-infected. At 68 h post-transfection, the culture medium was supplemented with IFN (IFN+) or left untreated (IFN-). Four hours later, cellular lysates were assayed for luciferase activity (measured in relative light units, RLU), which was normalised to β-galactosidase activity ($OD_{420\ nm}$ units). These are representative results from a set of independently repeated experiments with equivalent findings.

at 24 h post-transfection the cells were infected with $rSV5_{N>D}$ or W3. At 44 h or 68 h post-transfection (different transfection methods were used for human or murine cells, respectively) the culture medium was supplemented with IFN, or left untreated, and the cells were lysed 4 hours later to measure reporter gene activity levels. As illustrated in Figure 3.8, the results from this analysis showed that $rSV5_{N>D}$ blocks IFN signalling efficiently in both human and murine cells (Panels A and B, respectively), in contrast to W3 that blocks signalling in human (Panel A) but not murine cells (Panel B). Thus, in full agreement with the mci-2 V analysis described in the previous section, this experiment demonstrated that the single $N_{100}D$ substitution in V is sufficient to enable wild-type SV5 to disrupt IFN signalling in murine cells, while not affecting its ability to block signalling in human cells.

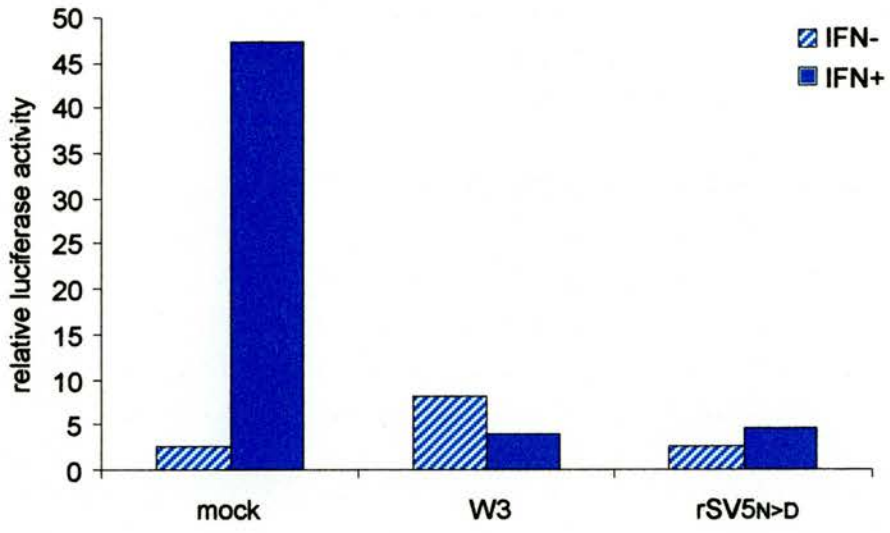
Figure 3.8: The recombinant virus that contains the $N_{100}D$ mutation ($rSV5_{N>D}$) blocks IFN signalling efficiently in murine cells, while retaining its ability to block IFN signalling in human cells, as does W3.

Human (2fTGH) cells were transfected (electroporation) with control plasmids (pJATlacz, 0.1 μg; pEFplink2, 0.3 μg) and the IFN-α/β-responsive plasmid (pISRE, 0.1 μg). At 24 h post-transfection, the cells were infected with either W3 or $rSV5_{N>D}$. At 44 h post-transfection, the culture medium was supplemented with IFN (IFN+) or left untreated (IFN-). Four hours later, cellular lysates were assayed for luciferase activity (measured in relative light units, RLU), which was normalised to β-galactosidase activity ($OD_{420\ nm}$ units).

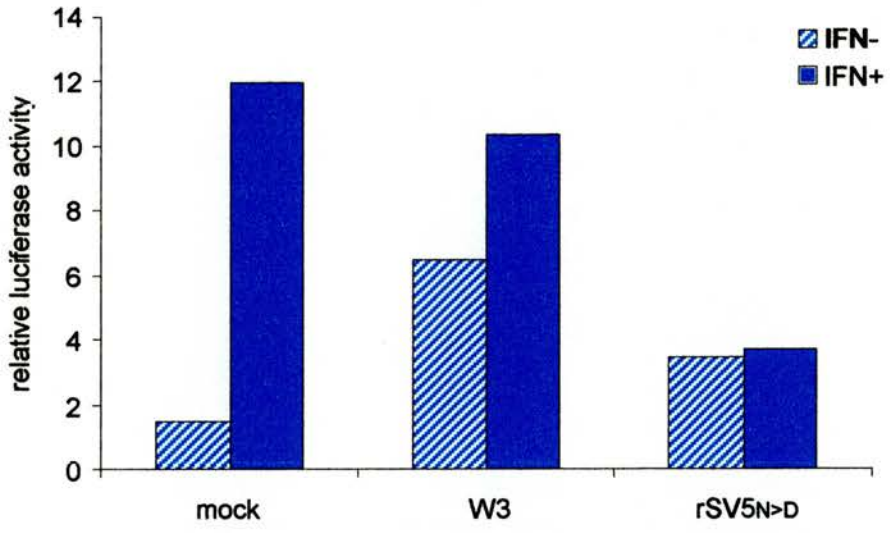
Murine (BF) cells were transfected (lipid-based method) with control plasmids (pJATlacz, 0.02 μg; pEFplink2, 0.06 μg) and the IFN-α/β-responsive plasmid (pISRE, 0.02 μg). At 24 h post-transfection, the cells were infected with either W3 or $rSV5_{N>D}$. At 68 h post-transfection, the culture medium was supplemented with IFN (IFN+) or left untreated (IFN-). Four hours later, cellular lysates were assayed for luciferase activity (measured in relative light units, RLU), which was normalised to β-galactosidase activity ($OD_{420\ nm}$ units).

These are representative results from a set of independently repeated experiments with equivalent findings.

A) Human cells



B) Murine cells



3.2 SV5 CPI+ and CPI-: Two closely related isolates with distinct biological properties

3.2.1 CPI+ and CPI- differ in their ability to degrade STAT1

3.2.1.1 Observations on STAT1 degradation

Why CPI+ and CPI-

CPI+ and CPI- are two closely related canine SV5 isolates, with CPI- having been derived experimentally from CPI+, as described in Chapter 1. Nevertheless, these viruses have distinct phenotypic characteristics and, using immunofluorescence analysis, they can be distinguished easily *in vitro* by the difference in their ability to bind the anti-Pk mAb. Thus, of the two isolates, only CPI+ is recognised by the anti-Pk mAb, whereas CPI- does not bind it. As presented in the previous section, a single amino acid substitution in the Pk epitope of W3 ($rSV5_{N>D}$ virus) is sufficient to alter dramatically the ability of the virus to block IFN signalling in human and murine cells. Consequently, it was also of interest to compare the ability of CPI+ and CPI- to degrade STAT1 in human, canine and murine cells, in order to investigate whether the difference(s) in the Pk epitope of these isolates had any effect on their ability to block IFN signalling.

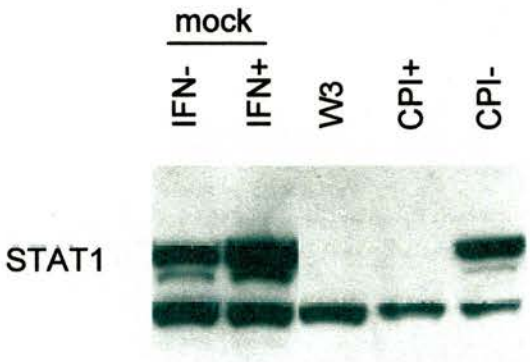
STAT1 levels in infected cells

STAT1 levels were examined in human (2fTGH), canine (Madin-Darby canine kidney; MDCK) and murine (BF) cells that had been infected with CPI+ or CPI- at a high m.o.i. Cell proteins were analysed by SDS-PAGE, followed by immunoblot analysis using polyclonal anti-STAT1 antibody. This analysis was performed by Dan Young (Chatziandreou *et al.*, 2002). As shown in Figure 3.9 (Panel A and B), CPI+ degrades STAT1 as efficiently as W3 in both human and canine cells, in contrast to CPI- that evidently fails to degrade STAT1 in cells of human and canine origin. However, when the immunoblot analysis was performed in murine cells (Figure 3.9, Panel C), it was revealed that neither of the two isolates induce the degradation of STAT1 in cells of murine origin. The degradation of STAT1 by CPI+ but not CPI- in canine cells was further confirmed at three different time points following infection (24, 48 and 72 h p.i.), as illustrated in Figure 3.10. Furthermore, in all three cell lines, infection with CPI- upregulated the levels of STAT1, which is known to be induced in response to virus infection, whilst the same effect was also observed in murine cells infected with CPI+, as with W3 (see previous section).

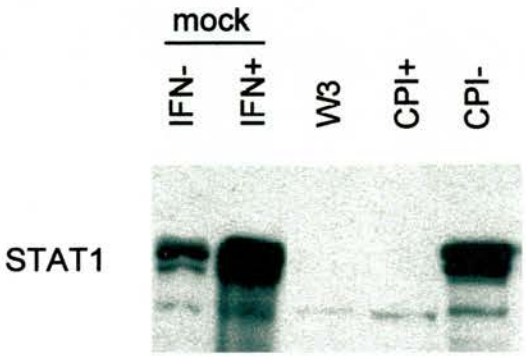
Figure 3.9: CPI+ induces the degradation of STAT1 in human and canine cells but not in murine cells, behaving like W3. CPI- does not degrade STAT1 in any of these cells.

Human (2fTGH), canine (MDCK) and murine (BF) cells were infected with CPI+ or CPI- at a high m.o.i. At 24 h p.i. the cells were lysed and cellular proteins were separated by SDS-PAGE. Immunoblot analysis was subsequently performed using polyclonal anti-STAT1 antibody.

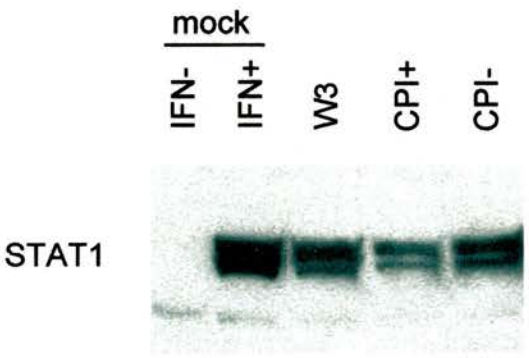
A) Human cells



B) Canine cells



C) Murine cells



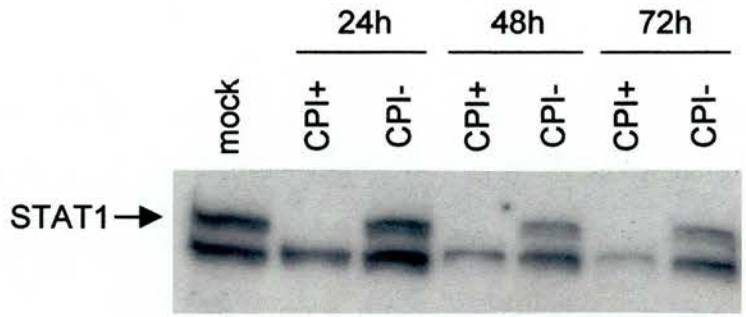


Figure 3.10: CPI+ induces the degradation of STAT1 in canine cells, whereas CPI- does not, as confirmed at 24, 48 and 72 h p.i.

Canine (MDCK) cells were infected at a high m.o.i. with CPI+ or CPI- and lysed at 24, 48 or 72 h p.i. Cellular proteins were analysed by SDS-PAGE and immunoblotted using anti-STAT1 pAb.

3.2.1.2 Examination of the V/P gene sequences

Sequence data of the V/P gene

The nucleotide sequence of the V/P genes of CPI+ and CPI- has been previously determined (Southern *et al.*, 1991). Nevertheless, since it was necessary to re-sequence parts of these genes in the laboratory, sequence data for CPI+ and CPI- V were re-examined. Thus, data analysis showed that, compared to W3, CPI+ has three amino acid differences that map to the V/P common amino-terminal domain and two substitutions that affect the unique carboxy-terminal domain of the P protein. In addition to these five amino acid substitutions, CPI- has an additional three amino acid changes in the V/P common amino-terminal domain. These additional mutations are tyrosine to histidine at residue 26 ($Y_{26}H$) and leucine to proline at residues 50 and 102 (designated $L_{50}P$ and $L_{102}P$, respectively). A schematic overview of the position and effect of the mutations in the V/P gene of CPI+ and CPI is shown in Figure 3.11, and complete nucleotide and amino acid residue information about the illustrated mutations is presented in Table 3.3.

The results obtained from this sequence analysis partially contradicted the published data (Southern *et al.*, 1991), as follows:

- There is not an A to G nucleotide change in CPI+ or CPI- at position 179 of the SV5PVA locus, GenBank accession no. J03142 (Southern *et al.*, 1991, presented in Fig.2, position 145)

- There is not a predicted K to R amino acid substitution in the V/P common amino-terminal domain at position 57 (SWISS-PROT primary accession no. P11208) (Southern *et al.*, 1991, presented in Fig.3)

- There is not a predicted T to K amino acid substitution affecting the P protein at position 293 (SWISS-PROT primary accession no. P11208) (Southern *et al.*, 1991, Fig.3), as there is not a corresponding nucleotide change in Fig.2.

3.2.2 CPI+ and CPI- differ in their ability to block IFN signalling

CPI+ vs. CPI-

As presented above, sequence analysis showed that CPI- V differs from that of CPI+ at three amino acid residue positions, all of which map to the amino-terminal domain of the protein. In an effort to establish the significance of these substitutions and confirm

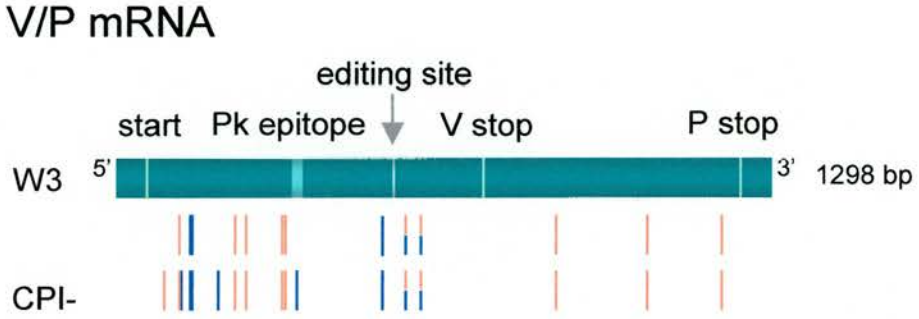


Figure 3.11: Gene map showing the relative position of the mutations identified in the V/P gene of CPI+ and CPI-.

The entire mRNA is represented schematically by the green band and mutations present in CPI+ and CPI- are marked below, with blue lines representing amino acid changes and orange lines indicating silent mutations. Mutations that are silent in the V ORF but affect codons in the P ORF are shown in orange and blue (|). Annotation on the mRNA reveals the relative position of the mutations with regard to the V and P ORFs and the Pk epitope.

Table 3.3: Nucleotide and amino acid differences between the V/P genes and proteins of W3, CPI+ and CPI-.

Nucleotide and amino acid changes are shown in black, whilst grey letters indicate silent mutations. Note that nucleotide changes at positions 579 and 609 affect the two ORFs of the gene differently, as they are silent in the V ORF (grey) but result in amino acid substitutions in the P ORF (black). Position numbers refer to the nucleotide residue information available for the V/P gene of SV5 W3 (SV5PVA locus, GenBank Accession no. J03142).

nucleotides				amino acids			
position	W3	CPI+	CPI-	W3	CPI+	CPI-	position
102	T	T	**C**	N	N	N	14
132	A	**G**	**G**	V	V	V	24
136	T	T	**C**	Y	Y	**H**	26
154	G	**A**	**A**	V	**I**	**I**	32
158	C	**T**	**T**	T	**I**	**I**	33
209	T	T	**C**	L	L	**P**	50
243	G	**A**	**A**	E	E	E	61
264	C	**T**	**T**	G	G	G	68
336	A	**G**	**G**	T	T	T	92
342	C	**T**	**T**	P	P	P	94
365	T	T	**C**	L	L	**P**	102
530	C	**T**	**T**	S	**F**	**F**	157
579	G	**A**	**A**	R/G	R/**E**	R/**E**	173/4
609	A	**G**	**G**	E/K	E/**R**	E/**R**	183/4
877	T	**G**	**G**	T	T	T	273
1057	T	**C**	**C**	I	I	I	333
1204	C	**T**	**T**	D	D	D	382

that they are responsible for the difference in the ability of these isolates to degrade STAT1 in different host species, the ability of CPI+ and CPI- to block IFN signalling in human, canine and murine cells was examined in a reporter (luciferase) assay experiment.

Outline of experiment

To prepare the appropriate test constructs for this assay, the portions of the genes containing the CPI+ or CPI- amino acid substitutions were introduced into the W3 V/P gene, which had been previously engineered into the constitutive eukaryotic expression vector pEF-plink2 (see Chapter 2). A schematic overview of this cloning strategy is presented in Figure 3.5. The plasmid constructs pEF.W3/CPI+/V and pEF.W3/CPI-/V were thus produced and used to express the V proteins of CPI+ and CPI- in mammalian cells. Human (2fTGH), canine (Dog134) and murine (BF) cells were co-transfected with either one of the CPI plasmids or pEF.SV5-V that expresses W3 V, together with a type I IFN-responsive luciferase reporter plasmid (pISRE). At 16 h post-transfection, the cells were treated with type I IFN and lysed 4 hours later to measure relative luciferase activity.

CPI+ blocks IFN signalling, CPI- does not

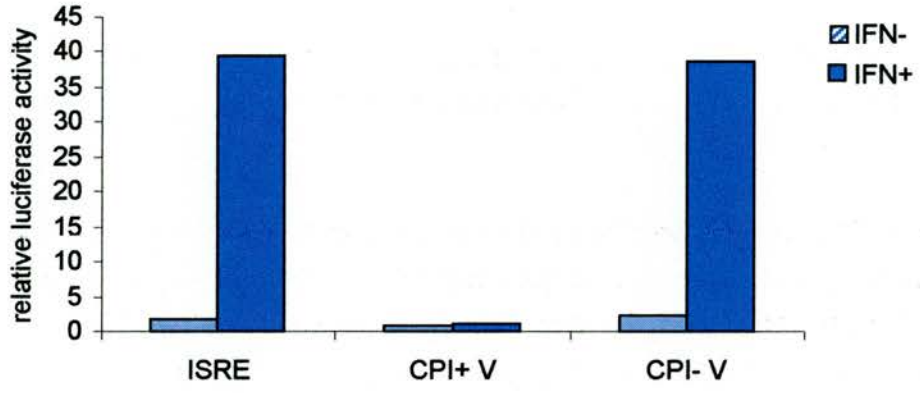
Consistent with the STAT1 degradation data presented in the previous section, the results from this experiment revealed that there are major differences in the ability of CPI+ and CPI- to block IFN signalling, as presented in Figure 3.12. CPI+ can efficiently block IFN signalling in human and canine cells, as shown in Panels A and B respectively, in contrast to CPI- that lacks this ability. Nevertheless, both isolates failed to block signalling in murine cells, as shown in Panel C. Thus, in conjunction with the STAT1 degradation observations, this experiment confirmed that CPI+ behaves like W3, blocking STAT1-mediated IFN signalling in human and canine cells but not in murine cells, while CPI- does not have the ability to block signalling in any of these cell lines.

Figure 3.12: CPI+ V blocks IFN signalling in human and canine cells but not in murine cells, resembling W3. CPI- does not block IFN signalling in any of these cells.

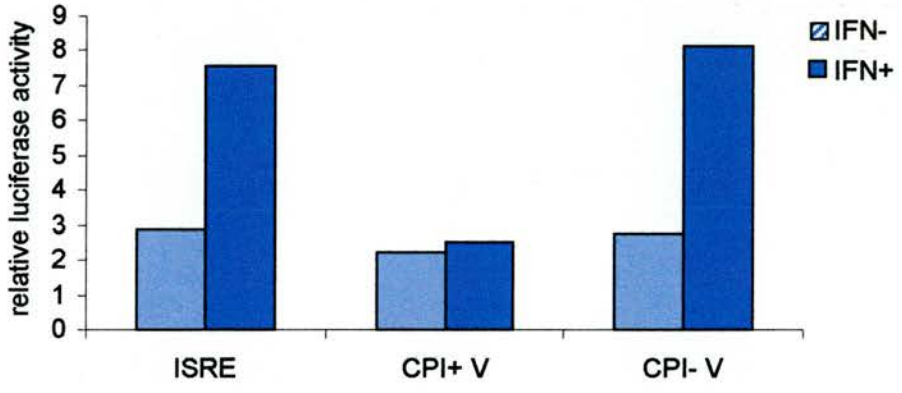
Human (2fTGH), canine (Dog134) and murine (BF) cells (Panels A, B and C, respectively) were transfected (electroporation) with 2 μg of the β-gal reporter plasmid (pJATlacz), 2 μg of the IFN-α/β-responsive plasmid (pISRE) and 6 μg of either the control plasmid (pEFplink2 vector), the CPI+ V-encoding construct (pEF.SV5-V/CPI+) or the CPI- V-encoding construct (pEF.SV5-V/CPI-). At 16 h post-transfection, the culture medium was supplemented with IFN (IFN+) or left untreated (IFN-). Four hours later, the cells were lysed and luciferase activity was measured (relative light units, RLU) and normalised to β-galactosidase activity ($OD_{420\ nm}$ units).

These are representative results from a set of independently repeated experiments with equivalent findings.

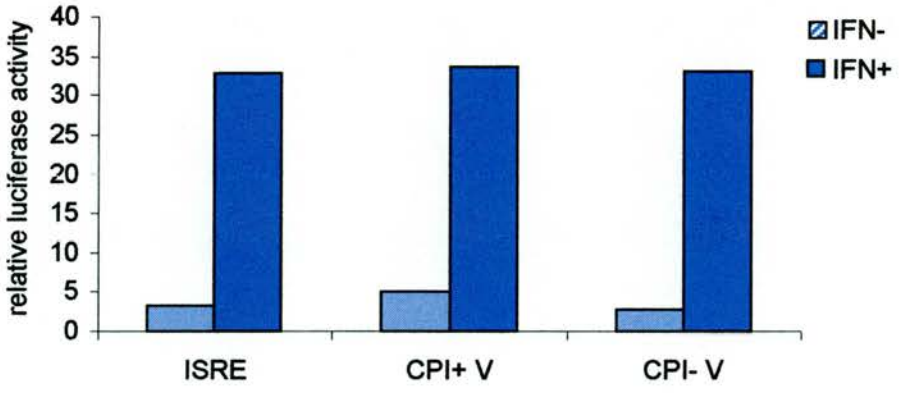
A) Human cells



B) Canine cells



C) Murine cells



3.2.3 Effect of the three amino acid residue differences between the V proteins of CPI+ and CPI- on their ability to block IFN signalling

3.2.3.1 Construction and examination of single mutants

Strategy for single mutants

To determine the effect of the three mutations found in the amino-terminal domain of the V protein of CPI- on its ability to block IFN signalling, a set of V mutants was produced. By site-directed mutagenesis, each of the three substitutions ($Y_{26}H$, $L_{50}P$ and $L_{102}P$) were introduced into the CPI+ V plasmid construct (pEF.W3/CPI+/V). Thereby, the plasmid constructs pEF.W3/CPI+/V-($Y_{26}H$), pEF.W3/CPI+/V-($L_{50}P$) and pEF.W3/CPI+/V-($L_{102}P$) were produced, which encoded the CPI+ V protein with the $Y_{26}H$, $L_{50}P$ and $L_{102}P$ substitution, respectively. A schematic overview of the plasmid construction strategy is provided in Figure 3.5.

$L_{50}P$ is important

In order to examine the ability of the mutant V proteins to block IFN signalling, human (2fTGH) cells were co-transfected with one of the prepared plasmid constructs or either one of the control plasmids (pEF.W3/CPI+/V and pEF.W3/CPI-/V), together with a type I IFN-responsive luciferase reporter plasmid (pISRE). At 18 h post-transfection, luciferase expression was induced by treatment with IFN. Cells were lysed 4 hours later and relative luciferase activity was measured and normalised to β-galactosidase activity. As shown in Figure 3.13, of the three mutations, only the $L_{50}P$ substitution had a major effect on the function of CPI+ V, as it reduced significantly the ability of the protein to block IFN signalling. Nevertheless, this effect was partial and, compared to the control protein (CPI- V), IFN signalling remained at relatively low levels reproducibly, indicating that $L_{50}P$ alone was not sufficient to eliminate totally the function of the protein. Mutation $L_{102}P$ was found to have a small effect, whereas mutation $Y_{26}H$ had no effect whatsoever.

3.2.3.2 Construction and examination of double mutants

Construction of double mutants

To determine further the effect of the three CPI- V amino acid substitutions $Y_{26}H$, $L_{50}P$ and $L_{102}P$ on the ability of the protein to block IFN signalling, another set of V mutants was produced. Using the single-mutant plasmid constructs (described above) as

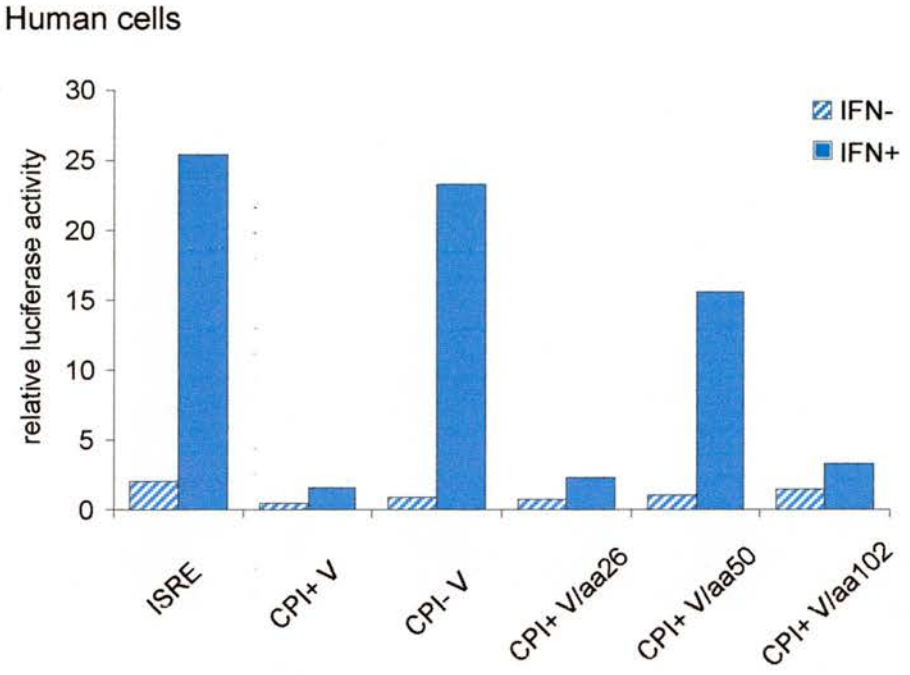


Figure 3.13: Analysis of the CPI+/CPI- single-mutant V proteins (each containing one of the three amino acid substitutions found exclusively in CPI- V; $Y_{26}H$, $L_{50}P$ and $L_{102}P$) reveals that mutation $L_{50}P$ has the greatest effect on the function of V.

Human (2fTGH) cells were transfected (electroporation) with 2 μg of the β-gal reporter plasmid (pJATlacz), 2 μg of the IFN-α/β-responsive plasmid (pISRE) and 6 μg of either the control plasmid (pEFplink2 vector) or the appropriate test plasmid (pEF.SV5-V/CPI/x) encoding CPI+ V, CPI- V or the CPI+/CPI- mutant V proteins with substitutions at amino acid position 26, 50, 102 (single-mutants). At 16 h post-transfection, the culture medium was supplemented with IFN (IFN+) or left untreated (IFN-). Four hours later, the cells were lysed and luciferase activity was measured (relative light units, RLU) and normalised to β-galactosidase activity ($OD_{420\ nm}$ units). These are representative results from a set of independently repeated experiments with equivalent findings.

templates in a further step of site-directed mutagenesis, the mutations were combined pairwise in CPI+ V, giving rise to three constructs that contained substitutions $Y_{26}H$ and $L_{50}P$ [termed pEF.W3/CPI+/V-($Y_{26}H/L_{50}P$)], $L_{50}P$ and $L_{102}P$ [pEF.W3/CPI+/V-($L_{50}P/L_{102}P$)] and $Y_{26}H/L_{102}P$ [pEF.W3/CPI+/V-($Y_{26}H/L_{102}P$)]. To test the properties of these double mutants, human (2fTGH) cells were co-transfected with one of the prepared plasmid constructs or either one of the control plasmids (encoding CPI+ or CPI- V), together with a type I IFN-responsive luciferase reporter plasmid (pISRE). At 44 h post-transfection, the cells were induced with type I IFN and 4 hours later the cells were lysed and the relative luciferase activity was measured.

$L_{50}P$ is required for blocking

This analysis revealed that, in confirmation of the observations from the single mutants, it is the $L_{50}P$ substitution that affects the function of CPI+ V to the greatest extent. As presented in Figure 3.14, in constructs where $L_{50}P$ was present, i.e. mutant proteins CPI+V/aa26/50 and CPI+V/aa50/102, a significant decrease in the ability of CPI+ V to block signalling was observed, with the latter having the greatest effect. Therefore, of the three double mutants, it was the combination of $L_{50}P$ and $L_{102}P$ (the latter mapping to the Pk epitope) that had the most detrimental effect on the function of V. However, this combination was not sufficient to abolish fully the ability of the protein to block IFN signalling, compared to CPI- V. The significance of the $L_{50}P$ substitution was also confirmed by the CPI+V/aa26/102 mutant (i.e. CPI- V with residue 50 changed back to the wild-type residue), which blocked signalling efficiently, despite the $Y_{26}H$ and $L_{102}P$ substitutions.

3.2.3.3 Overview of results from the mutants

Synergistic effect

In conclusion, mutational analysis of the amino-terminal domain of CPI+ revealed that, on their own, none of the three amino substitutions exclusively found in CPI- V can fully abolish the function of the protein. Amino acid substitution at residue position 50 appeared to be the most critical mutation, nevertheless failing to confer the full phenotype of CPI- V on CPI+ V, even when combined with either of the other two substitutions. This analysis therefore made clear that all three CPI- V unique amino acid substitutions are required for blocking of IFN signalling to be eliminated completely.

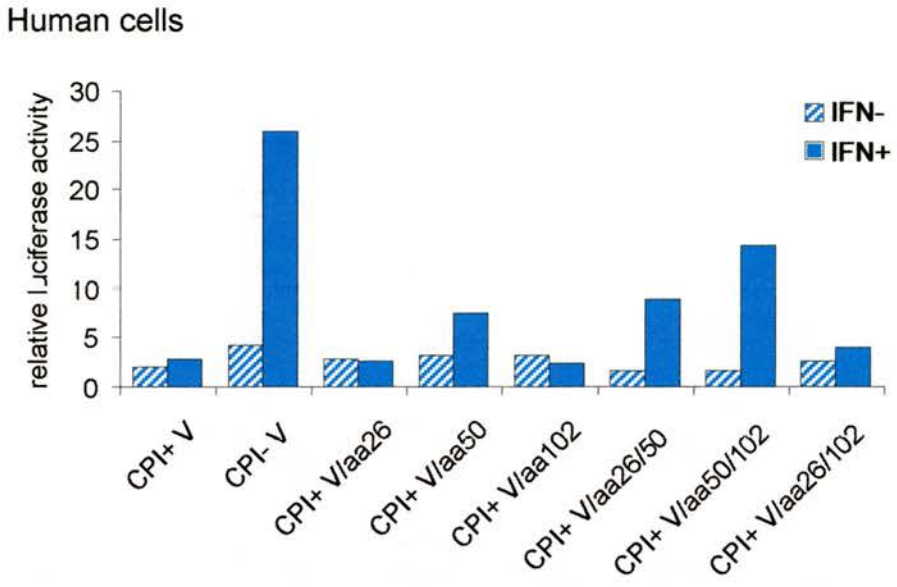


Figure 3.14: Analysis of the CPI+/CPI- double-mutant V proteins (containing two of the three amino acid substitutions found exclusively in CPI- V [$Y_{26}H$, $L_{50}P$ and $L_{102}P$] in different combinations) confirms that, although mutation $L_{50}P$ has the greatest effect on the function of V, the lack of ability of CPI- V to block IFN signalling is due to the synergistic effect of all three mutations.

Human (2fTGH) cells were transfected (lipid-based method) with 0.4 μg of the β-gal reporter plasmid (pJATlacz), 0.4 μg of the IFN-α/β-responsive plasmid (pISRE) and 1.2 μg of either the control plasmid (pEFplink2 vector) or the appropriate test construct (pEF.SV5-V/CPI/x). The test constructs encode CPI+ V, CPI- V or the CPI+/CPI- mutant V proteins with substitutions at amino acid position 26, 50, 102 (single-mutants) or their combinations 26/50, 50/102, 26/102 (double-mutants). At 44 h post-transfection, the culture medium was supplemented with IFN (IFN+) or left untreated (IFN-). Four hours later, the cells were lysed and luciferase activity was measured (relative light units, RLU) and normalised to β-galactosidase activity ($OD_{420\ nm}$ units). These are representative results from a set of independently repeated experiments with equivalent findings.

3.2.4 Viral protein synthesis levels of CPI+ and CPI- in cells that produce and respond to IFN

Having established that CPI+ blocks IFN signalling whereas CPI- does not, due to its failure to degrade STAT1, it was of interest to compare the efficiency of replication of these viruses under the effect of IFN. For this reason, the protein synthesis profiles of the two isolates were examined in canine (MDCK) cells, which are known to produce and respond to IFN upon viral infection. Cells were infected with CPI+ or CPI- at a high m.o.i. and metabolically labelled with [^{35}S]-methionine at 10 to 12 h, 16 to 18 h, 22 to 24 h, and 46 to 48 h p.i. At the end of the radiolabelling incubation period, the cells were lysed and the HN, NP, F, P and M proteins were immune precipitated with a mixture of appropriate mAbs. The immune precipitates and a portion of the total cell extracts (sampled before the immune precipitation process) were subsequently analysed by SDS-PAGE and the profiles of the radiolabelled proteins were visualised by phosphorimage analysis, the results of which are presented in Figure 3.15. At 12 and 48 h p.i. the profiles for CPI+ and CPI- were very similar, however, at 18 and 24 h p.i. there was a small but significant difference between the two isolates. Specifically, NP levels were higher in CPI+ than in CPI- infected cells (Panel A), in addition to an evident difference in the expression levels of surface glycoproteins HN and F (Panel B). Similarly, there was a difference in the expression levels of the M protein, but to a smaller extent. Overall, the results from this analysis indicated that at 18 and 24 h p.i. levels of viral protein synthesis were higher in cells infected with CPI+ than in those infected with CPI-.

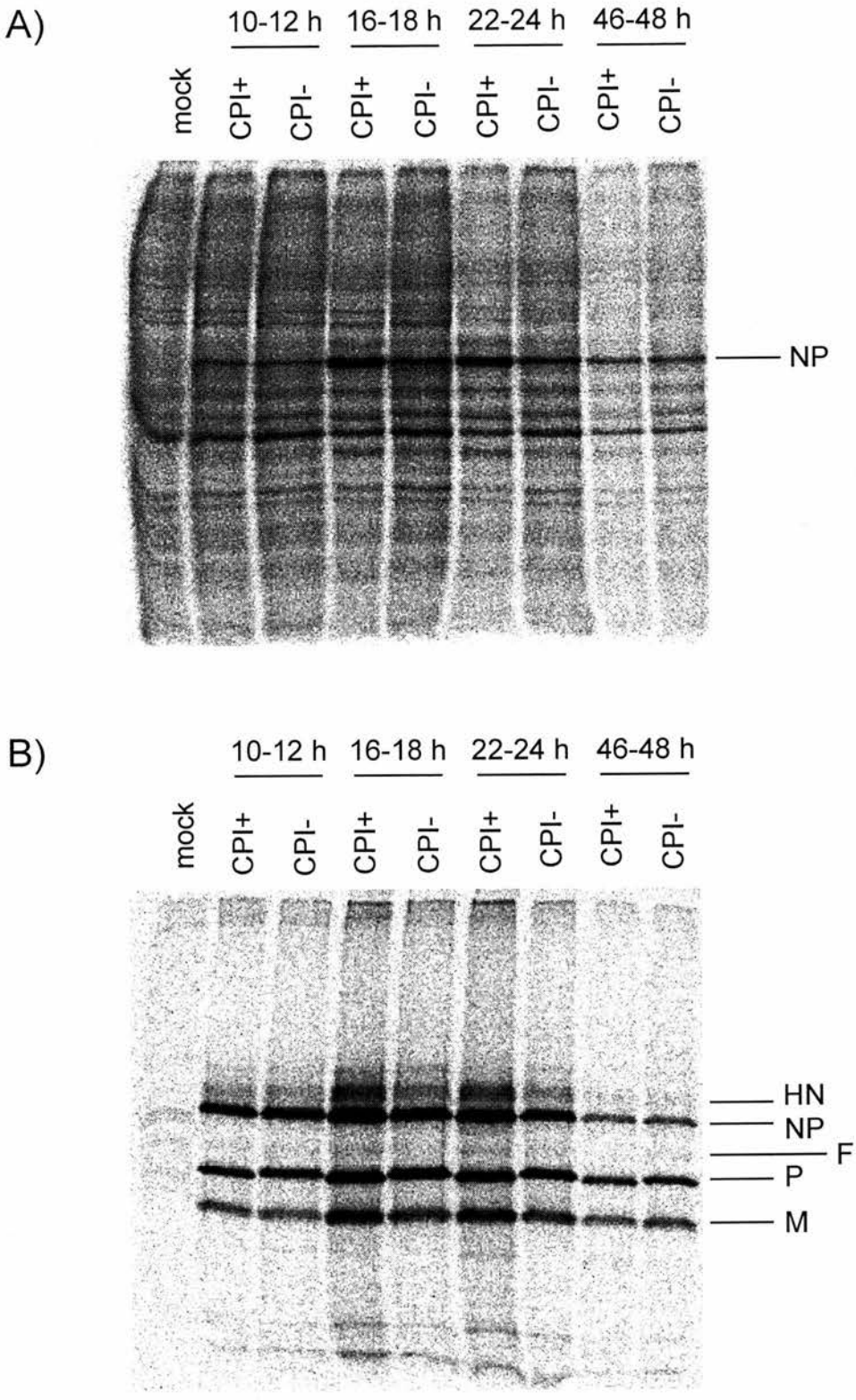
3.2.5 Differences in the ability of CPI+ and CPI- to spread within a cell monolayer

Given that CPI- does not block IFN signalling and that its viral protein synthesis levels are reduced by the IFN response of the host, in contrast to CPI+ that blocks signalling and can sustain high levels of protein synthesis, it was of interest to compare the ability of these isolates to spread in a cell monolayer in the presence of IFN. For this purpose, canine (MDCK) cells were infected with CPI+ or CPI- at a low m.o.i. (0.01 pfu/cell) and the culture medium was supplemented with IFN-α/β (or left untreated) at 12 h p.i. This later time of IFN treatment was to allow a round of replication to be completed.

Figure 3.15: Viral protein synthesis levels at 18 and 24 h p.i. are higher in canine cells infected with CPI+, compared to cells infected with CPI-.

Canine (MDCK) cells were infected with CPI+, CPI- or mock-infected and metabolically labelled with [^{35}S]-methionine from 10 to 12 h, 16 to 18 h, 22 to 24 h, and 46 to 48 h p.i. The cells were lysed at the end of the radioisotope incubation period and the protein content of the total cell extract was analysed by SDS-PAGE (Panel A). Viral polypeptides were also immune-precipitated from the cell extracts using monoclonal antibodies specific for the SV5 HN, NP, F, P and M proteins. The precipitated proteins were subsequently dissociated from the mAbs and analysed by SDS-PAGE (Panel B). Protein profiles were visualised by phosphorimage analysis.

Fig. 3.15


A)
10-12 h
16-18 h
22-24 h
46-48 h
mock
CPI+
CPI-
CPI+
CPI-
CPI+
CPI-
CPI+
CPI-
NP
B)
10-12 h
16-18 h
22-24 h
46-48 h
mock
CPI+
CPI-
CPI+
CPI-
CPI+
CPI-
CPI+
CPI-
HN
NP
F
P
M

The infected monolayers were examined at 24 and 72 h p.i. by immunofluorescence, using anti-NP (SV5-NP-a) and anti-P (SV5-P-e) mAbs.

CPI+ spreads, CPI- is restricted

The results from this analysis, which are illustrated in Figure 3.16, revealed that in canine cells, in the presence of IFN, CPI+ can spread more efficiently, compared to CPI- which is restricted. Thus, although in the absence of IFN both CPI+ and CPI- had infected the entire monolayer by 72 h p.i., in cultures that had been supplemented with exogenous IFN the spread of CPI- was blocked completely. In contrast, CPI+ had infected approximately 30% of the cell population by 72 h p.i., indicating that its spread was delayed but not prevented by the action of IFN.

3.2.6 Distribution patterns of NP and P in cells infected with CPI+ and CPI-

CPI- forms inclusion bodies

In addition to confirming the differences in the ability of CPI+ and CPI- to spread in a cell monolayer, an experiment similar to the one presented in the previous section also revealed that, in the presence of IFN, there are major differences between the distribution patterns of NP and P in cells infected with CPI+ and CPI-. Thus, as shown in Figure 3.17, by 72 h p.i., in the absence of IFN, cells that had been infected with CPI+ or CPI- at a low m.o.i. showed a diffuse distribution of the NP and P proteins throughout the cytoplasm. Noticeably, these proteins were also aggregated in cytoplasmic inclusion bodies, with this type of distribution being more evident in cells infected with CPI-. In striking contrast, in the presence of IFN, CPI- infected cells contained inclusion bodies only, whereas cells infected with CPI+ showed a distribution pattern similar to that observed in the absence of IFN.

3.2.7 Differences in cells persistently infected with CPI+ or CPI-

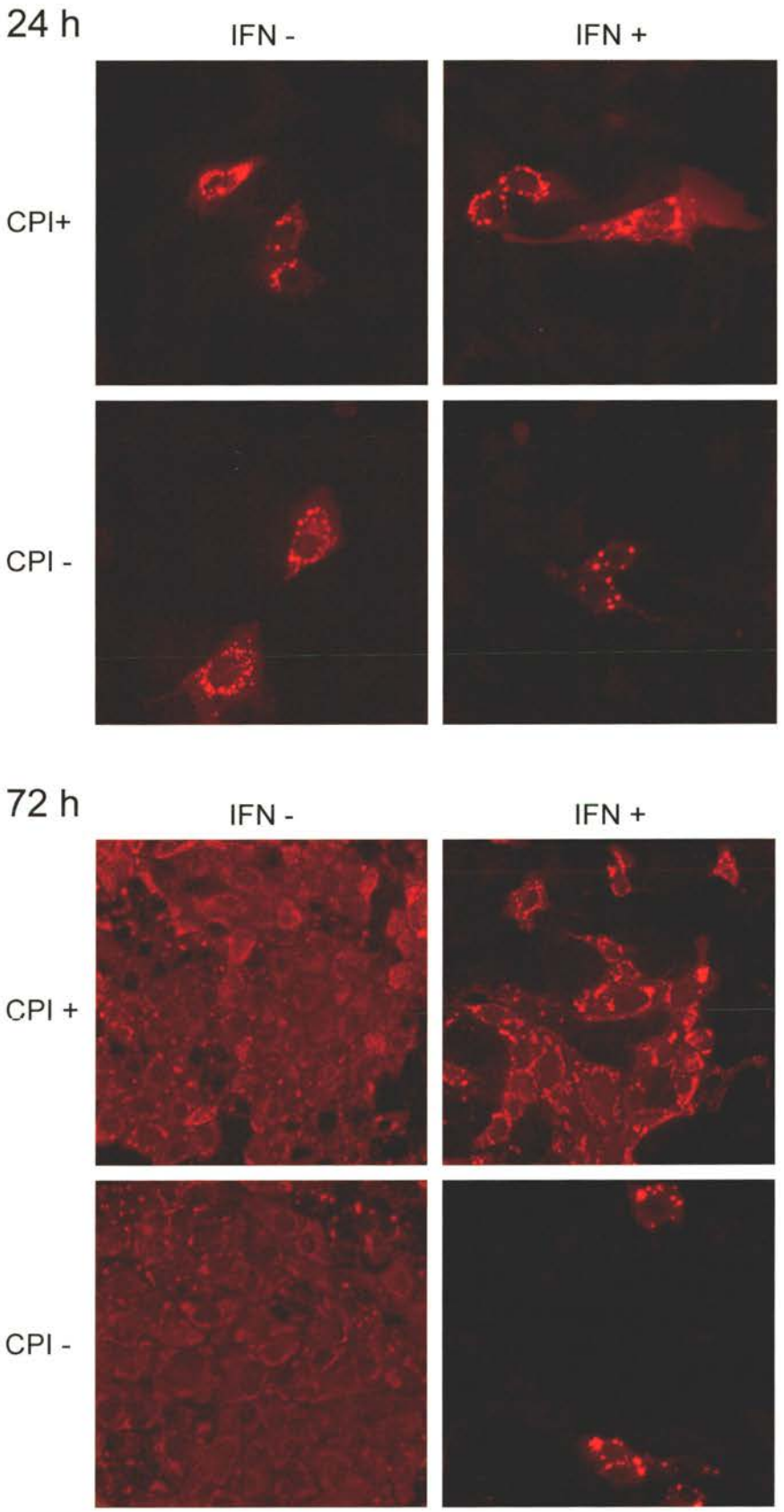
W3 in murine cells

Didcock and colleagues (1999a) have reported previously that W3 does not lead to the degradation of STAT1 in murine cells, thus failing to block IFN signalling. As a result, W3 is IFN-sensitive and consequently restricted in cells of this species origin. Following infection of murine cells with W3 at a high m.o.i., there is an initial phase of high viral protein synthesis that is reduced as soon as the IFN response is elicited and

Figure 3.16: In the presence of IFN, CPI+ spreads within a monolayer of canine cells more efficiently than CPI-.

Canine (MDCK) cells were infected with CPI+ or CPI- at a low m.o.i. (approximately 0.01 pfu/cell), and incubated with exogenous IFN at 12 h p.i. (IFN+) or not (IFN-). The cells were fixed at 24 and 72 h p.i. and stained with monoclonal anti-NP (SV5-NP-a) and anti-P (SV5-P-e) antibodies, followed by a secondary anti-mouse Ig Texas Red-conjugated antibody.

Fig. 3.16



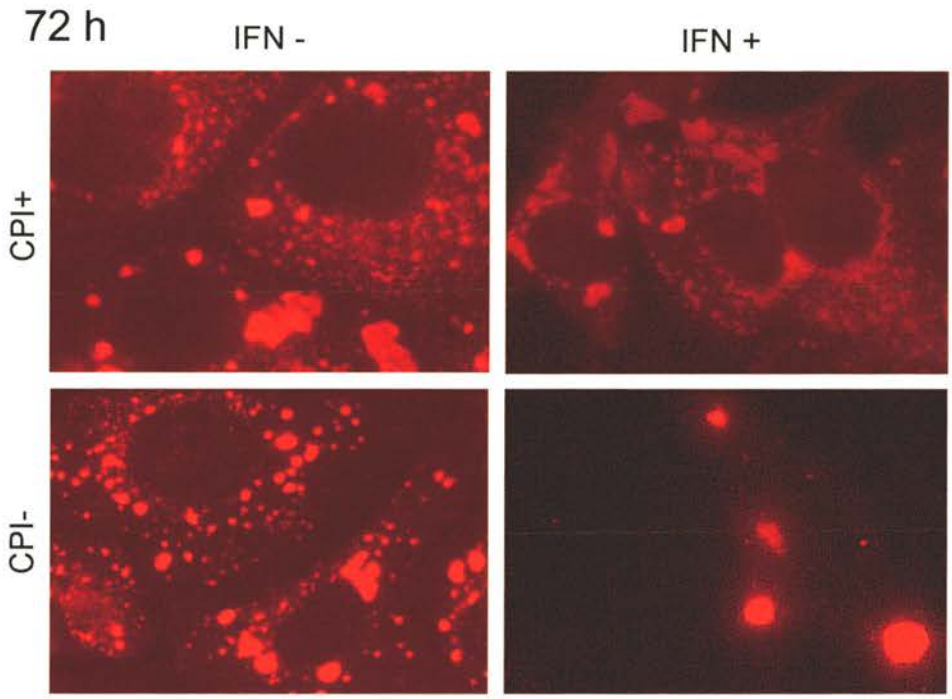


Figure 3.17: Immunofluorescence analysis of NP and P protein distribution in canine cells infected with CPI+ or CPI-, in the presence or absence of IFN.

Canine (MDCK) cells were infected with CPI+ or CPI- at a high m.o.i. and at 12 h p.i. the culture medium was supplemented with exogenous IFN (IFN+) or left untreated (IFN-). The cells were fixed at 72 h p.i. and stained with anti-NP (SV5-NP-a) and anti-P (SV5-P-e) mAbs, followed by a secondary anti-mouse Ig Texas Red-conjugated antibody.

the cellular antiviral mechanisms become activated. As a result, in an infected population, the virus is cleared effectively in the majority of cells. In the part of the population that remains infected, any fluctuation of the local concentration of IFN affects the state of the virus, since the ability of the cells to clear the virus is IFN-based. Thus, the virus is repressed when IFN levels are high and becomes active when IFN levels drop, such as when the culture media are changed upon passaging of the monolayers (Young *et al.*, 1997).

CPI- in canine cells

According to the observations described in the previous sections, CPI- is sensitive to IFN in canine cells, similar to W3 in murine cells, as it fails to induce STAT1 degradation and block IFN signalling. Therefore, it was of interest to investigate whether CPI- behaves in canine cells as W3 does in murine cells. With regard to CPI+, it was anticipated that this isolate would phenotypically resemble W3 in canine cells, given its ability to degrade STAT1 and block signalling efficiently in those cells. To compare the phenotypes of the two isolates, canine (MDCK) cells were infected with CPI- or CPI+ at a high m.o.i. and passaged regularly over a period of three weeks. Monolayers were fixed at one and 20 days p.i. and examined following staining with the appropriate a-NP and a-P mAbs. The results from this immunofluorescence analysis are presented in Figure 3.18. It was clearly shown that, although at one day p.i. all of the cells were infected with CPI+ or CPI-, at 20 days p.i. there was a major difference in the number of cells that remained infected by the two isolates. Thus, in contrast to the CPI+ infected cell population all of which stained positive for viral proteins NP and P, in cells infected with CPI- there was noticeably less fluorescence, as approximately 40% of the cells had cleared the infection.

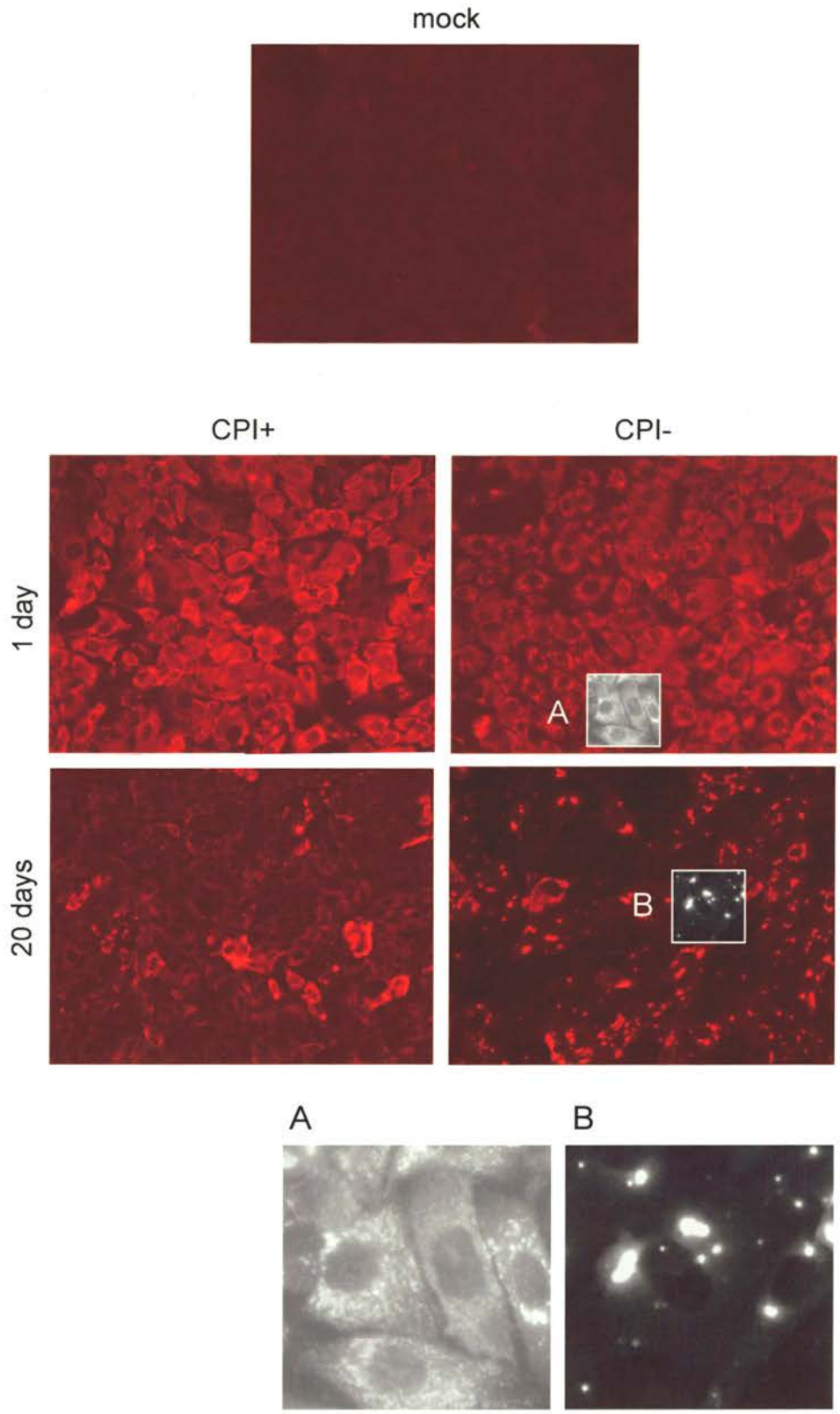
Protein distribution

In addition to the difference in the number of cells that stained positive for CPI+ and CPI- proteins, there was also a striking difference in the intracellular distribution patterns of the viral proteins in the two cell populations. At one day p.i., diffuse cytoplasmic staining patterns were observed both in cells infected with CPI+ and in cells infected with CPI-, as shown in Figure 3.18, Panel A. However, at 20 days p.i., the majority of the cell population that remained infected with CPI- was marked by distinctive cytoplasmic inclusion bodies and had little or no diffuse protein distribution, as presented in Figure 3.18, Panel B. Inclusion bodies of NP and P were also found in cells that had been infected with CPI+, but to a far lesser extent. Thus, these cells were

Figure 3.18: Immunofluorescence analysis showing that, in persistently infected canine cells, the majority of cells have cleared CPI- infection by 20 days p.i., whereas all cells that had been infected with CPI+ remain infected. The intracellular distribution patterns of the stained proteins differ significantly between the two isolates.

Canine (MDCK) cells that had been infected with CPI+ or CPI- at a high m.o.i. (10 pfu/cell), or mock infected, were passaged regularly following infection. The cells were fixed at one and 20 days p.i. and treated with anti-NP (SV5-NP-a) and anti-P (SV5-P-e) mAbs, followed by staining with a secondary anti-mouse Ig Texas Red-conjugated antibody. The intracellular distribution patterns of NP and P of CPI- are shown in the magnified areas (close-up panels A and B).

Fig. 3.18



mostly characterised by diffuse cytoplasmic protein distribution, showing a staining pattern similar to that observed in CPI+ infected cells at one day p.i.

3.2.8 Distribution of HN and NP/P proteins in cells infected with CPI+ and CPI-

NP and P vs. HN

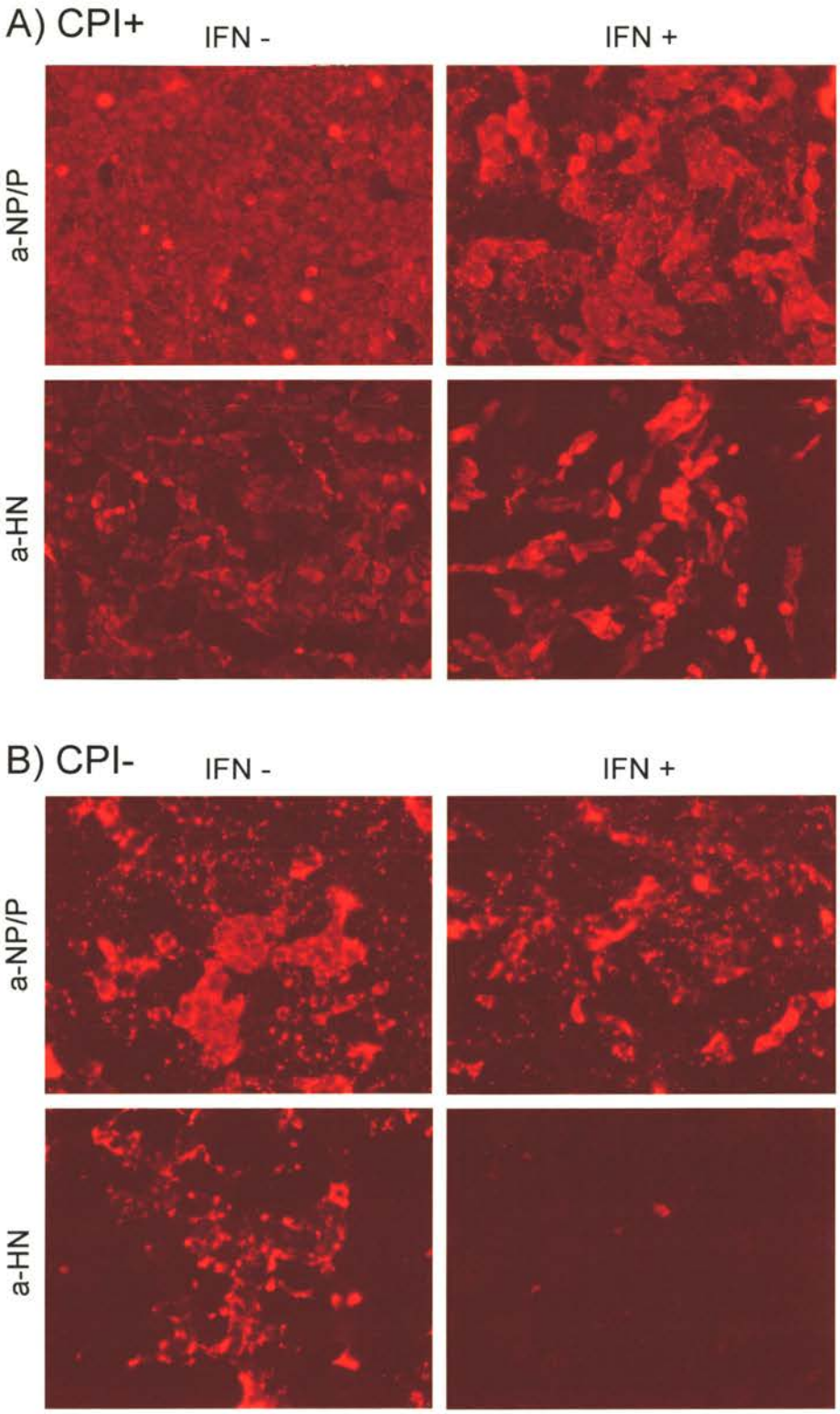
Immunofluorescence experiments performed previously by Young and colleagues (1997) have shown that in murine (BF) cells infected with W3, in the presence of IFN, the NP protein aggregates in cytoplasmic inclusion bodies, whereas only a very low percentage of infected cells stains positive for the HN glycoprotein. In the present study, results showed that there is a significant difference in the distribution of NP and P in cells infected with CPI+ and CPI-, with the distribution pattern in CPI- infected cells being reminiscent of that of NP in murine cells infected with W3. Thus, it was of interest to perform immunofluorescence analysis on cells infected CPI+ and CPI- and compare HN expression levels in those cells.

Cells were infected with CPI+ and CPI- at a low m.o.i. (0.5 to 1.0 pfu/cell) and treated with IFN at 12 h p.i. (or left untreated). The monolayers were examined by immunofluorescence at 24 and 72 h p.i. using the appropriate a-HN mAb or a combination of a-NP and P mAbs, to compare the staining of HN and NP/P, respectively. At 24 h p.i. both CPI+ infected cells and cells infected with CPI- stained positive for HN and NP proteins (data not shown). However, by 72 h there was a significant difference between CPI+ and CPI-, as presented in Figure 3.19. Following the addition of exogenous IFN, there was an evident reduction in HN levels in cells that had been infected with CPI-, whereas cells infected with CPI+ exhibited a far smaller decrease in HN surface expression. Moreover, in confirmation of the results from the experiment described above, in the presence of IFN, NP levels dropped and protein distribution changed significantly in cells infected with CPI-. Thus, whilst there was a decrease in the number of cells that showed diffuse staining, a greater proportion of them contained NP aggregated in cytoplasmic inclusion bodies. In contrast, in CPI+ infected cells, IFN seemed to have a significantly smaller effect on NP, which had diffuse cytoplasmic distribution in the majority of cells.

Figure 3.19: Immunofluorescence analysis of HN and NP/P proteins in canine cells infected with CPI+ or CPI-.

Canine (MDCK) cells were infected with CPI+ (Panel A) or CPI- (Panel B) at a low m.o.i. (0.5 to 1.0 pfu/cell). At 12 h p.i. the culture medium was supplemented with exogenous IFN (IFN+) or left untreated (IFN-). The cells were fixed at 72 h p.i. and stained with a combination of monoclonal anti-NP (SV5-NP-a) and anti-P (SV5-P-e) antibodies, or with monoclonal anti-HN antibody (SV5-HN-4a). Primary antibody treatment was followed by a secondary anti-mouse Ig Texas Red-conjugated antibody.

Fig. 3.19



3.2.9 CPI- is rescued from IFN in cells that permanently express the V protein of W3

The results presented above indicated that due to the amino acid differences between the V proteins of CPI+ and CPI-, the latter fails to block IFN signalling, thus being restricted in cells that produce and respond to IFN. In contrast, CPI+ can overcome the IFN response and proliferate efficiently, as its V protein can degrade STAT1. In order to confirm that the lack of ability of CPI- V to mediate the degradation of STAT1 is a critical factor for the observed restricted replication of this isolate, the behaviour of CPI- was examined in cells that expressed the wild-type (W3) V protein. Human cells that constitutively expressed the W3 V protein (2fTGH.SV5-V cell line) or not (2fTGH) were infected with CPI+ or CPI- at a low m.o.i. The culture medium was supplemented with IFN at 12 h p.i. (or left untreated) and at 72 h p.i. the cells were immunostained with anti-NP (SV5-NP-a) and anti-P (SV5-P-e) mAbs.

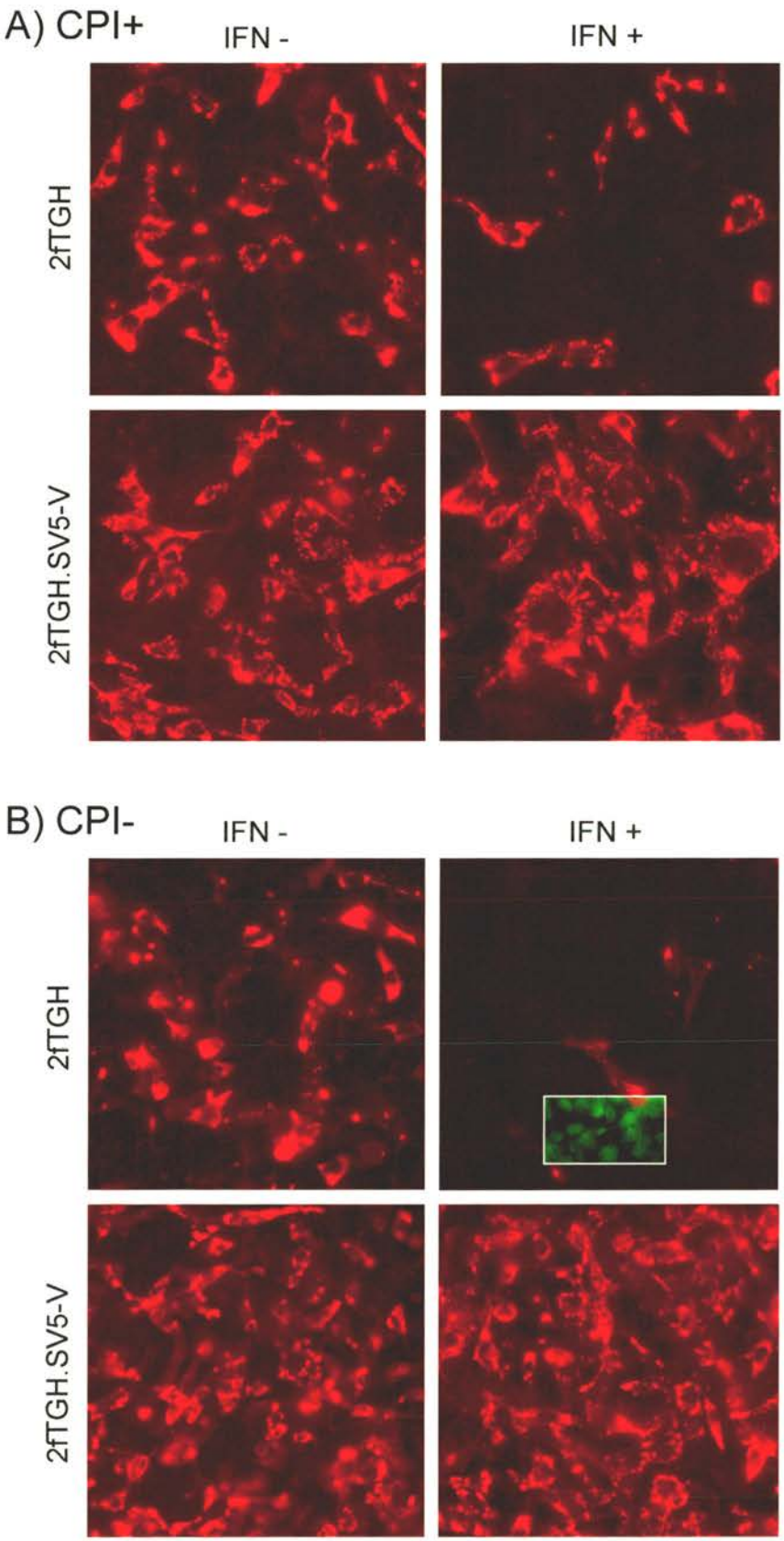
The spread of the isolates is favoured in V-expressing cells

The results from this analysis made clear that CPI- is rescued from the antiviral action of IFN in cells that constitutively express wild-type V. As shown in Figure 3.20, the spread of CPI+ and CPI- (Panels A and B, respectively) was restricted in human (2fTGH) cells in the presence of IFN. This effect was more evident in the case of CPI- which was eventually cleared in the majority of infected cells (see Figure 3.16). In striking contrast, cells that constitutively expressed V (2fTGH.SV5-V) favoured the spread of both strains dramatically. Thus, in V-expressing cells, even in the presence of IFN, both CPI+ and CPI- replicated and spread as efficiently as in cells that had not been treated with IFN. This result indicated that the V protein expressed in these cells rescued the isolates from the constraints usually posed by IFN. Moreover, whilst treatment with IFN appeared to lead to the accumulation of NP and P aggregates in the cytoplasm of 2fTGH cells infected with CPI-, the distribution of the same proteins was markedly different (diffuse cytoplasmic) in V-expressing cells infected with the same virus.

Figure 3.20: Constitutive expression of W3 V in human cells enables CPI+ and CPI- to spread in the presence of IFN as efficiently as in the absence of IFN.

Human cells that constitutively express W3 V (2fTGH.SV5-V) or not (2fTGH) were infected with CPI+ or CPI- at a low m.o.i. At 12 h p.i. the culture medium was supplemented with exogenous IFN (IFN+) or left untreated (IFN-) and the cells were fixed at 72 h p.i. and immunostained using anti-NP (SV5-NP-a) and anti-P (SV5-P-e) mAbs, followed by a secondary anti-mouse Ig Texas Red-conjugated antibody. The number of cells in the monolayer that stained lightly for NP and P is indicated by a superimposed image of DAPI staining (same field of view) of the same monolayer (Panel B, 2fTGH, IFN+).

Fig. 3.20



3.2.10 Cell lines expressing permanently the V proteins of CPI+ and CPI-

Experimental outline

Having established the importance of the amino acid differences between CPI+ and CPI- V in their ability to degrade STAT1 and block IFN signalling, it was of interest to obtain cell lines that stably express the V proteins of the two viruses. The aim was to investigate whether there were any differences in the interaction of CPI+ and CPI- V with host cellular proteins, which would further elucidate the molecular mechanism of STAT1 degradation. To produce these cell lines, the V genes from CPI+ and CPI- V were re-cloned into an appropriate selectable vector (pEF.IRES.neo; see Chapter 2) and the prepared constructs (termed pEF.W3/CPI+/V.IRES.neo and pEF.W3/CPI-/V.IRES.neo, respectively) were transfected into human (2fTGH) cells. Transformed clones were selected in media supplemented with geneticin (a neomycin analogue) and screened by immunofluorescence to ascertain whether they expressed the proteins of interest. The monolayers were immunostained with mAb 14E8 or a mixture of mAbs 11C6 and 31F6, which all recognise the unique carboxy-terminal domain of V.

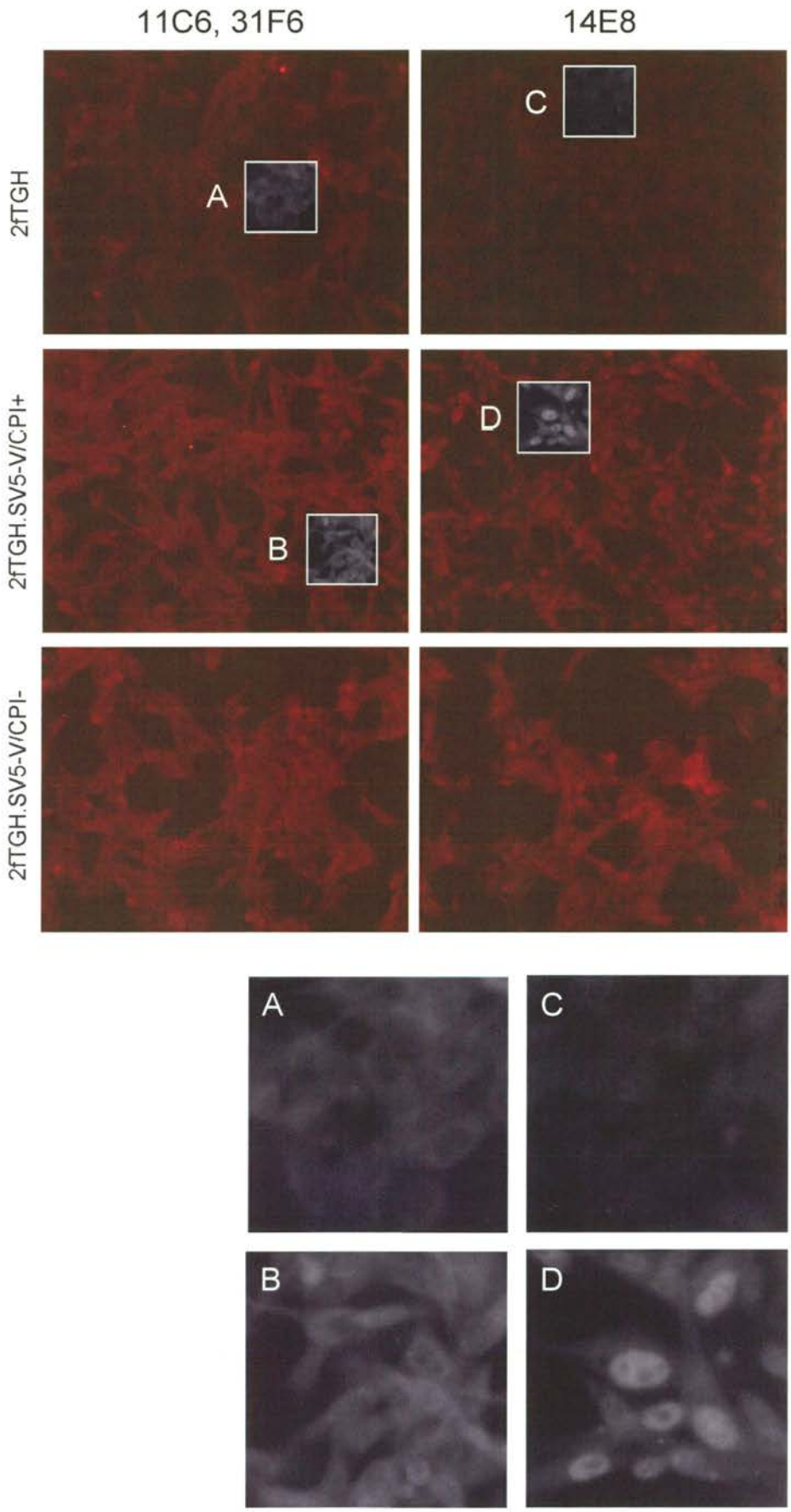
Immunofluorescence analysis

As shown in Figure 3.21, immunostaining confirmed that the CPI+ and CPI- V proteins were expressed in the 2fTGH.SV5-V/CPI+ and 2fTGH.SV5-V/CPI- cell lines, respectively. However, there was a striking difference in the staining patterns obtained by the two different treatments. Thus, in both CPI+ and CPI- V cell lines, staining with mAbs 11C6 and 31F6 revealed that V localised in the cytoplasm in the majority of cells (Figure 3.21, Panel B), in contrast to mAb 14E8 which showed that V was present primarily in the cell nuclei (Figure 3.21, Panel D). Negative control treatments (staining of non-expressing cells with mAbs 11C6 and 31F6, and 14E8) are shown in Figure 3.21, Panels A and C, respectively. Thus, this immunofluorescence analysis demonstrated that masking of certain V epitopes with mAbs can affect significantly the intracellular distribution of the protein. Although characterisation of these cell lines was not possible to complete in this study, examination of the interaction of CPI+ and CPI- V with host cellular proteins by Dr. J. Andrejeva revealed that there are differences between the two proteins, as discussed in Chapter 4.

Figure 3.21: Immunofluorescence analysis of CPI+ and CPI- V expression and localisation in human cells that express permanently these proteins (cell lines 2fTGH.CPI+V and 2fTGH.CPI-V, respectively).

Human (2fTGH) cells were transfected (lipid-based method) with 3 μg of either the CPI+ or CPI- V-encoding plasmid constructs (pEF.IRES.neo.CPI+V or pEF.IRES.neo.CPI-V, respectively). Permanently transfected clones were selected in culture medium supplemented with geneticin. To confirm the expression of V in the selected clones, cells were fixed and stained with a combination of mAbs (11C6 and 31F6) or mAb 14E8, all of which specifically recognise the carboxy-terminal domain of SV5 V. The intracellular localisation of V in cells treated with different antibodies is shown in the magnified image areas (close-up panels B and D), in comparison to immunofluorescence analysis of non-expressing cells (close-up panels A and C, respectively).

Fig. 3.21



3.3 SV5-hPIV2 Hybrid V proteins

3.3.1 Construction of the hybrid V/P genes

Focussing on domains – SV5 vs. hPIV2

In order to examine the V protein further, it was of interest to examine the properties of the amino- and carboxy-terminal domains of V. In contrast to SV5 V which degrades STAT1, experimental evidence has shown that hPIV2 V leads to the degradation of STAT2 (Young *et al.*, 2000; Parisien *et al.*, 2001). In both proteins, the carboxy-terminal domains are highly conserved, having a significant degree of homology, whilst the amino-terminal domains are variable (see Chapter 1). In order to investigate whether the carboxy-terminal domain has the catalytic activity (mediation of degradation) and the amino-terminal domain defines which of the STAT proteins is degraded (specificity), SV5-hPIV2 hybrid V proteins were produced so as to bring the amino-terminal or carboxy-terminal domain of SV5 together with the carboxy-terminal or amino-terminal domain of hPIV2, respectively, as presented schematically in Figure 3.22.

Construction of the hybrids

The strategy for the construction of the SV5-hPIV2 and hPIV2-SV5 V hybrids is illustrated in Figure 3.23 (Panels A and B, respectively). The construction process involved two main steps. Firstly, the gene regions coding for the domains of interest were amplified from the original virus V/P genes by PCR. In these reactions, the appropriate oligonucleotide primers contained not only part of the sequence of the virus gene being amplified, but also a short stretch of the V/P sequence from the other virus. For example, for the amplification of the hPIV2 amino-terminal domain, the reverse direction, internal primer contained (in 5'-3' order) a short stretch of the SV5 V/P sequence (gene region downstream the SV5 editing site) followed by a portion of the hPIV2 amino-terminal domain (gene region upstream the hPIV2 editing site). Thus, the products of PCR I and II were portions of the amino- and carboxy-terminal domain-encoding regions from the V/P genes of SV5 and hPIV2, fused to short sequence extensions of reciprocal virus origin.

PCR I and II products were subsequently subcloned, grown in larger quantities and purified, and used in the second step (PCR III). In this reaction, the purified fragments primed strand extension reciprocally, thus giving rise to the complete template (hybrid

Figure 3.22: Schematic representation of the hybrid V constructs showing their derivation from the amino- and carboxy-terminal domains[1] of the SV5 and hPIV2 V proteins.

The SV5/hPIV2 hybrid V protein consists of the SV5 V amino-terminal domain and the hPIV2 V carboxy-terminal domain, whereas the hPIV2/SV5 hybrid V protein comprises the hPIV2 V amino-terminal domain and the SV5 V carboxy-terminal domain.

[1] "Domain" is a term used to describe the region of the protein between the first (amino-terminal) or last (carboxy-terminal) amino acid of the protein and the editing site of the protein of the same virus. For example, the hPIV2/SV5 V hybrid contains the amino-terminal domain of hPIV2 V (which starts at the amino-terminal amino acid and ends at the hPIV2 V editing site) followed by the SV5 V carboxy-terminal domain (i.e. the portion of the protein between the SV5 V editing site and the carboxy-terminal amino acid).

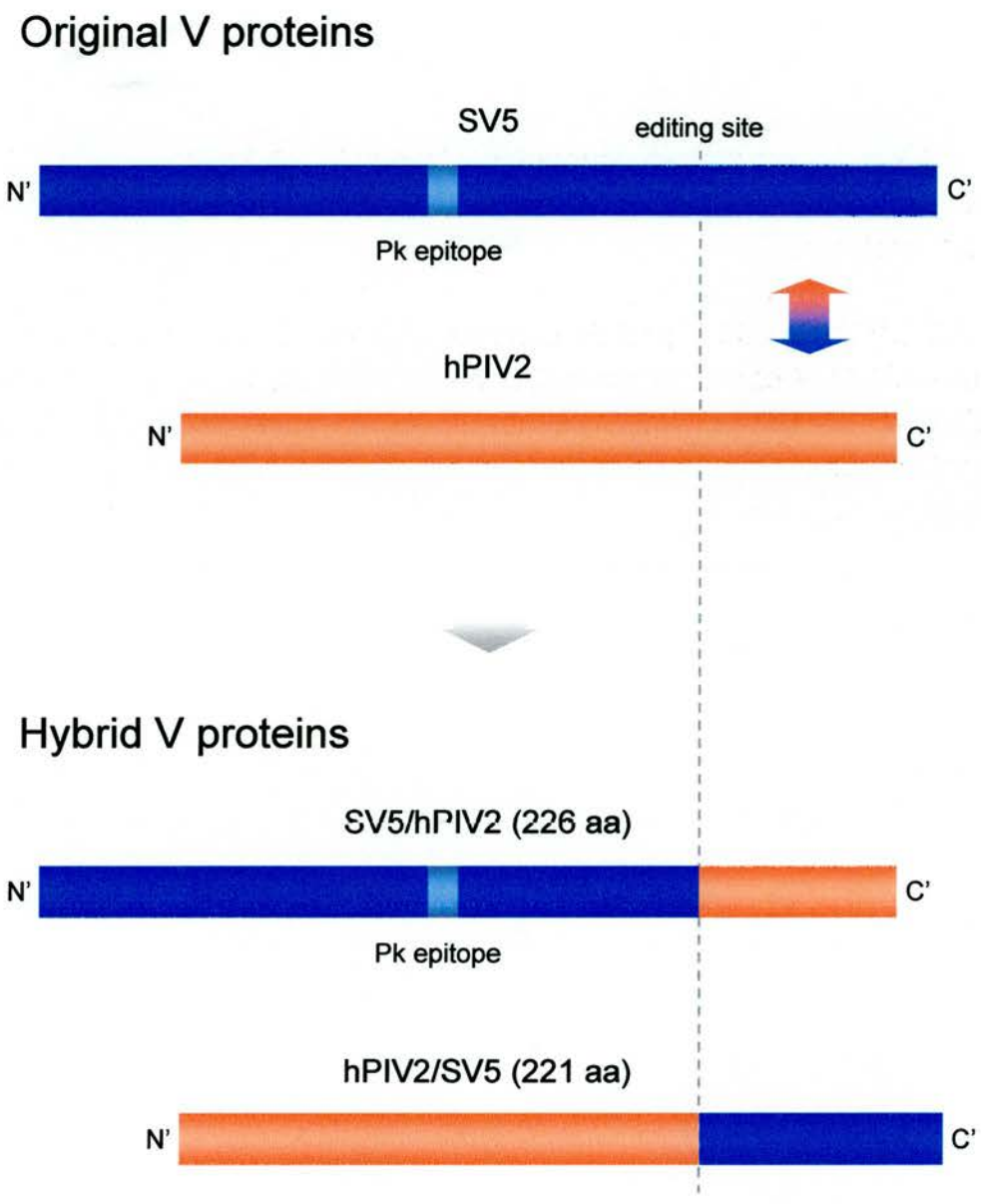

Original V proteins
SV5
editing site
N'
C'
Pk epitope
hPIV2
N'
C'
Hybrid V proteins
SV5/hPIV2 (226 aa)
N'
C'
Pk epitope
hPIV2/SV5 (221 aa)
N'
C'

Figure 3.23: Schematic presentation of the construction strategy for the hybrid V-encoding genes.

The regions of interest (amino- or carboxy-terminal domains) were initially amplified from the original virus V/P genes by PCR (PCR I and II) using SV5-hPIV2 hybrid oligonucleotides as internal primers. The resulting fragments were subsequently combined in a new PCR (PCR III), in which they primed the synthesis of the complete template (the overlapping sequence regions of the PCR I and II products enabled reciprocal annealing and strand extension). In the presence of oligonucleotide primers that annealed on either ends of it, the hybrid gene template was finally amplified to quantities sufficient for gene cloning.

This method was followed for the construction of both the SV5/hPIV2 and the hPIV2/SV5 hybrid genes (Panels A and B, respectively).

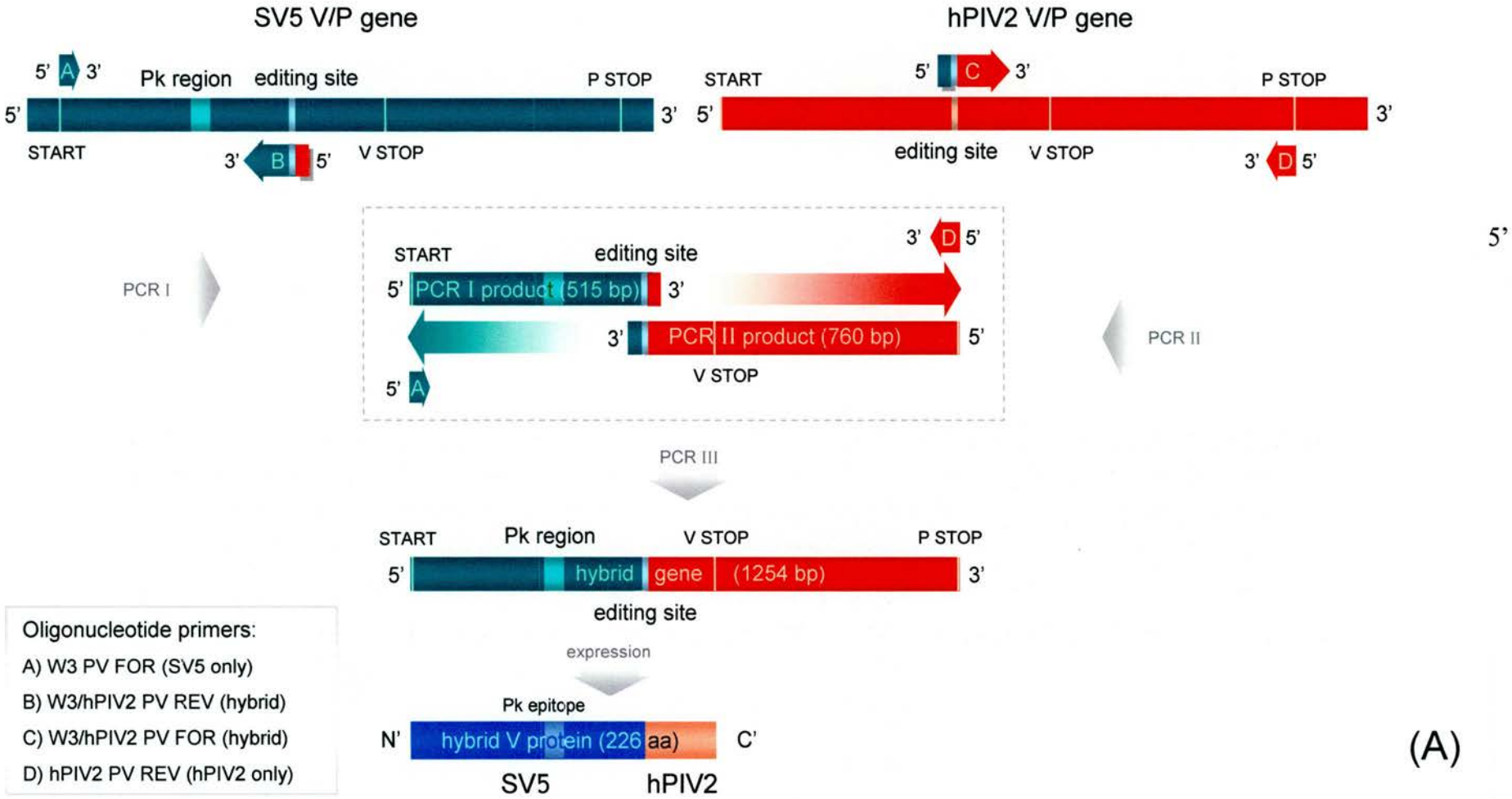
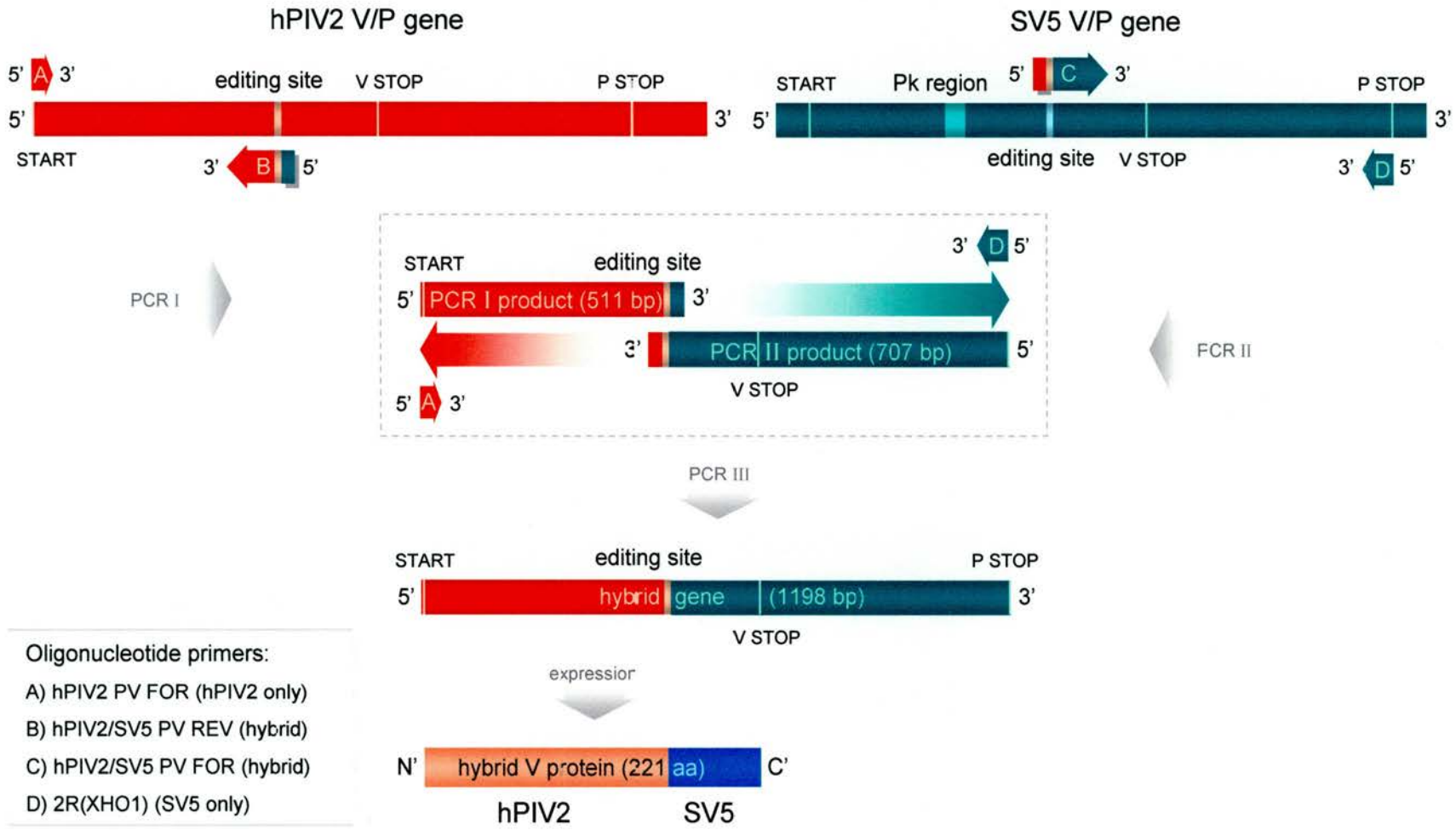

SV5 V/P gene
hPIV2 V/P gene
5' A 3'
Pk region
editing site
P STOP
5'
3'
START
3' B 5'
V STOP
START
5' C 3'
P STOP
editing site
V STOP
3' D 5'
PCR I
START
editing site
3' D 5'
5' PCR I product (515 bp) 3'
3' PCR II product (760 bp) 5'
V STOP
5' A
PCR II
5'
PCR III
START
Pk region
V STOP
P STOP
5' hybrid gene (1254 bp) 3'
editing site
expression
Pk epitope
N' hybrid V protein (226 aa) C'
SV5
hPIV2
Oligonucleotide primers:
A) W3 PV FOR (SV5 only)
B) W3/hPIV2 PV REV (hybrid)
C) W3/hPIV2 PV FOR (hybrid)
D) hPIV2 PV REV (hPIV2 only)
(A)

Fig. 3.23


hPIV2 V/P gene
5' A 3'
editing site
V STOP
P STOP
5'
3'
START
3' B 5'
SV5 V/P gene
START
Pk region
5' C 3'
P STOP
5'
3'
editing site
V STOP
3' D 5'
PCR I
START
editing site
3' D 5'
5' PCR I product (511 bp) 3'
3'
PCR II product (707 bp) 5'
V STOP
5' A 3'
FCR II
PCR III
START
editing site
P STOP
5'
hybrid gene (1198 bp)
3'
V STOP
expressior
N'
hybrid V protein (221 aa)
C'
hPIV2
SV5
Oligonucleotide primers:
A) hPIV2 PV FOR (hPIV2 only)
B) hPIV2/SV5 PV REV (hybrid)
C) hPIV2/SV5 PV FOR (hybrid)
D) 2R(XHO1) (SV5 only)

(B)

Fig. 3.23

gene). In the presence of oligonucleotide primers that annealed on either ends of it, this hybrid gene was finally amplified (in the same reaction) to a quantity sufficient for insertion into the appropriate expression vector (pEF-plink2).

3.3.2 Analysis of the hybrid V proteins

Immunofluorescence analysis

The composition of the final plasmid constructs was confirmed by sequence analysis, as described above. Furthermore, to determine whether the hybrid proteins were expressed in cells, human (2fTGH) cells were transfected with the constructs (as above), fixed and stained with mAbs SV5-P-k (anti-Pk) and hPIV2-P-a, which recognise the amino-terminal domains of SV5 V and hPIV2 V, respectively (see Chapter 2, Table 2.1). The results from this immunofluorescence analysis are illustrated in Figure 3.24. As shown in Panel B, staining of the amino-terminal domain of the SV5/hPIV2 hybrid indicated that the hybrid protein was expressed in transfected cells. Similarly, immunostaining of the amino-terminal domain of the hPIV2/SV5 protein showed that this hybrid was also expressed, as presented in Panel D. The native SV5 (W3) and hPIV2 V proteins were stained with the same mAbs, as positive controls (Panels A and C, respectively).

Further analysis

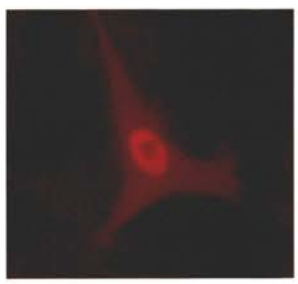
The plasmid constructs, termed pEF.SV5/hPIV2-V and pEF.hPIV2/SV5-V, were each co-transfected into human (2fTGH) cells, either with the type I (pISRE) or type II (pGAS) IFN-responsive reporter plasmids. The culture media were supplemented with IFN-α/β (type I) or IFN-γ (type II), in order to activate the ISRE or GAS promoter respectively, and the activity of the reporter (luciferase) gene was measured. Although the expression of the hybrid V proteins in transfected cells was confirmed by immunofluorescence analysis (see above), this experiment yielded inconclusive results in all repeated assays, as the obtained luciferase reporter activation data seemed to be inconsistent and the control treatments did not always respond properly to IFN stimulation (data not shown). Nevertheless, time being a limiting factor, further analysis of these hybrids was not performed and their characteristics remained subject to investigation.

Figure 3.24: Immunofluorescence analysis showing SV5/hPIV2 and hPIV2/SV5 hybrid V protein expression in transiently transfected human cells.

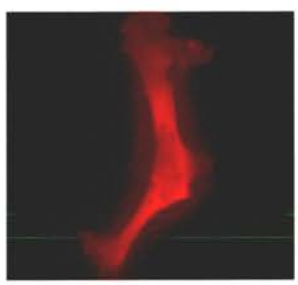
Human (2fTGH) cells were transfected (lipid-based method) with 0.4 μg of the β-gal reporter plasmid (pJATlacz), 0.4 μg of the IFN-α/β-responsive (pISRE) or IFN-γ-responsive plasmid (pGAS) and 1.2 μg of one of the test plasmids. Test plasmids pEF.SV5-V, pEF.hPIV2-V, pEF.SV5/hPIV2-V or pEF-hPIV2/SV5-V, encoded SV5 (W3), hPIV2, SV5/hPIV2 hybrid or hPIV2/SV5 hybrid V proteins, respectively.

The cells were stained with antibodies that recognise the V proteins. Each photomicrograph is accompanied by a schematic diagram that indicates the composition of the protein expressed and the domain recognised by the antibody used. SV5 V was stained with the monoclonal anti-Pk antibody (SV5-P-k), as shown in Panel A (control treatment). The same antibody was used to detect the SV5/hPIV2 hybrid protein (recognition of the amino-terminal domain; Panel B). hPIV2 V (control treatment) and the hPIV2/SV5 V hybrid protein (Panels C and D, respectively) were detected with mAb hPIV2-P-a which recognises the amino-terminal domain of hPIV2 V.

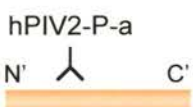
A) SV5 V

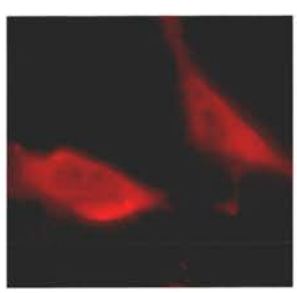


B) SV5/hPIV2 V

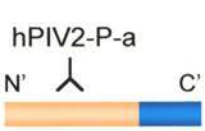


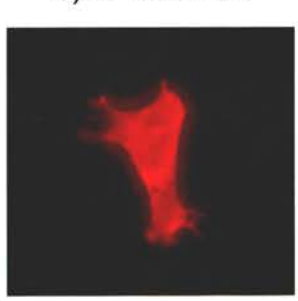
C) hPIV2





D) hPIV2/SV5





3.4 Characterisation of SV5 isolates of human, canine and porcine origin

3.4.1 The V/P genes of different SV5 isolates

Looking at human SV5 isolates

Past reports have linked SV5 to persistent infections in humans (reviewed in Randall & Russell, 1991) and several strains (termed MIL, DEN, LN, MEL and RQ) have been isolated from bone marrow cells of multiple sclerosis patients (Goswami *et al.*, 1984; see Chapter 1). Given that the V protein is directly involved in the ability of the virus to overcome the IFN response, and that a single mutation can dramatically change the phenotype of the virus as shown in the case of mci-2, it was of interest to analyse the V/P genes of these isolates. The primary aim was to investigate whether these isolates are IFN-sensitive, in which case, such a finding could probably explain their ability to escape recognition by the host immune system and establish persistent infections. The potential role of IFN sensitivity in the establishment of persistent infections by SV5 is discussed in Chapter 4. Furthermore, sequence variation within the V/P genes of these isolates could provide evidence of virus fluxing (switching between an active and inactive state) in response to IFN, which would also support the proposed model of persistent SV5 infection (Chapter 4).

Looking at canine SV5 isolates

In addition to the human isolates, it was of also of interest to examine two more canine isolates (H221 and 78524; viruses obtained from Prof. O. Jarrett, Faculty of Veterinary Medicine, University of Glasgow, Glasgow, UK), in order to determine their sensitivity to IFN in cells of different host species origin. Moreover, by analysing the sequence of the V/P genes from these isolates and comparing them to those of CPI+ and CPI-, it was intended to determine the degree of variation within these geographically distant strains (CPI+ and CPI- were isolated in Germany, whereas H221 and 78524 were isolated in the UK) and thus investigate whether they represent viruses adapted in their host species. To further complement this analysis, the sequence of the V/P gene of a porcine isolate was also analysed (virus isolate obtained from Dr. Klenk H.D., Institut fuer Virologie, Philipps-Universitaet Marburg, Germany) .

Experimental outline

The V/P genes of the isolates mentioned above were cloned by RT-PCR, inserted in pGATA plasmid vectors and sequenced as described previously. This work was done in

collaboration with Andrea Kinver (Kinver, 2000). The obtained nucleotide sequences were compared to that of W3 and mutations affecting the V ORF were identified.

3.4.1.1 Sequence analysis of the V/P genes of the different SV5 isolates

Low sequence variation

Sequence analysis of the V/P gene of the human, canine, murine and porcine isolates revealed that there is a low degree of variation, with the V ORF percentage amino acid variation ranging from 0.0 to 2.7% (0.5 to 2.0% in the P ORF). A comparative overview of the sequence analysis results from the V/P gene of all SV5 isolates is presented schematically in Figure 3.25. Mutations were identified in the V/P common amino-terminal domain, as compared to W3. In contrast, amongst all isolates, only three mutations were found to affect the unique carboxy-terminal domain of the P protein (one present in all of the human isolates and two found in CPI+ and CPI-), all three of which are silent in the V ORF. None of the isolates were found to have mutations in the Pk epitope, with the exception of mci-2 and CPI- (which have already been discussed in the previous sections), neither were any mutations in the carboxy-terminal domain of V, confirming that this part of the protein is conserved. Although there are few amino acid substitutions in the V protein, there are considerably more (silent) nucleotide changes within the V ORF, the patterns of which correlate with the species origin of the isolates. Full nucleotide and amino acid residue information about the mutations identified in the V/P genes of all examined isolates is provided in Table 3.4. Please note that incomplete sequence data were obtained for the V/P gene of RQ, as indicated in Figure 3.25.

3.4.2 STAT1 degradation by the SV5 isolates in human and canine cells

Experimental outline

In order to determine the sensitivity of the human and canine isolates to IFN, their ability to degrade STAT1 was examined in different cell lines. STAT1 levels were examined by immunoblotting in extracts of human (2fTGH) and canine (MDCK) cells that had been infected with the different isolates at a high m.o.i. (approximately 100 pfu/cell). Prior to immunoblot analysis, the infected cells were tested by immunofluorescence (staining with anti-NP and anti-P mAbs), in order to confirm that >95% cells were positive for infection by these virus (data not presented).

Figure 3.25: Gene map (one-nucleotide resolution) showing the relative position and effect of all the mutations identified in the V/P mRNA of the human (MIL, DEN, LN, MEL, RQ), canine (CPI+, CPI-, H221, 78524), murine (mci-2) and porcine (SER) isolates of SV5.

Mutations that lead to amino acid substitutions are shown with blue lines. Silent mutations are indicated by orange lines. Annotation of the mRNA (top band) and V and P proteins (bottom two bands) reveal the relative position of the mutations with regard to the V and P ORFs, the V- or P- unique carboxy-terminal domains and the Pk epitope. Mutations that are silent in the V ORF but affect codons in the P ORF are shown in orange and blue (|). The portion of the RQ V/P sequence that remains to be determined (from ORF start to Pk epitope) is indicated by grey shading.

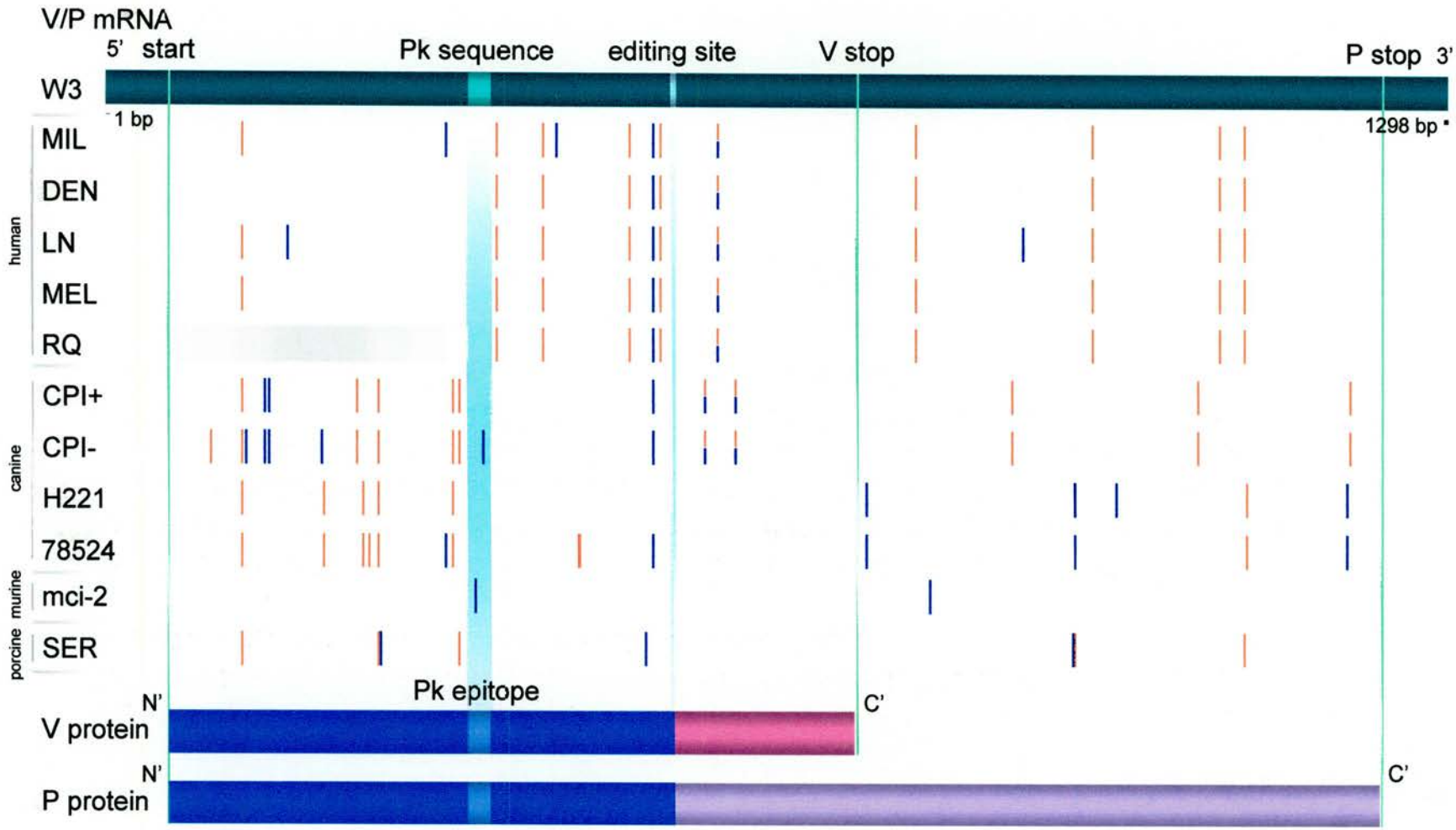

V/P mRNA
5' start
Pk sequence
editing site
V stop
P stop 3'
W3
1 bp
1298 bp
MIL
DEN
LN
MEL
RQ
human
CPI+
CPI-
H221
78524
canine
mci-2
murine
SER
porcine
Pk epitope
N'
C'
V protein
N'
C'
P protein

Fig. 3.25

Table 3.4: Nucleotide changes (top, acetate layer) and amino acid substitutions (bottom page) in the V/P gene and products of the human (MIL, DEN, LN, MEL, RQ), canine (CPI+, CPI-, H221, 78524), murine (mci-2) and porcine (SER) isolates of SV5.

Table cell border colour and shading indicate the relative position of the mutations, with regard to the V/P common amino-terminal domain (red border), the Pk epitope (residue numbers in grey-shaded cells), the region between the editing site and the V stop codon (i.e. the V-unique carboxy-terminal domain and part of the P carboxy-terminal domain; yellow border), and the (rest of the) P carboxy-terminal domain (blue border). Nucleotide residues and substitutions are shown in black and their position numbers correspond to the the sequence information available for the SV5PVA locus (GenBank Accession no. J03142). Amino acid position numbers, residues and substitutions are printed in red colour, whereas amino acid residue positions that are not affected by nucleotide changes (silent mutations) are shown in grey. Note that the mutations identified between the editing site and the V stop codon do not affect the V carboxy-terminal domain (grey residues), but only the P carboxy-terminal domain (red residues).

nt	W3	MIL	DEN	LN	MEL	RQ	CPI+	CPI-	H221	78524	mci-2	SER
102	T							C				
132	A	G		G	G	G	G	G	G	G		G
136	T							C				
154	G						A	A				
158	C						T	T				
176	G			A								
209	T							C				
211	C								T	T		
243	G						A	A				
249	T								C	C		
255	T									C		
264	C						T	T	T	T		T
266	C											T
329	A	G								G		
336	A						G	G	G	G		
342	C						T	T				T
358	A										G	
365	T							C				
378	C	T	T	T	T	T						
423	A	T	T	T	T	T						
436	T	A										
459	G									A		
507	A	G	G	G	G	G						
523	A											C
526	A											
530	C	T	T	T	T	T	T	T		T		
537	C	T	T	T	T	T						
579	G						A	A				
591	C	T	T	T	T	T						
609	A						G	G				
734	G								A	A		
784	C	T	T	T	T	T						
798	A										G	
877	T						G	G				
888	T			C								
936	C								A	A		A
938	C											T
955	T	C	C	C	C	C						
976	T								A			
1057	T						C	C				
1078	G	A	A	A	A	A						
1102	T	C	C	C	C	C			C	C		C
1199	A								C	C		
1204	C						T	T				
nt	W3	MIL	DEN	LN	MEL	RQ	CPI+	CPI-	H221	78524	mci-2	SER

W3	MIL	DEN	LN	MEL	RQ	CPI+	CPI-	H221	78524	mci-2	SER	aa
N												14
V												24
Y							H					26
V						I	I					32
T						I	I					33
G			E									39
L							P					50
L												51
E												61
T												63
H												65
G												68
S											L	69
D	G								G			90
T												92
P												94
N										D		100
L							P					102
D												106
T												121
S	T											126
A												133
R												149
T											P	155
S												156
S	F	F	F	F	F	F	F		F			157
I												159
R/G						R/E	R/E					173/4
I/S	I/L	I/L	I/L	I/L	I/L							177/8
E/K						E/R	E/R					183/4
V								M	M			226
R												242
Q										R		247
T												273
M			T									277
T								K	K		K	293
L												294
V												299
N								K				306
I												333
Q												340
I												348
I								L	L			381
D												382
W3	MIL	DEN	LN	MEL	RQ	CPI+	CPI-	H221	78524	mci-2	SER	aa

nt	W3	MIL	DEN	LN	MEL	RQ	CPI+	CPI-	H221	78524	mci-2	SER	aa
102	T N							C					14
132	A V	G		G	G	G	G	G	G	G		G	24
136	T Y							C H					26
154	G V						A I	A I					32
158	C T						T I	T I					33
176	G G			A E									39
209	T L							C P					50
211	C L								T	T			51
243	G E						A	A					61
249	T T								C	C			63
255	T H									C			65
264	C G						T	T	T	T		T	68
266	C S											T L	69
329	A D	G G								G G			90
336	A T						G	G	G	G			92
342	C P						T	T				T	94
358	A N										G D		100
365	T L							C P					102
378	C D	T	T	T	T	T							106
423	A T	T	T	T	T	T							121
436	T S	A T											126
459	G A									A			133
507	A R	G	G	G	G	G							149
523	A T											C P	155
526	A S												156
530	C S	T F	T F	T F	T F	T F	T F	T F		T F			157
537	C I	T	T	T	T	T							159
579	G R/G						A R/E	A R/E					173/4
591	C I/S	T I/L	T I/L	T I/L	T I/L	T I/L							177/8
609	A E/K						G E/R	G E/R					183/4
734	G V								A M	A M			226
784	C R	T	T	T	T	T							242
798	A Q										G R		247
877	T T						G	G					273
888	T M			C T									277
936	C T								A K	A K		A K	293
938	C L											T	294
955	T V	C	C	C	C	C							299
976	T N								A K				306
1057	T I						C	C					333
1078	G Q	A	A	A	A	A							340
1102	T I	C	C	C	C	C			C	C		C	348
1199	A I								C L	C L			381
1204	C D						T	T					382
nt	W3	MIL	DEN	LN	MEL	RQ	CPI+	CPI-	H221	78524	mci-2	SER	aa

All isolates but CPI- degrade STAT1

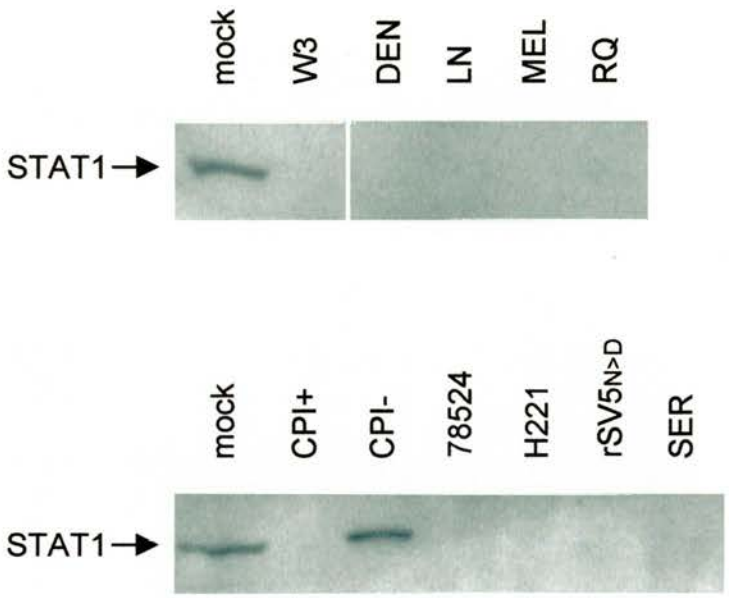
As shown in Figure 3.26, the results obtained from the analysis of human cell extracts revealed that all variants induced complete degradation of STAT1, with the exception of CPI-. Furthermore, the band of STAT1 in CPI- infected cells was more intense than that in mock-infected cells, indicating that virus infection triggered IFN production and a consequent STAT1 upregulation in these cells. Examination of MIL-infected monolayers by immunofluorescence showed that only a very low percentage of cells was infected by this isolate. For this reason, MIL was excluded from this immunoblot panel.

Similar results were obtained when the isolates were tested in canine cells. As presented in Figure 3.27, CPI- was the only isolate that failed to degrade STAT1 in MDCK cells. In contrast, the rest of the variants led to STAT1 degradation as efficiently as W3. To examine MIL in this immunoblot analysis, extracts were obtained from cells that had been infected with the isolate and passaged further, to ensure that all cells had been infected prior to immunoblotting (infected cell percentage was confirmed by immunofluorescence analysis). However, although the same procedure was followed for RQ, the majority of cells stained negative for viral proteins and this isolate was therefore excluded from this set of results (see next section).

3.4.3 Distribution pattern of NP and P in canine cells infected with RQ

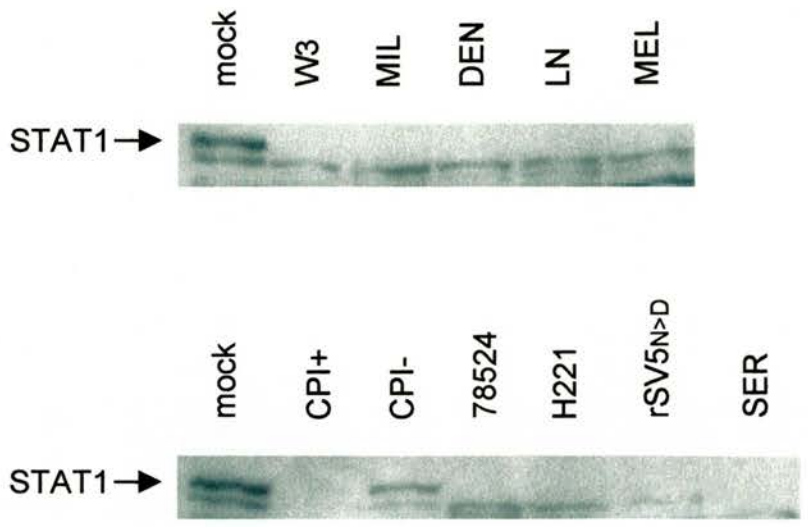
RQ forms cytoplasmic inclusion bodies

For the purposes of the immunoblot analysis discussed in the previous section, canine (MDCK) cells were infected with RQ at a high m.o.i. and examined by immunofluorescence, in order to confirm that all cells had been infected prior to immunoblotting. A preliminary examination of the cells soon after infection revealed that only a small number of cells were positive for viral proteins. For this reason, the same cells were passaged so as to allow the virus to proliferate and spread within the cell monolayer. Following passaging, a second immunofluorescence examination (several days after infection) revealed that a few cells showed bright cytoplasmic staining, as presented Figure 3.28. However, on closer examination, distinctive cytoplasmic inclusion bodies of aggregated NP and P proteins were observed in a higher percentage of cells, as shown in Figure 3.28, Panel A. Thus, since not all cells were



Figures 3.26: Immunoblot analysis showing the relative levels of STAT1 in human cells infected with the human SV5 isolates DEN, LN, MEL, RQ, the canine isolates CPI+, CPI-, H221, 78524, the recombinant $rSV5_{N>D}$ virus (derived from mci-2), or the porcine SER isolate.

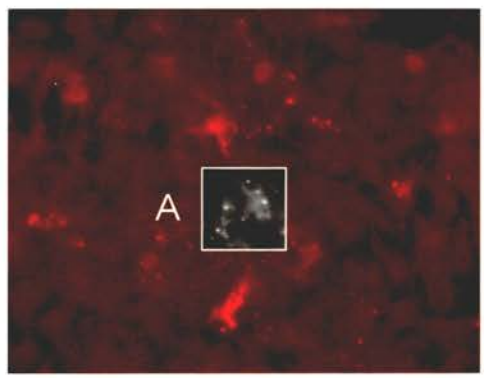
Human (2fTGH) cells were infected with these isolates (or mock infected) at a high m.o.i. At 29 h p.i. the cells were lysed and cellular proteins were separated by SDS-PAGE. Immunoblot analysis was subsequently performed using anti-STAT1 pAb.



Figures 3.27: Immunoblot analysis showing the relative levels of STAT1 in canine cells infected with the human SV5 isolates MIL, DEN, LN, MEL, the canine isolates CPI+, CPI-, H221, 78524, the recombinant $rSV5_{N>D}$ virus (derived from mci-2), or the porcine isolate SER.

Canine (MDCK) cells were infected with these viruses (or mock infected) at a high m.o.i. and lysed at 24 h p.i. Cellular proteins were analysed by SDS-PAGE and immunoblotted using anti-STAT1 pAb. Cells that had been infected with MIL were additionally passaged to ensure that all of the cells were positive for virus infection prior to harvesting.

MDCK, RQ



A

Figure 3.28: Immunofluorescence analysis of NP and P proteins in canine cells infected with RQ.

Canine cells (MDCK) were infected with RQ at a high m.o.i. and passaged once, in order to ensure that all of the cells had been infected by the virus. The cells were stained with anti-NP (SV5-NP-a) and anti-P (anti-P-e) mAbs, followed by a secondary anti-mouse Ig Texas Red-conjugated antibody. The cytoplasmic distribution pattern of NP and P is shown in greater detail in the magnified image area (close-up panel A).

infected with RQ and viral protein distribution seemed similar to that in CPI- infected cells, this observation suggested that RQ may be IFN-sensitive in canine cells. Nevertheless, this is something that requires further investigation.

3.4.4 Sequence analysis of the F genes of the different SV5 isolates

Focussing on the F gene

The origin of the human SV5 strains (MIL, DEN, LN, MEL and RQ), which were isolated from bone marrow tissue of human patients (see Chapter 1), has been previously questioned, with claims that their isolation only represents an experimental artefact from laboratory contamination. Assuming that the surface fusion protein (external protein) would be more variable than the V/P gene (internal protein), it was therefore of interest to sequence the F genes of these isolates, in order to complement the sequence variation data obtained from their V/P genes and obtain a further insight into their origin. Furthermore, since previous findings have linked the length of the F protein of a canine (T1) and a porcine (SER) isolate to differences in their fusogenic activity (Ito *et al.*, 2000; Seth *et al.*, 2002), it was also of interest to characterise further the human and canine isolates and determine the length of their F proteins.

Experimental outline

The analysis of the F genes of the examined SV5 isolates was done in collaboration with Dr. J. Andrejeva. In order to examine the variation in the length of the F gene, the F genes were amplified by RT-PCR from genomic RNA isolated from purified virus particles (ultracentrifugation), using oligonucleotide primers that annealed on the intergenic regions that flank the F gene (please refer to Chapter 1, Figure 1.4). The cloned genes were subsequently inserted into pGEM® T-Easy (see Chapter 2) and sequenced, as described above. The obtained data were analysed and, to extend the comparison of the sequences, the F proteins of the canine T1 (Ito *et al.*, 2000) and porcine SER (accession no. CAC07422; Klenk & Klenk, 2000) isolates were also included in the amino acid sequence alignment. Human RQ was excluded from this analysis, as no RT-PCR product was obtained from the experimental procedure followed.

Mutations Sequence analysis results from the F genes of the different SV5 isolates are shown in Figure 3.29. Comparison of the obtained sequences revealed a low degree of variation, with percentage amino acid variation ranging from 1.5 to 2.4% (or 1.1 to 2.1% without the 22-amino acid cytoplasmic tail extension). Furthermore, similar to the V gene sequence data (Figure 3.25), it was found that some mutations are exclusively present in canine or human isolates, whilst others are shared by all isolates regardless of their origin. Thus, exclusive to all human isolates are four identical amino acid changes, namely substitutions T_3I, $S_{19}G$, $L_{498}F$, whilst all canine isolates are marked by I_4R, $N_{92}Y$, $E_{132}K$, $A_{135}V$, $M_{310}I$ and $S_{438}T$. It is only canine H221 and 78524 which contain $A_{17}S$ and $Y_{377}F$, the former position, however, also being mutated in canine T1, but to a different amino acid ($A_{17}T$). Common to all examined isolates are substitutions $S_{443}P$ and $K_{529}N$. Mutation $V_{516}A$ is also present in all human and canine isolates, but not in the porcine SER isolate, which instead has $V_{516}T$ at this position. Strikingly, of the substitutions shared by all the canine isolates, $E_{132}K$, $M_{310}I$ and $S_{438}T$ are also present in porcine SER. In addition to these changes, there is a number of sporadic mutations that are unique for particular isolates, namely substitutions $I_{271}M$ and $L_{366}F$ in human LN and $M_{346}R$ in human MEL. Substitutions $V_{76}M$ and $V_{428}I$ are uniquely found in canine T1, whilst $A_{149}T$, $I_{489}M$ and $V_{370}M$ are only present in porcine SER.

F protein length With regard to the length of F, this sequence analysis revealed that the F proteins of all isolates is longer than that of W3, as the stop codon in the F genes of all isolates maps downstream that of wild-type F, resulting in a longer ORF. In almost all isolates, this accounts for a cytoplasmic extension that is identical (in length) to that reported for the canine SV5 isolate T1. Thus, compared to W3 F, all examined F proteins have a 22-amino acid extension at their carboxy-terminus, with the exception of the F protein from human MEL which have five additional residues. This result agreed with previously reported data (Randall *et al.*, 1987) from SDS analyses of radiolabelled virus polypeptides immune precipitated with mouse polyclonal anti-SV5 serum or anti-F mAb, which showed that, amongst the human isolates examined, MEL (referred to as MT in that paper) is the only isolate that has a shorter F protein. The present sequence alignment additionally revealed that there are amino acid differences between these short extensions, as all human isolates have Q_{530} and Q_{536} (the latter not being present in the shorter extension of MEL), whereas the examined canine isolates, as well as porcine SER, contain S_{530} and R_{536} at the respective residue positions.

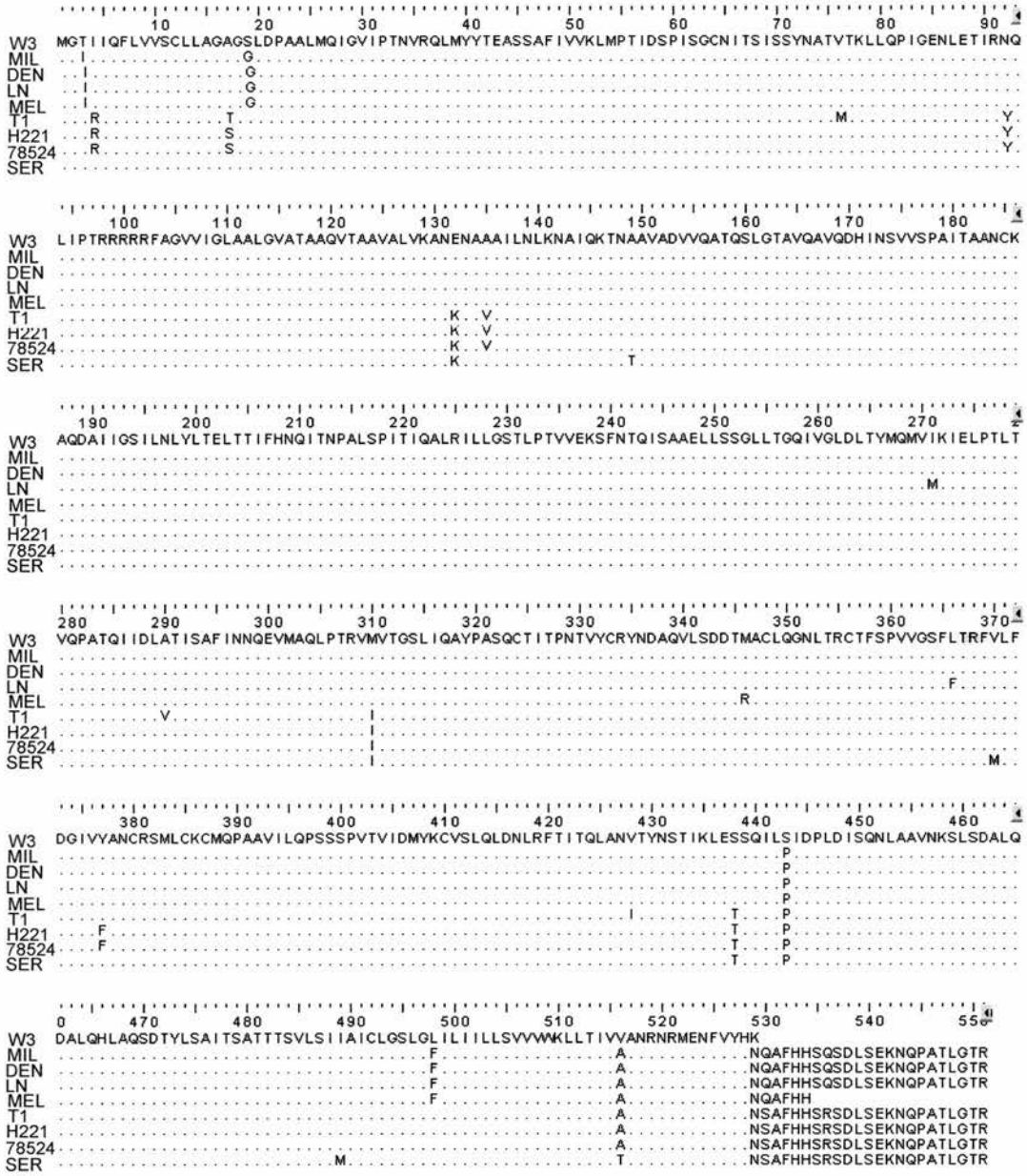


Figure 3.29: Amino acid sequence alignment of the F proteins of the human (MIL, DEN, LN, MEL), canine (H221, 78524, T1) and porcine (SER) SV5 isolates.

The F protein of W3 (wild-type strain) has been included in this analysis as a reference sequence. Amino acid residues that are identical to W3 F have been omitted, whereas residue substitutions are indicated at their respective positions. All aligned proteins (except W3 F) have a carboxy-terminal extension (amino acid position 530 onwards).

Chapter 4

Discussion

4.1 Insights from mci-2 and the CPI isolates

4.1.1 The amino-terminal domain of V is important for STAT1 degradation

4.1.1.1 Focussing on the amino-terminal domain

The carboxy-terminal domain

Previous reports have highlighted the importance of the unique, carboxy-terminal domain of V in blocking IFN signalling. This domain is highly conserved amongst paramyxoviruses and experiments have illustrated its counter-IFN properties particularly in rubulaviruses. Thus, Kubota and colleagues (2001) showed that the carboxy-terminal domain alone of the V protein from mumps virus is able to prevent the establishment of an IFN-induced antiviral state. Moreover, work on hPIV2, which is also closely related to SV5, revealed that a recombinant version of the virus, expressing a truncated form of V that lacks the carboxy-terminal domain, exhibited an IFN-sensitive phenotype (Kawano *et al.*, 2001), in contrast to wild-type hPIV2 which normally overcomes the IFN response.

The amino-terminal domain

Although the central role of the carboxy-terminal domain of V in blocking IFN-induced mechanisms is established, the experimental data presented in this report have demonstrated that the amino-terminal domain of V is also highly significant for the function of the protein. Thus, as revealed in the cases of the murine isolate mci-2 and the canine strain CPI-, amino acid substitutions in the amino-terminal domain of V can be sufficient to differentiate the ability of the protein to block IFN signalling, broadening research enquiry to the role of this domain in STAT1 targeting. A schematic overview of the V protein, indicating the regions involved in its known functions, is shown in Figure 4.1.

4.1.1.2 The Pk epitope of V

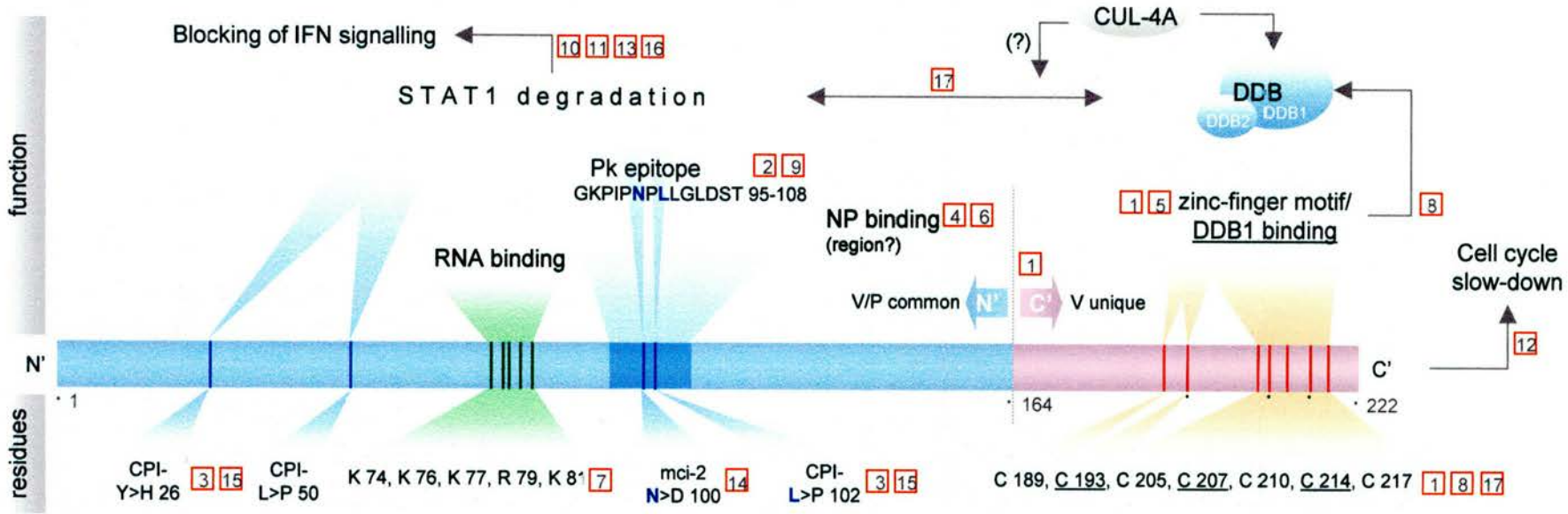
Murine mci-2 and canine CPI-, which have altered properties in terms of their ability to block IFN signalling, were both identified by the failure of their V (and P) protein to bind the anti-Pk antibody (mAb SV5-P-k). The Pk epitope, which maps to the V/P common amino-terminal domain of V and P, comprises eight amino acid residues (Dunn *et al.*, 1999; Southern *et al.*, 1991) and the contained asparagine amino acid

Figure 4.1: Schematic representation of the V protein of SV5 showing regions and amino acids that are involved in known properties of the protein.

The entire length of the protein is represented by a band which is divided in two parts, the amino-terminal domain (left-hand portion) and the carboxy-terminal domain (right-hand portion). Important residue positions that are known to be critical for certain properties of the protein (indicated in the upper part of the diagram) are schematically marked with vertical lines, the colour of which varies according to group (denoted function). Amino acid residues and position numbers are indicated in the lower part of the diagram, whilst substitutions of wild-type residues by different residues (identified in mutant virus isolates) are also marked accordingly (for example, the leucine to proline substitution at residue position 50 identified in the V protein of CPI- is indicated as $^{\text{CPI-}}_{\text{L>P 50}}$.)

All relevant literature references are listed below the diagram (in chronological order), with entry numbers pointing to relevant residues and properties of the protein.

SV5 V protein



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4 Precious, B., Young, D.F., Bermingham, A., Fearns, R., Ryan, M., Randall, R.E. (1995). *Journal of Virology* **69** (12), 8001-8010
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13 Young, D.F., Didcock, L., Goodbourn, S., Randall, R.E. (2000). *Virology* **269**, 383-390
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15 Chatziandreou, N., Young, D., Andrejeva, J., Goodbourn, S., Randall R.E. (2002). *Virology* **293**, 234-242
16 Andrejeva, J., Young, D.F., Goodbourn, S., Randall, R.E. (2002). *Journal of Virology* **76** (5), 2159-2167
17 Andrejeva, J., Poole, E., Young, D.F., Goodbourn, S., Randall, R.E. (2002). *In press*

Fig. 4.1

residue (residue N_{100}) has been shown to be essential for recognition by the anti-Pk mAb (Dunn *et al.*, 1999).

Pk in mci-2 In agreement with the failure of mci-2 and CPI- viruses to bind the anti-Pk antibody, sequence data for the Pk region of these viruses confirmed that these isolates have changes in their Pk epitope. In the case of mci-2 V, it was the substitution of the asparagine residue with aspartic acid (D) at position 100 (designated $N_{100}D$) which accounted for the observed failure of the anti-Pk mAb to stain infected cells. Strikingly, characterisation of mci-2 revealed that it has altered phenotypic properties in that, unlike W3 (wild-type strain), it blocks IFN signalling both in human and murine cells.

Pk in CPI- CPI- is another SV5 isolate that has an amino acid substitution mapping to the Pk epitope, but at a different residue (change of leucine (L) to proline (P) at residue 102, designated $L_{102}P$). This virus was also found to be different to W3, as well as to its parental strain CPI+, in terms of its ability to block IFN signalling. Nevertheless, in contrast to mci-2, CPI- was shown to have completely lost its ability to block IFN signalling in all examined cell lines. Mutagenesis-based examination of the role of the three mutations that are uniquely present in CPI- V revealed that, although the mutation in the Pk epitope is not sufficient to abolish the ability of the protein to block IFN signalling, it is certainly required for the synergistic effect of complete loss of function (all three substitutions, $Y_{26}H$, $L_{50}P$ and $L_{102}P$ are required for CPI- V to lose its ability to block IFN signalling). Thus, similar to the case of mci-2 V, it was demonstrated that alterations within the Pk epitope can have an impact on V function.

Conclusion Taking into account the above, and although the mechanism through which SV5 V targets STAT1 for degradation remains subject to experimental investigation, it appears that the Pk epitope of the V protein may have a central role in the molecular interactions that ultimately eliminate STAT1 in SV5-infected cells. It is noteworthy that, although the Pk epitope maps to the V/P common amino-terminal domain of both V and P, only V blocks IFN signalling, whilst P does not have this property (Didcock *et al.*, 1999b). Nevertheless, given that P forms tetramers and has a completely different carboxy-terminal domain to V, it is possible that the Pk epitope may be altered or sterically concealed in this protein. Therefore, apart from the role of the V amino-terminal domain

in STAT1 degradation, it seems certain that the V-unique carboxy-terminal domain is required for function.

4.1.2 Point mutations in V can change the phenotype of SV5

The most striking finding regarding the role of SV5 V, and particularly its amino-terminal domain, in counteracting the IFN response was that only few or, astonishingly, even a single amino acid substitution in the protein can dramatically affect the ability of the virus to block IFN signalling and thereby lead to major phenotypic changes. Thus, as mentioned above, three amino acid substitutions in CPI- V (residue substitutions $Y_{26}H$, $L_{50}P$ and $L_{102}P$) were shown to be responsible for the failure of this protein to block IFN signalling. Nevertheless, the simplest demonstration of the susceptibility of V function to amino acid changes came from the murine isolate mci-2. Characterisation of this isolate revealed that even a single point mutation in the amino-terminal domain of V (residue substitution $N_{100}D$) can profoundly alter the properties of the protein and, in this case, enable it to block signalling in murine cells, without affecting the wild-type ability to block ISRE activation in human cells. Remarkably, when $N_{100}D$ was introduced into the genome of W3, the recovered recombinant virus (rSV5-V/P $N_{100}D$) gained the ability to block IFN signalling in murine cells, while retaining its previous ability to produce the same effect in human cells. Moreover, further characterisation of rSV5-V/P $N_{100}D$ revealed that, compared to W3, this virus was also able to sustain longer periods of protein synthesis in murine cells (although expression levels eventually dropped; see section below). These observations thus not only highlighted the critical effect that a point mutation can have on the function of V, but also reflected the influential role of the ability of a virus to block IFN signalling in its metabolism. Interestingly, in the C proteins of Sendai virus (SeV), which are known to be the functional counterpart of the rubulavirus V as they also block IFN signalling (Didcock *et al.*, 1999a; Garcin *et al.*, 1999; Garcin *et al.*, 2000; Gotoh *et al.*, 1999; Komatsu *et al.*, 2000), a single amino acid substitution ($F_{170}S$) was again shown to be able to adversely influence their anti-IFN function, giving rise to a virus phenotype that was attenuated in mice (natural host) (Garcin *et al.*, 1997).

4.1.3 Changes in V affect viral protein synthesis levels and virus spread within a cell monolayer

Protein synthesis

In terms of their sensitivity to IFN, CPI+ is very different to CPI-, in that it blocks IFN signalling and thereby overcomes the IFN response. However, comparison of their protein synthesis levels in canine cells that produce and respond to IFN (high m.o.i.) revealed that there was not a major difference between the profiles of the two viruses. Thus, although at 18 and 24 h p.i. the protein synthesis level of CPI+ was higher than that of CPI-, by 48 h p.i. they reached the same levels, which indicated that, similar to CPI-, CPI+ protein synthesis was also restricted in cells that produce and respond to IFN. Apparently, the same effect of protein synthesis reduction was also observed in murine cells infected with the recombinant rSV5-V/P $N_{100}D$ virus (Young *et al.*, 2001), which, nevertheless, also blocks IFN signalling. Thus, by 72 h p.i., the protein synthesis levels of rSV5-V/P $N_{100}D$ in murine cells dropped dramatically, again indicating that a restricting factor must have acted upon the virus to drastically reduce its protein expression. Given that in both cases the examined viruses (CPI+ and rSV5-V/P $N_{100}D$ virus) are known to block IFN signalling in the particular cell lines (of canine and murine origin, respectively), it may be suggested that the observed inhibition of protein synthesis was either due to a cellular constraint, such as the induction of an antiviral state through an alternative pathway (via IRF-1 activation, for example), or due to a viral shift from transcription to replication caused by the rise of NP concentration above a critical metabolic threshold. Moreover, in the case of rSV5-V/P $N_{100}D$, a possible induction of an antiviral state in murine cells could also be due to a low level of signalling that was observed both in cells infected with rSV5-V/P $N_{100}D$ and in cells that expressed mci-2 V.

Virus spread

As presented in Chapter 3, CPI+ was able to spread more readily than CPI- within a cell population in the presence of exogenous IFN. However, IFN still had an inhibitory effect on CPI+ which appeared to replicate less efficiently and spread at a lower rate (than in cells that had not been treated with IFN). Therefore, it would be reasonable to assume that, although replication of CPI+ may be initially suppressed in cells that are in an antiviral state, its ability to degrade STAT1 leads gradually to the deterioration of the IFN response, which essentially allows the virus to thrive thereafter. In support of this assumption, Didcock and colleagues (1999b) have previously shown that SV5 is indeed

able to eliminate STAT1 in cells that are in an antiviral state, thus ultimately lifting the constraints posed by the IFN system on the virus and rendering the intracellular environment permissive for normal virus protein synthesis. Furthermore, virus replication seemed to be alleviated in cells that permanently express W3 V, indicating that V prevents the establishment of an IFN-induced antiviral state and thus enables the virus to spread at a higher rate. In confirmation of this, Andrejeva and colleagues (2002) have shown that W3 V-expressing human cells fail to respond to IFN and that the induction of an antiviral state is impaired in these cells.

4.1.4 Remarks on the investigation from mci-2 and CPI-

4.1.4.1 Interference with IFN signalling is a significant but not absolute advantage for SV5

mci-2 and $N_{100}D$

The fact that mci-2 was isolated from murine cells after a large number of passages suggests that the susceptibility of V and P function to mutations may act as a constraint on the selection of new variants, and that only few mutations in the V/P gene may be able to improve the performance of V without adversely affecting the life cycle of the virus. Nevertheless, despite the selection of such favourable mutations, the ability to block IFN signalling seems not to be the only factor that influences SV5 replication. Thus, although $N_{100}D$ undoubtedly conferred on the virus an obvious advantage, the recombinant rSV5-V/P $N_{100}D$ seemed to be subject to other constraints, as indicated by its reduced ability to generate infectious particles in murine cells (compared to human or simian cells). It is therefore possible that such constraints, which have an inhibitory effect on SV5, may also account for the attenuated *in vivo* phenotype of rSV5-V/P $N_{100}D$ which, despite its ability to block IFN signalling, was shown to be nonpathogenic in immunocompetent mice (unpublished observations).

4.1.4.2 Permanent cell lines as tools for the identification of molecular interactions of V with cellular components

The CPI isolates and permanent cell lines

Cell lines with altered properties have been used extensively to address questions in research. In the case of STAT1 degradation by SV5, the cells that express permanently the V protein from CPI+ and CPI- were designed for the dissection of the molecular

interactions involved in STAT1 targeting. Thus, comparison of the interaction of CPI+ and CPI- V with host cell proteins would enable further molecular characterisation of the protein. Furthermore, analysis of proteomes from cell lines that differ in the expression of a single protein would be a very powerful technique that could provide an insight into the effect of V on the cell, thus indicating new directions for experimental investigation. Time being a limiting factor, closer examination and characterisation of the stable CPI+/CPI- V cell lines was not possible to complete in this study. However, experimental data from CPI+ and CPI- V obtained recently by Dr. J. Andrejeva have shown that there is a difference in the ability of these proteins to bind DDB1, as, in contrast to the V proteins from W3 and CPI+, CPI- V appears to bind to this cellular protein very poorly, which correlates with its failure to block IFN signalling (Andrejeva *et al.*, in press). Since CPI+ and CPI- V differ only by three mutations in the amino-terminal domain, this finding further supported the belief that alterations in this domain may directly affect interactions of V with cellular proteins. Similarly, it would be of interest to take the examination of the SV5/hPIV2 hybrid-V cell lines further and try to determine differences in the ability of these proteins to interact with cellular components.

4.2 SV5 persistence and overview of the V protein

4.2.1 Model of persistent SV5 infection

The evidence collected from the present and previous reports have highlighted certain features of SV5 which can throw light on the mechanics of the establishment of persistent infections by this virus. As shown by the results from the experimental characterisation of the two canine strains, the key difference between CPI+ and CPI- is that CPI+ blocks IFN signalling, whereas CPI- does not. Nevertheless, CPI- was isolated 12 days p.i. from tissue that had been infected with CPI+. This fact may simply mirror that, under certain circumstances, the sensitivity to IFN may be beneficial for the survival of the virus in its host, as explained in the proposed model of persistent infection described further below. The fundamental principles and findings that allow the construction of such a model are first discussed in the following sections.

4.2.1.1 SV5 quasispecies populations

RNA virus variability

Paramyxoviruses are RNA viruses and give rise to 'quasispecies' virus populations (in virology, the term 'quasispecies' is used to refer to "a population of viruses that share a common origin but which have distinct genomic sequences as a result of mutation, drift and the impact of selection"; Smith *et al.*, 1997). Thus, it possible that many distinct variants may arise in an individual host, and these variants together form a 'quasispecies distribution'. The dynamics of such a population are characterised by high evolution rates, which are an immediate consequence of the occurrence of high population numbers, the high replication speed of the virus, as well as the tendency of the RNA replication machinery to introduce errors in the genome. Thus, high mutation rates ultimately lead to the generation of a pool of variants with new features which can prove beneficial for the fitness of the virus in its environment. In paramyxoviruses, the occurrence of the V/P gene, as an example of a complex strategy that dramatically increases the coding potential of the viral genome, has been linked to RNA virus quasispecies dynamics (Jordan *et al.*, 1999), and it is possible that different variant phenotypes of SV5, regarding the ability to circumvent the IFN system, are also highly likely to arise during virus proliferation.

4.2.1.2 Immune recognition of SV5-infected cells

SV5 avoids detection by the immune system

The ability of paramyxoviruses to establish persistent infections has been previously linked to evasion of immune recognition of virus-infected cells. Studies by Young and colleagues (1990) on mice infected with SV5 showed that *in vivo* clearance of persistent paramyxovirus infections is mediated by cellular immune responses but not serum-neutralising antibody. Thus, depletion of $CD8^+$ cells in mice resulted in a dramatic decrease in their ability to clear SV5 infection, whereas depletion of B cells did not have such an effect. Experimental demonstration of MHC class I-restricted CTL activity additionally suggested that the antiviral action of $CD8^+$ cells may involve cytolytic activity. Examination of the role of serum-neutralising antibody in virus clearance from immunodeficient mice showed that it had only a small effect. Moreover, studies on murine cells persistently infected with SV5 showed that neutralising antibody had a minimal influence on the cultures (Young *et al.*, 1997). Interestingly, similar findings were obtained by Canon and colleagues (1987) for another paramyxovirus, revealing

that persistent infection with respiratory syncytial virus (RSV) is cleared primarily by $CD8^+$ but not $CD4^+$ cells. Therefore, the ability of SV5 (and possibly of other paramyxoviruses) to maintain a persistent infection depends heavily on the ability of the virus to avoid immune recognition and elimination of virus-infected cells by cytolytic effector cells. Furthermore, since presentation of viral antigen on the surface of infected cells is a function of viral protein synthesis (via the cytosolic antigen-processing pathway and MHC class I-dependent antigen presentation), it seems that the ability of SV5 to persist in its host may involve the ability to remain transcriptionally inactive and quiesce the expression of virus sequences in the cell.

4.2.1.3 SV5 quiescence and inclusion bodies

SV5 can remain quiescent in the cell

The ability of SV5 to establish quiescent infections by remaining inactive in cells has been documented previously (Fearns *et al.*, 1994). As described in Chapter 1, experimental evidence from murine cells infected with SV5 revealed that nondefective virus could be recovered from cells that did not show signs of virus replication by immunofluorescence, and the cytoplasm of those cells was marked by the presence of inclusion bodies. In this same report, it was proposed that SV5 genomes may remain inactive in those tight cytoplasmic formations and that SV5 can be reactivated occasionally, once conditions become permissive for virus replication. In the present study, cytoplasmic inclusion bodies were also observed in canine cells infected with CPI-, which is sensitive to IFN, as well as in cells infected with the human strain RQ (although the latter remains to be further characterised). Taking into account the behaviour of SV5 in murine cells (in which it does not block IFN signalling), it would be reasonable to assume that the occurrence of inclusion bodies in cells infected with CPI- is linked to IFN sensitivity (see below), and that it may signify that the virus has entered a quiescent state.

4.2.1.4 IFN sensitivity can be subject to a single amino acid change in V

A single mutation can have a profound phenotypic effect

As mentioned above, it has become clear that few or even one mutation can be sufficient to differentiate the ability of SV5 V to block IFN signalling and thus drastically change the sensitivity of the virus to IFN. Whilst this finding reinforces the

notion that a quasispecies population can easily give rise to a phenotype with altered anti-IFN properties (discussed above), it also highlights the ease with which a single amino acid substitution may also revert an IFN-sensitive phenotype back to its previous state and restore its ability to circumvent the IFN response.

4.2.1.5 SV5 sensitivity to IFN and fluxing

SV5 can switch between active and inactive states

The suggestion that SV5 can exit quiescence and be reactivated (Fearns *et al.*, 1994) has been demonstrated experimentally in murine cells, in which SV5 went through multiple stages of inactivation and activation (Young *et al.*, 1997). In that study, although the majority of cells cleared the virus infection (8 to 15 days p.i.), some cells remained infected and the cell line became persistently infected. Virus fluxing between active and repressed states was observed upon passaging of the persistently infected cells as a result of IFN production, thus providing a link between inhibition of SV5 replication and IFN production in murine cells. Staining of the infected cell monolayers yielded irregular patterns of HN and NP expression, presumably revealing areas of repressed or ongoing virus protein synthesis in response to local IFN levels. Similarly, as presented in Chapter 3, there was an apparent inhibitory effect of exogenous IFN on HN and NP expression in cells infected with CPI- (IFN-sensitive) but not in cells infected with CPI+ (IFN-resistant). Thus, it may be assumed that fluctuations in IFN concentration *in vivo* may also cause localised repression or activation of virus replication, depending on the IFN sensitivity of the virus phenotype.

4.2.1.6 Proposed model of persistent SV5 infection

The model

The proposed model of persistent SV5 infection is schematically presented in Figure 4.2 (please also refer to figure legend). This model, which is based upon the principles and findings described above, provides a possible explanation of how SV5 escapes clearance by the host immune system. The isolation of the IFN-sensitive CPI- from an animal infected with CPI+ probably suggests that the former was selected for its ability to evade adaptive immunity. Thus, in the presence of IFN, although CPI+ would be able to sustain protein synthesis and become an obvious target for acquired immune responses (mediated by CTL cytotoxicity or antibody-dependent inactivation), CPI-

Figure 4.2: Schematic presentation of the persistent SV5 infection model proposed on the basis of findings from the canine CPI strains and the murine mci-2 isolate (see text).

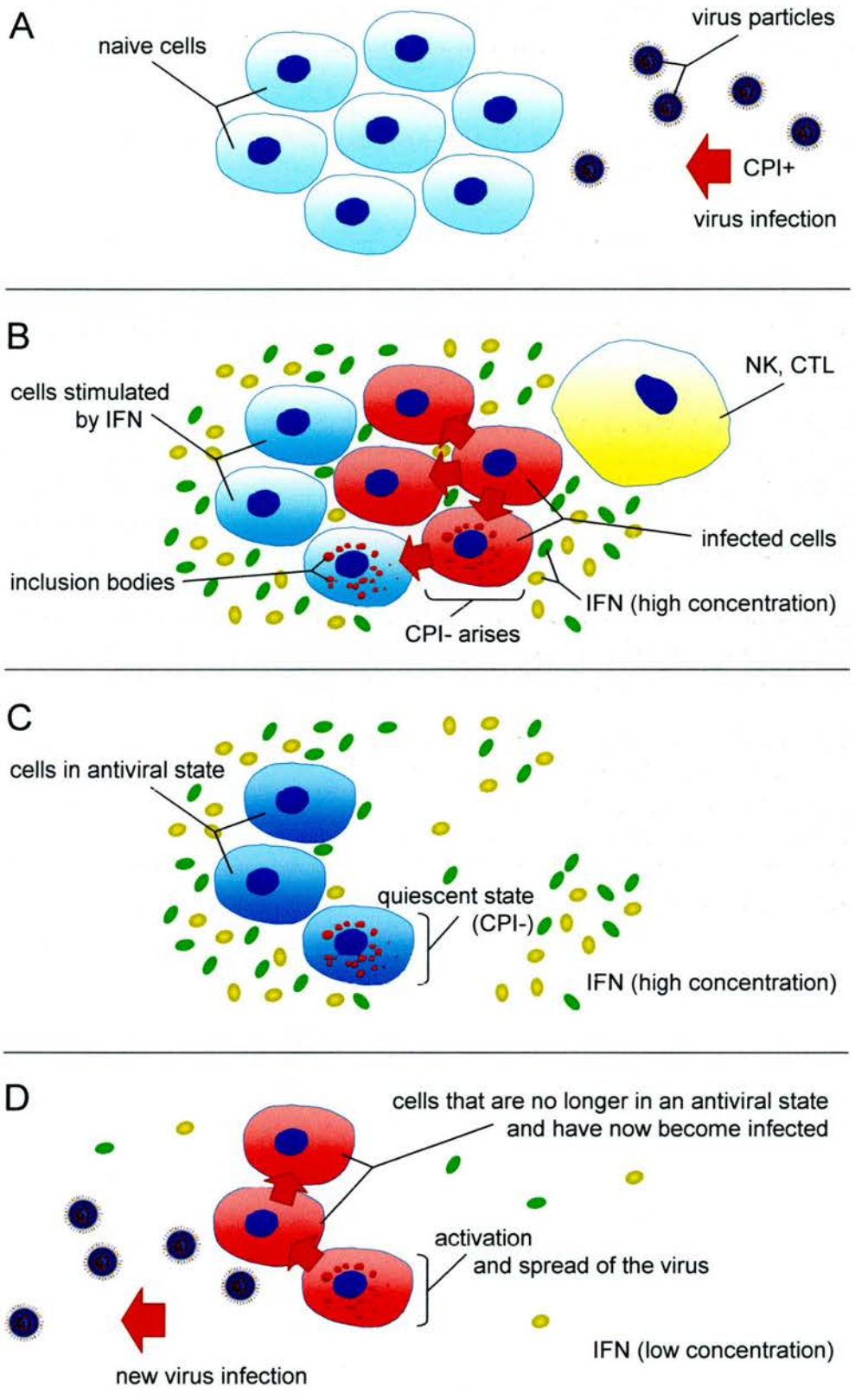
Panel A: Viral infection begins when infectious particles of CPI+ (IFN-resistant) come in contact with susceptible, naive host cells.

Panel B: Upon infection, IFNs are secreted (yellow and green particles) and their levels rise in the vicinity of the infection. Being IFN-resistant, CPI+ proliferates productively in cells (cells with red shading) and spreads to adjacent cells (red arrows). Productive infections result in viral antigen processing and presentation on the surface of infected cells, which in turn leads to activation of natural killer (NK) cells and cytotoxic T lymphocytes (CTL) (cell with yellow shading). In response to stimulation with IFN, neighbouring cells begin to enter an antiviral state (blue shading) that is nonpermissive for virus replication. CPI-, which arose during CPI+ proliferation (see text for details), is IFN-sensitive and enters a quiescent state in IFN-stimulated cells, becoming transcriptionally inactive and forming cytoplasmic inclusion bodies (red cytoplasmic particles).

Panel C: Cells that were infected productively in the previous phase have been cleared by NK cells and CTLs. IFN levels remain high, cells are in an antiviral state (dark blue shading), whilst CPI- remains quiescent.

Panel D: Following CPI+ clearance and inactivation of immune mechanisms (viral antigen is not displayed on the surface of cells), IFN levels drop. Cells that had previously entered an antiviral state return to their normal state, which is permissive for virus replication. CPI-, which remained quiescent in those cells thus escaping immune recognition, is activated once IFN constraints are withdrawn and starts to replicate productively (red shading), giving rise to new infectious virus particles.

Fig. 4.2


A
naive cells
virus particles
CPI+
virus infection
B
cells stimulated
by IFN
NK, CTL
infected cells
inclusion bodies
IFN (high concentration)
CPI- arises
C
cells in antiviral state
quiescent state
(CPI-)
IFN (high concentration)
D
cells that are no longer in an antiviral state
and have now become infected
activation
and spread of the virus
IFN (low concentration)
new virus infection

protein expression would be affected dramatically. In cells infected with CPI-, nucleocapsids would aggregate in cytoplasmic inclusion bodies and the viral genome would become unavailable for viral processes (Fearns *et al.*, 1994). In such a repressed state, the generation of foreign antigen by CPI- in the cell would be reduced, thus also preventing the expression and display of virus glycoproteins on the cell surface. Consequently, the effect of IFN on CPI- would essentially conceal the virus from the adaptive immune system, thereby saving it from clearance (Young *et al.*, 1990). Moreover, given the ability of SV5 to flux between repressed and active states in response to local IFN production (Young *et al.*, 1997), it would be reasonable to assume that the virus would be able to resume active replication once the acute immune response and inflammatory restrains were withdrawn from the vicinity of the infected cells. Since the acquisition of point mutations has been shown to be able to change the sensitivity of the virus to IFN, an active replication phase would be likely to give rise to revertant IFN-resistant variants that would be able to maintain productive infections in the absence of acute immune responses. The virus would therefore be able to further spread and probably be released in the environment and infect another individual.

4.2.1.7 Remarks on the proposed model

In the above model of persistent SV5 infection, virus survival is mediated by the occurrence of different variants that respond differently to the host antiviral measures, depending on their sensitivity to IFN and their ability to maintain protein expression. An attenuated virus such as CPI- may have been selected for its ability to escape immune recognition, thus not only providing a possible explanation of how certain paramyxoviruses establish persistent infections *in vivo*, but also suggesting that RNA viruses may be able to use the host IFN response to their own benefit. It therefore seems possible that, under certain conditions, the ability of a virus to block IFN signalling may not always be an absolute advantage. Furthermore, the ability of SV5 to mutate is a fundamental concept in the proposed model, as mutations in the V/P gene can have a dramatic impact on V function and the virus phenotype. Today, RNA viruses are indeed believed to be able to escape immune responses on the basis of their ability to mutate, and antigenic variation is an established example of this strategy.

4.3 SV5 isolates of different host species origin

4.3.1 V/P gene sequence analysis and examination of STAT1 degradation

Sequence analysis of the V/P genes from all the isolates examined revealed a low degree of variation (less than 3%), with the mutation patterns correlating with the host species origin of the viruses (see Chapter 3). Thus, taking into account both amino acid substitutions and silent mutations, there were mutations that were unique for all human isolates, whilst others were uniquely found in the V/P genes from canine isolates. Strikingly, there were not any amino acid substitutions mapping to the carboxy-terminal domain of the V protein (mutations that mapped to this gene region resulted in residue changes in the P ORF but were silent in the V ORF), confirming that this domain is highly conserved. This observation thus showed that the carboxy-terminal domain of V must play a critical role in the function of the protein, as any changes occurring in it were evolutionarily rejected. Interestingly, of the isolates examined, only CPI- failed to induce STAT1 degradation, suggesting that all other amino acid substitutions identified in the V proteins of the other isolates do not inhibit protein function. Furthermore, sequencing of the V/P genes from the different human isolates showed that there are mutations that are unique for certain strains (MIL, LN), thus disproving the previous notion that these isolates may represent a laboratory contamination artefact. This was further supported by the results from the analysis of the F gene sequences, as discussed in the following section.

4.3.2 F gene sequence analysis

Similar to the results from the V/P genes of the examined isolates, sequence analysis of the F gene sequences from the same isolates revealed a low degree of variation (with a percentage amino acid variation range of 1.5 to 2.4%). Strikingly, this analysis also showed that all F proteins are longer than W3 F, thus being similar to that of the canine T1 strain (Ito *et al.*, 2000). This therefore suggests that the W3 strain may only represent a SV5 variant with a truncated F protein, which either arose later than the variants isolated from nonsimian hosts (it seems possible that W3 lost part of its F carboxy-terminal domain through evolution), or which occurred in the laboratory as a result of repeated virus growing. Furthermore, with regard to glycoprotein sequence

variation in CPI+ and CPI-, the F genes of which were not included in this analysis, it is noteworthy that Baty and colleagues (1991) have reported previously that the HN proteins of these isolates also show a low degree of variation, with a maximum of 2.4% (CPI+). Thus, in agreement with the findings from the present analysis, the same researchers noted that variation in external glycoproteins and internal nucleocapsid-associated proteins occurs at equivalent rates, which probably represents the error rate of the virus RNA polymerase. With regard to the origin of the human isolates, and similar to the results from the V/P gene sequence analysis, the present analysis also showed that, although all human isolates share common mutations, some of them (namely LN and MEL) have unique amino acid substitutions. Thus, together with the V/P gene sequence data, this investigation essentially confirmed that these viruses represent genuinely different isolates.

4.3.3 Human SV5 isolates and persistence

The ability of the examined SV5 isolates to induce STAT1 degradation showed that these isolates are not sensitive to IFN, indicating that they are not likely to have persisted on the basis of IFN sensitivity. In addition, sequence analysis showed a low degree of variation in the V/P (and F) genes of these viruses, particularly in the less conserved amino-terminal domain of V, thus not providing any evidence of virus fluxing in response to IFN. Therefore, although IFN sensitivity may have played a critical role in the selection of CPI- in a dog infected with CPI+ (see model of persistent SV5 infection above), it seems possible that different phenotypic characteristic(s) must have enabled the human isolates to persist in their hosts and avoid clearance by the immune system. Furthermore, whereas RQ appeared to be IFN-resistant in human cells, in canine cells its replication seemed to be restricted, with its viral proteins being aggregated in inclusion bodies and resembling those observed in cells infected with CPI-. Whilst further experiments are required to investigate this case, it seems reasonable to suggest that RQ may be IFN-sensitive in cells of canine origin, which, if true, would reinforce the role of IFN as a major constraint on SV5 host species range. Should more SV5 isolates of different origin become available for laboratory studies, it would also be possible to determine whether the presence of mutations in SV5 isolates represents a random event or a product of virus adaptation in their respective host species.

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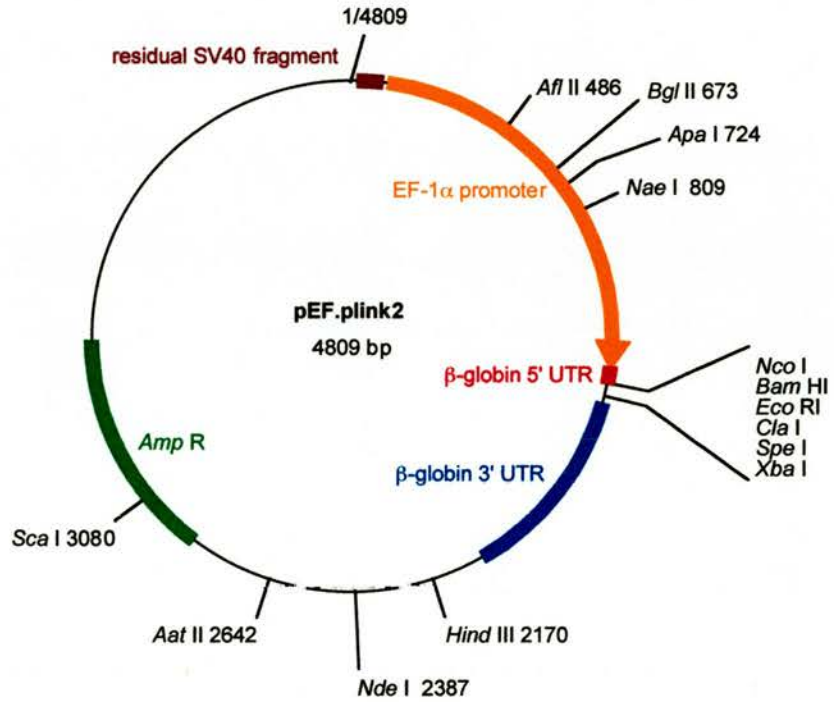
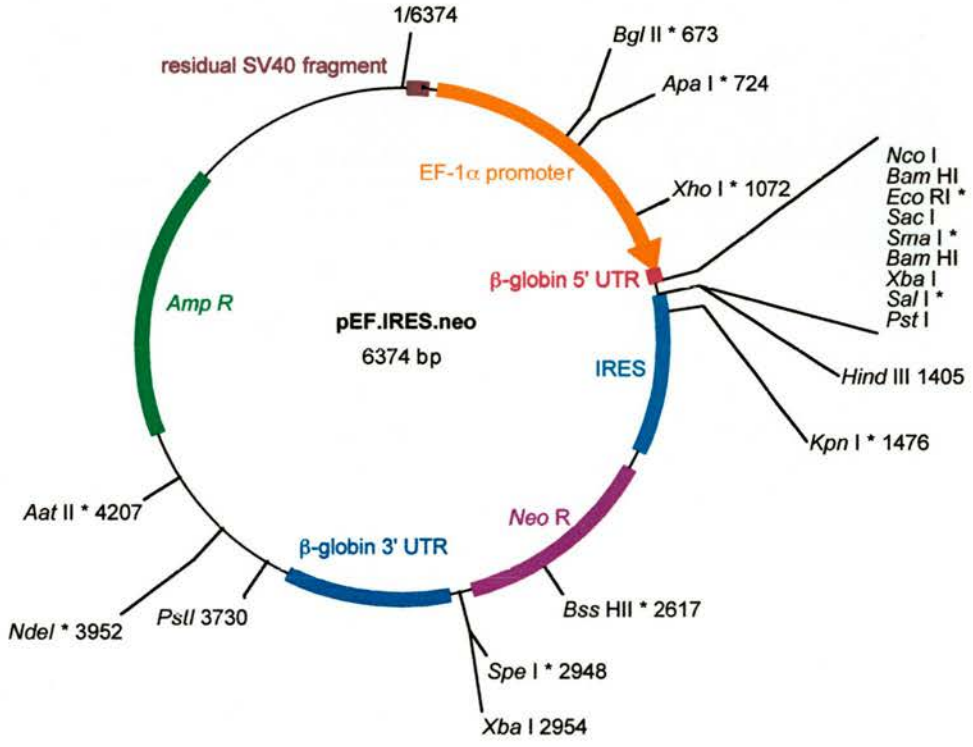
Appendix 1: Map of the pEF-plink2 expression vector (constructed by Dr. S. Goodbourn, St. George's Hospital Medical School, London). (diagram adapted from Nicola Stock)



Appendix 2: Map of the pEF-IRES.neo expression vector (constructed by Dr. S. Goodbourn, St. George's Hospital Medical School, London).

The internal ribosomal entry site (IRES) enables translation of the *Neo* R gene and the insert of interest from the same transcript. Asterisks indicate unique restriction endonuclease sites. (diagram adapted from Nicola Stock)



Appendix 3: Oligonucleotide primers used in DNA sequencing, gene cloning, mutagenesis, and hybrid-gene construction.

target DNA	direction	lab id code	length (nt)	Tm (°C)	sites	nucleotide sequence (5'-3')
pEF-plink2 vector	fwd	PEF-VECT	19	46.6		GCT TAC ATT TGC TTC TGA C
HN gene (SV5)	fwd	HN FOR (#198)	32	59.2	***Bgl* II**	CCT AG**A ' GAT CT**C ACA TAC AAT ACA CTA CAA TG
	rev	HN REV (#199)	20	51.7		CCT GAA GGC TTC TGC CTT TA
		HN #85 (INT)	20			
		HN #87 (INT)				
F gene (SV5)	fwd	FOR F-gene	17	46.9		CTG GAA TCA CCA GCT TG
	fwd	F FOR (#200)	34	65.6	***Xho* I**	CCT AG**C ' TCG AG**C CCA TAT CGT CCT TCA AAT CAT G
	fwd	SV5 F FOR INT	20	57.8		AGG AGA CGC CGG TTT GCA GG
	fwd	SV5 F INT FOR 2	23	58.7		GAT CCT ACT GGG GAG TAC CTT GC
		F #55 (INT)	21			
	rev	F REV (#201)	30	67.0	***Apa* I**	CTA C**GG GCC ' C**CG TGA GTG GTG GAA TGC TTA
	rev	REV F-gene	15	44.5		GAT TGA CCG AGA CGG
V/P gene (SV5)	fwd	P/V-FWD	32	64.3	***Bgl* II**	GGA AGA **A ' GA TCT** CAG CGC AAT CCA CAA TCC AC
	fwd	1S(ET)	33	66.8	***Eco*RI**	TCC CC**G ' AAT TC**C ATG GAT CCC ACT GAT CTG AGC
	fwd	W3 PV FOR 2	38	69.8	***Kpn* I**, *Nco* I	CCC CTT TT**G GTA C " C**A TGG CAG CGC AAT CCA CAA TCC AC
	fwd	PK-PVINT1	19	53.1		GCC ATT GTG CCA GCA GAT G
	rev	PK-PVINT2	19	48.8		CCA CAG TAA CAT TAC TGG G
	rev	2R(XHO1)	36	67.8	***Xho* I**	TGG TGC **C ' TC GAG** TCA AAT TGC ACT GCG GAT GAT TGC
	rev	P/V-REV	36	66.7	***Xba* I**, ***Xho* I**	CGG CG**T ' CTA GAC ' TCG AG**T GTA ATT GGG TGT TTC TTG
	rev	W3 PV - N' STOP	36	64.4	***Eco*R I**, **STOP**	GGG AAA **G ' AA TTC** TTA GCC CCT CTT AAA ATC GAT GGG
	fwd	W3C term FOR	33	66.8	***Eco*R I**, **START**	CCC TTT **G ' AA TTC** ATG AGG GAT ACC GGC GGG TTC
V/P gene mutagenesis (SV5)	fwd	MUTCPIPV136FOR (CM1 FOR)	56	70.4	**nt #136 (+)**	CTC ATA GAG ACA GGC CTG AAT ACT GTG GAG **c** AT TTT ACT TCC CAA CAA ATC ATA G
	rev	MUTCPIPV136REV (CM1 REV)	56	70.4	**nt #136 (-)**	CCT ATG ATT TGT TGG GAA GTA AAA T **g** C TCC ACA GTA TTC AGG CCT GTC TCT ATG A
	fwd	MUTCPIPV209FOR (CM2 FOR)	46	73.4	**nt #209 (+)**	CCA GGG GTC ACA GGA C **c** A CTA ACC AAT GCT GCA GAG GCA AAG ATC C
	rev	MUTCPIPV209REV (CM2 REV)	46	73.4	**nt #209 (-)**	GGA TCT TTG CCT CTG CAG CAT TGG TTA GT **g** GTC CTG TGA CCC CTG G
	fwd	MUTCPIPV365FOR (CM3 FOR)	38	72.0	**nt #365 (+)**	CCG ATC CCA AAC CCT C **c** A TTA GGT CTG GAC TCC ACC CC
	rev	MUTCPIPV365REV (CM3 REV)	38	72.0	**nt #365 (-)**	GGG GTG GAG TCC AGA CCT AAT **g** GA GGG TTT GGG ATC GG
V/P gene (hPIV2)	fwd	PIV2 PV FOR	29	65.7	***Kpn* I**, *Nco* I	CCC TTT **GGT AC " C** ATG GCC GAG GAA CCA AC
	rev	PIV2 PV REV	38	65.5	***Eco*R I**	GGG AAA **G ' AA TTC** TGG ATG CAT GTT GAG TGT CTC TTG TG
V/P gene hybrid (SV5/hPIV2)	fwd	W3/PIV2 PV FOR	37	67.7	**SV5** / hPIV2	**TAA GAG GGG C** / GG AGC TAA TAG AGA AAG AGC AAG AGG C
	rev	W3/PIV2 PV REV	37	67.7	**hPIV2** / SV5	**CTA TTA GCT CC** / G CCC CTC TTA AAA TCG ATG GGG GAA C
V/P gene hybrid (hPIV2/SV5)	fwd	PIV2/W3 PV FOR	37	71.0	**hPIV2** / SV5	**TAA GAG GGG G** / AG GGA TAC CGG CGG GTT CCA TAG AAG G
	rev	PIV2/W3 PV REV	35	69.0	**SV5** / hPIV2	**CGG TAT CCC T** / CC CCC TCT TAA AGT TGG GGT CTT GG

Appendix 4: Publications

Young, D. F., **Chatziandreou**, N., He, B., Goodbourn, S., Lamb, R. A., and Randall, R. E. (2001). Single amino acid substitution in the V protein of simian virus 5 differentiates its ability to block interferon signaling in human and murine cells. *J Virol* **75**, 3363-70.

Chatziandreou, N., Young, D., Andrejeva, J., Goodbourn, S., and Randall, R. E. (2002). Differences in interferon sensitivity and biological properties of two related isolates of simian virus 5: a model for virus persistence. *Virology* **293**, 234-42.

Comparative analysis of the V/P and F genes of simian virus 5 isolates of human and canine origin. (In preparation)