Interferon modulation of paramyxovirus infections

by

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This PhD Thesis is dedicated to my mum Regina

Abstract

The paramyxovirus, parainfluenza virus 5 (simian virus 5 [SV5]), is the prototype species of the genus *Rubulavirus* within the family *Paramyxoviridae*. It naturally infects humans and dogs and was used as a model paramyxovirus in this research project. Like many other paramyxoviruses, SV5 has evolved molecular mechanisms to circumvent the interferon (IFN)-induced antiviral response by blocking IFN signalling and by reducing the amount of IFN released by infected cells. However, its ability to circumvent the IFN response is not absolute. Therefore, the aim of this thesis was to examine the effects of IFN on SV5 infection. A model system was developed in which Vero cells, which do not produce but can respond to IFN, were infected with CPI-, a strain of SV5 that does not block IFN signalling, to analyse the effects of the addition of IFN on infection in the absence of virus countermeasures. Evidence from the results presented in this thesis highlighted that IFN induces a rapid (within 6 hours) alteration in the pattern of SV5 transcription and protein synthesis, as well as a redistribution of virus proteins within infected cells that result in an enhanced formation of virus cytoplasmic inclusion bodies. IFN caused a change in the virus transcription gradient that led to augmented levels of mRNA from genes at the 3' end of the genome and decreased levels of mRNA from genes at the 5' end of the genome. It also induced the production of M gene mRNAs with longer poly (A) tails, suggesting that the processivity of the virus polymerase was altered in cells in an IFN-induced antiviral state. Although not in complete concordance with mRNA levels, IFN also changed the pattern of protein synthesis, causing a specific down-regulation in the expression levels of genes downstream of the V/P gene. A similar delay of virus replication and formation of virus cytoplasmic inclusion bodies was also observed when cells in an IFN-induced antiviral state were subsequently infected with strains of SV5, parainfluenza virus type 2, and mumps virus that block IFN signalling. However, STAT1/STAT2 are eventually degraded with such viruses, and these viruses ultimately re-establish a more normal pattern of virus replication. Additional evidence is presented which suggests that CPI- can give rise to persistent infections in Vero cells in the presence of IFN, by remaining in a quiescent state, possibly in large inclusion bodies that could be observed in the cytoplasm. The nature of the inclusion bodies, as well as the molecular events that underlie their formation was investigated, as was the relevance of viral inclusion bodies to SV5 persistence.

Declarations

(i) I, Teresa Sequeira Carlos, hereby certify that this thesis, which is approximately 54,000 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.

Date ..22/11/05.... Signature of candidate

(ii) I was admitted as a research student in January 2002 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 2002 and 2005.

Date ..22/11/05.. Signature of candidate

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

Date 22/11/05. Signature of supervisor

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Abbreviations

Units

Amp	ampere
bp	basepair
Ci	Curie (3.7×10^{10} disintegrations/sec)
Da	Dalton
×g	acceleration of gravity
h	hour
kb	kilobase
l	litre
m	metre
min	minute
mol	mole
nm	nanometre
pfu	plaque-forming-units
sec	second
V	volt

Chemicals and reagents

ATP	adenosine triphosphate
CTP	cytidine triphosphate
DAPI	4', 6'- diamidino-2- phenylindole
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
FCS	foetal calf serum
GDP	guanosine diphosphate
GTP	guanosine triphosphate
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MOPS	3-(N-Morpholino)-propanesulfonic acid
NCS	newborn calf serum
^{32}P	phosphorus-32 radioisotope
PA	polyacrylamide
PBS	phosphate-buffered saline
PVDF	polyvinylidene difluoride
^{35}S	sulphate-35 radioisotope
SDS	sodium dodecyl sulfate
SSC	sodium chloride-sodium citrate
Taq	*Thermus aquaticus*
TBE buffer	TRIS-borate EDTA
TRIS-HCl	TRIS-hydroxymethyl-aminomethane
TTP	thymidine triphosphate

Nucleic acids

RNA	ribonucleic acid
dsRNA	double-stranded RNA
mRNA	messenger RNA
ssRNA	single-stranded RNA
tRNA	transfer RNA
DNA	2' deoxyribonucleic acid
cDNA	complementary DNA
ssDNA	single-stranded DNA
A	adenosine
C	cytidine
G	guanosine
I	inosine
T	thymidine
U	uridine

Viruses

bPIV3	Bovine parainfluenza virus type 3
BUNV	Bunyamwera virus
BVDV	Bovine viral diarrhoea virus
CDV	Canine distemper virus
CMV	Cytomegalovirus
CPI	Canine parainfluenza virus
EBV	Epstein-Barr virus
EMCV	Encephalomyocarditis virus
FLUAV	Influenza A virus
FLUBV	Influenza B virus
HCV	Hepatitis C virus
HeV	Hendra virus
HHV	Human herpes virus
HIV	Human immunodeficiency virus
hPIV1/2/3	Human parainfluenza virus types 1, 2 and 3
HPV	Human papilloma virus
HSV	Herpes simplex virus
LACV	La Crosse virus
mci-1/2	Murine cell isolate types 1 and 2
MeV	Measles virus
MuV	Mumps virus
NDV	Newcastle disease virus
NiV	Nipah virus
RSV	Respiratory syncytial virus
RVFV	Rift Valley fever virus
SeV	Sendai virus
SV5	Simian virus types 5
SV41	Simian virus types 41
THOV	Thogoto virus
TPMV	Tupaia virus
VSV	Vesicular stomatitis virus

Proteins and complexes

Ab	antibody
ADAR	adenosine deaminase
AP-1	activator protein-1
ATF	activating transcription factor
CBP	CREB-binding protein
CRF2	cytokine receptor family 2
Cul4a	cullin 4a
DDB	damage-specific DNA binding protein
DDB1	127-kDa DDB subunit
E2F1	E2F transcription factor 1
eIF	eukaryotic initiation factor
F	fusion glycoprotein
GAF	gamma-activated factor
Gal-T	Galactosyltransferase
GBP-1	guanylate-binding protein-1
GTPase	guanosine triphosphatase
HMG-I/Y	high mobility group chromatin proteins
HN	haemagglutinin-neuraminidase
IFIT1	IFN-induced protein with tetratricopeptide repeats
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase complex
IL	interleukin
IPS-1	IFN-β promoter stimulator 1
IRF	IFN-regulatory factor
ISGF3	ISG factor 3
ISRE	IFN-stimulated response element
Jak	Janus kinase
JNK	c-jun amino-terminal kinase
L	large protein
LAMP-70	Lysosome associated membrane protein 1
M	matrix protein
M6PR	Mannose 6-Phosphate-Receptor
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MCM5	minichromosome maintenance deficient 5
mda-5	melanoma differentiation associated gene-5
MyD88	myeloid differentiation factor 88
NEMO	NF-κB essential modulator
NF-κB	nuclear factor κB
NMI	nMYC and STAT interactor
NOS	nitric oxide synthase
NP	nucleocapsid protein
NS	nonstructural protein
2-5 OAS	2'-5' oligoadenylate synthetase
P	phosphoprotein
pAb	polyclonal antibody
PACT	PKR-activating protein

PI3K	phosphatidylinositol 3-kinase
PIAS	protein inhibitor of activated STAT
PKC	protein kinase C
PKR	Protein kinase R
PML	promyelocytic leukaemia protein
PML-NBs	PML nuclear bodies
PMP-70	70-kDa peroxisomal membrane protein
POD	PML oncogenic domain
RIG-I	retinoic acid-inducible gene I
RIP-1	receptor-interacting protein 1
RLI	RNaseL inhibitor
RNaseL	endoribonuclease L
SH	small hydrophobic
SHP-1	SH2-containing tyrosine phosphatase-1
SOCS	suppressors of cytokine signalling
STAT	signal transducer and activator of transcription
SUMO	Small Ubiquitinrelated Modifier
TANK	TRAF family member-associated NF-κB activator
TAP	transporter
TBK1	TANK binding kinase I
TFIIH	general transcription factor IIH
TICAM-1	TIR-containing adaptor molecule-1
TIR	toll-interleukin 1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
TRIF	TIR domain-containing adaptor-inducing IFN-β
Tyk2	tyrosine kinase 2
VAK	virus activated kinase

Miscellaneous

APC	antigen-presenting cell
Asn	asparagine
BF	BALB/c fibroblast
CARD	caspase recruitment domain
ECL	enhanced chemiluminescence
GAS	gamma-activated sequence
GenBank	National Institutes of Health (USA) genetic sequence database
ICTV	International Committee on Taxonomy of Viruses
IRES	internal ribosome entry site
ISG	IFN stimulated gene
Le+	leader RNA
m.o.i.	multiplicity of infection
MALDI-TOF	Matrix Assisted Laser Desorption /Ionization- Time Of Flight
MHC	major histocompatibility complex
NK	natural killer cell
ORF	open reading frame
p.i.	post-infection
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
PRD	positive regulatory domain
RT	reverse transcription
SCID	severe combined immunodeficient
Ser	serine
SSPE	subacute sclerosing panencephalitis
SWISS PROT	European Bioinformatics Institute protein database
Th	T helper cells
TPR	tetratricopeptides repeat
Tyr	tyrosine
UV	ultraviolet
VLPs	virus-like particles
wt	Wild type
Zn	zinc

Contents

1. INTRODUCTION

1.1 The *Paramyxoviridae*

The *Paramyxoviridae* contains a wide variety of significant pathogens that infect vertebrates, causing a number of important diseases of both humans and animals that have been well recognized since early studies in virology. The term "paramyxovirus", to be used, here refers to any member of the *Paramyxoviridae*. Measles virus (MeV) is a paramyxovirus that is considered one of the most infectious viruses known, in particular to children, which in rare instances is followed by subacute sclerosing panencephalitis (SSPE). Mumps is a ubiquitous and serious disease of early childhood in many regions of the world, causing parotitis, meningitis, encephalitis, and orchitis and can lead to sterility and deafness. Viruses such as the human parainfluenza viruses and the human respiratory syncytial virus (RSV) are the main causative agents of acute respiratory disease of infancy and early childhood.

The *Paramyxoviridae* also includes animal viruses of great economic significance, including Newcastle disease virus (NDV), bovine RSV and turkey rhinotracheitis virus, which affect poultry and livestock. Simian virus type 5 (SV5), also referred to as canine parainfluenza virus (CPI) and parainfluenza virus 5, causes respiratory illness in dogs (kennel cough) and has been suspected to cause other complications in humans.

The family also includes newly emergent agents, which have been discovered over the last decade in a wide range of animals (rodent, bat, rat, mouse, pig, horse, penguin, snake, porpoise, dolphin, seal; reviewed in Wang & Eaton, 2001). Although individual paramyxoviruses are often very host-specific, these recently isolated viruses have demonstrated the ability of viruses to cross host species. The newly emergent Hendra and Nipah viruses, whose natural reservoir appears to be fruit bats, have caused infectious outbreaks in farm animals, domestic animals and humans.

1.1.1 Classification of paramyxoviruses

The *Paramyxoviridae* family is a large group of enveloped, non-segmented negative-sense (-) stranded RNA viruses, which is part of the virus order *Mononegavirales*, together with three other families, *Rhabdoviridae* (Rabies, vesicular stomatitis, bovine ephemeral fever viruses), *Filoviridae* (Marburg and Ebola viruses), and *Bornaviridae* (Bornavirus). The families in the order *Mononegavirales* share several features; particularly they all contain single-stranded, linear RNA genomes of negative polarity, which have a similar organization and gene order. The genomic RNA (-) of all negative-strand RNA viruses serves as a template for synthesis of mRNAs and as a template for synthesis of the antigenome (+; positive-sense) strand, and therefore these viruses encode and package their own RNA polymerase. The newly synthesised antigenome (+) strand then serves as a template for further copies of genomic (-) RNA. Furthermore, the viruses in the *Mononegavirales* order are distinguished from other viruses by their genome size, cellular site of genome replication and transcription, as well as the extent of mRNA processing. Additionally, the viruses in this order appear to have a distinguishable nucleocapsid structure and viral proteins that are similar among the four families. However, viruses from each of the four families in the *Mononegavirales* order have different hosts, ranging from plants to mammals, and distinct morphological and biological properties.

The *Paramyxoviridae* family is divided into two-subfamilies, *Paramyxovirinae* and *Pneumovirinae*, which are further sub-divided into genera according to characteristics such as genome organization, virus morphology, protein characteristics and relatedness of protein sequences (reviewed in Lamb & Kolakofsky, 2001). *Pneumovirinae* has two genera, *Pneumovirus* and *Metapneumovirus*, while *Paramyxovirinae* subfamily includes five genera, namely *Respirovirus*, *Morbillivirus*, *Rubulavirus*, *Avulavirus*, and *Henipavirus* (Table 1.1). The latter two were approved to be included into the *Paramyxovirinae* sub-family by the latest International Committee on Taxonomy of Viruses (ICTV) in 2002, which was updated and published in 2005 (The Eighth

Table 1.1 Classification of the *Paramyxoviridae* family according to the Eighth Report of ICTV (2005), and list of species in each genus.

Family ***Paramyxoviridae***

Subfamily ***Paramyxovirinae***

Genus ***Respirovirus***
- Sendai virus (ScV)
- 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)
- Mapuera virus (MPRV)
- Mumps virus (MuV)
- human Parainfluenza virus type 2 (hPIV2)
- human Parainfluenza virus types 4a & 4b (hPIV4a/4b)

Genus ***Morbillivirus***
- Measles virus (MeV)
- Cetacean Morbillivirus (CeMV)
- Canine Distemper virus (CDV)
- Peste-des-petits-ruminants virus (PPRV)
- Phocine distemper virus (PDV)
- Rinderpest virus (RPV)

Genus ***Henipavirus***
- Hendra virus (HeV)
- Nipah virus (NiV)

Genus ***Avulavirus***
- Newcastle Disease virus (NDV)
- Avian paramyxovirus 1 to 9 (APMV-1 to -9)

Genus ***"TPMV-like Viruses"***
- Tupaia virus (TPMV)

Subfamily ***Pneumovirinae***

Genus ***Pneumovirus***
- human respiratory syncytial virus (hRSV)
- bovine respiratory syncytial virus (bRSV)
- Murine pneumonia virus (MPV)

Genus ***Metapneumovirus***
- Turkey rhinotracheitis virus (TRTV)

Report of the International Committee on Taxonomy of Viruses, 2005). The Tupaia virus is still to be assigned to a genus, but has been categorized in an independent genus, named "*TPMV-like viruses*" genus. The *Paramyxoviridae* have a RNA genome of approximately 15 kb, with exception for the genomes of henipaviruses and TPMV-like viruses that are relatively long (ranging from 17.9 to 18.2 kb) and those of the *Metapneumovirus* that are relatively short (13.4 kb).

1.1.2 Virion structure

Paramyxovirus virions have typically spherical forms ranging from 150 to 350 nm in diameter, although pleomorphic and filamentous forms have been also observed (1000 to 10000 nm long). The *Paramyxoviridae* have a lipid bilayer envelope derived from the plasma membrane of the host cell in which the virus replicates, in which are embedded the glycoprotein spikes that extend about 8 to 12 nm from the surface of the membrane. The surface glycoproteins for membrane fusion (F) and attachment (HN, H or G) mediate virus entry into the host cell during infection and exit from the host cell during maturation and release. The fusion proteins are reasonably similar among the *Paramyxoviridae*, whilst the attachment glycoprotein is more variable, and is used as one of the criteria for the classification of virus species. Those of the *Respirovirus* and *Rubulavirus* genera have an attachment glycoprotein with both haemagglutinin and neuraminadase activities (termed HN protein), while those of the *Morbillivirus* genus have a protein with haemagglutinin activity only (termed H protein) and those of the *Henipavirus* genus and *Pneumovirinae* subfamily have an attachment protein with neither activity (termed G protein). Inserted into the lipid envelope are also copies of the SH (small hydrophobic) protein, although this protein is only present in some rubulaviruses (including SV5), and in members of the subfamily *Pneumovirinae*. The matrix (M) protein, which is one of the most abundant proteins in the virion, is located on the inner surface of the envelope and plays an important role in the integrity and architecture of the virus particle. The M protein is released from the virion during virus entry and is thought to be involved in the formation and budding of virus particles. Within the lipid envelope is the internal helical nucleocapsid core, which is remarkably

stable. This core structure consists of genomic RNA, which is encapsidated by the nucleoprotein (N or NP), forming helices that confer a "herring-bone" appearance on the helical ribonucleo-protein capsid when viewed by transmission electron microscopy, and negative staining (Figure 1.1). Associated with the nucleocapsid is the virus RNA-dependent RNA polymerase complex, consisting of the phospho (P) and large (L) proteins (reviewed in Lamb & Kolakofsky, 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.

Paramyxovirus helical nucleocapsid, rather than the free RNA genome, is the template for all RNA synthesis, and the genomic RNA complexed with the NP (or N), P and L proteins are thought to be the minimal unit of infectivity, and have the ability to transcribe mRNAs *in vitro*. Paramyxovirus helical nucleocapsids do not disassemble in the course of viral infection, although the helical structures do exist in a number of different forms of varying pitch. It is thought that RNAs complementary to the template are made without dissociation of the helical nucleocapsid, although to allow the viral polymerase access to the RNA bases it may be necessary to locally uncoil the nucleocapsid. Therefore, it is possible that the viral polymerase is able to uncoil and recoil the helix as it moves along the genome, allowing transcription and replication to occur. Depending on the helix to be in the uncoiled or coiled forms, the nucleocapsid has different values of helical pitch, and those differences are reflected on different values of diameter and consequently different nucleocapsid lengths. Differences in nucleocapsid morphology are also used as criteria for classification of *Paramyxoviridae*, and the *Pneumovirinae* are morphologically distinguished from the *Paramyxovirinae*, as the former contain narrower nucleocapsids.

In addition to the common six gene products essential for viral replication, paramyxoviruses encode a subset of accessory proteins, largely unique to each genus and some of these are present in the virion. These accessory proteins include (in the *Paramyxovirinae* subfamily) the various products of the P gene and (in pneumoviruses) the matrix protein 2 (M2) and nonstructural proteins

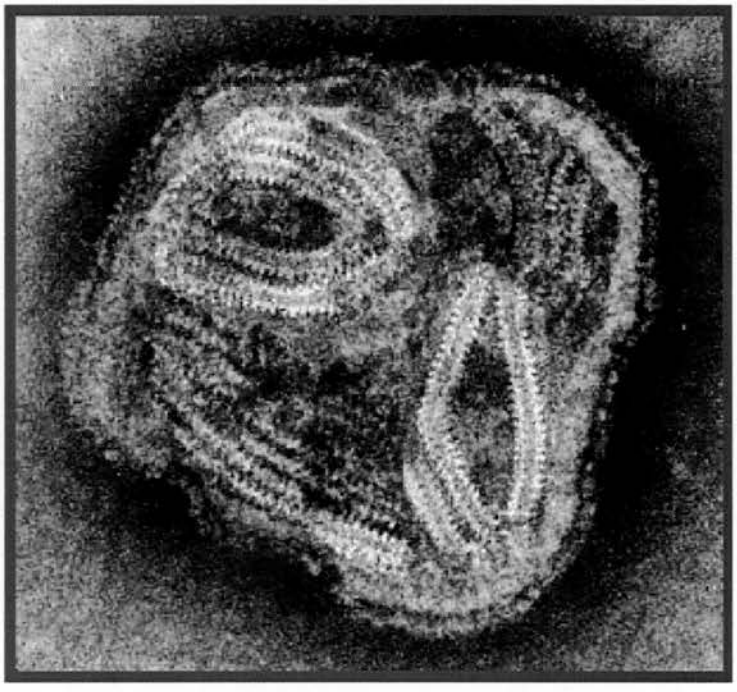
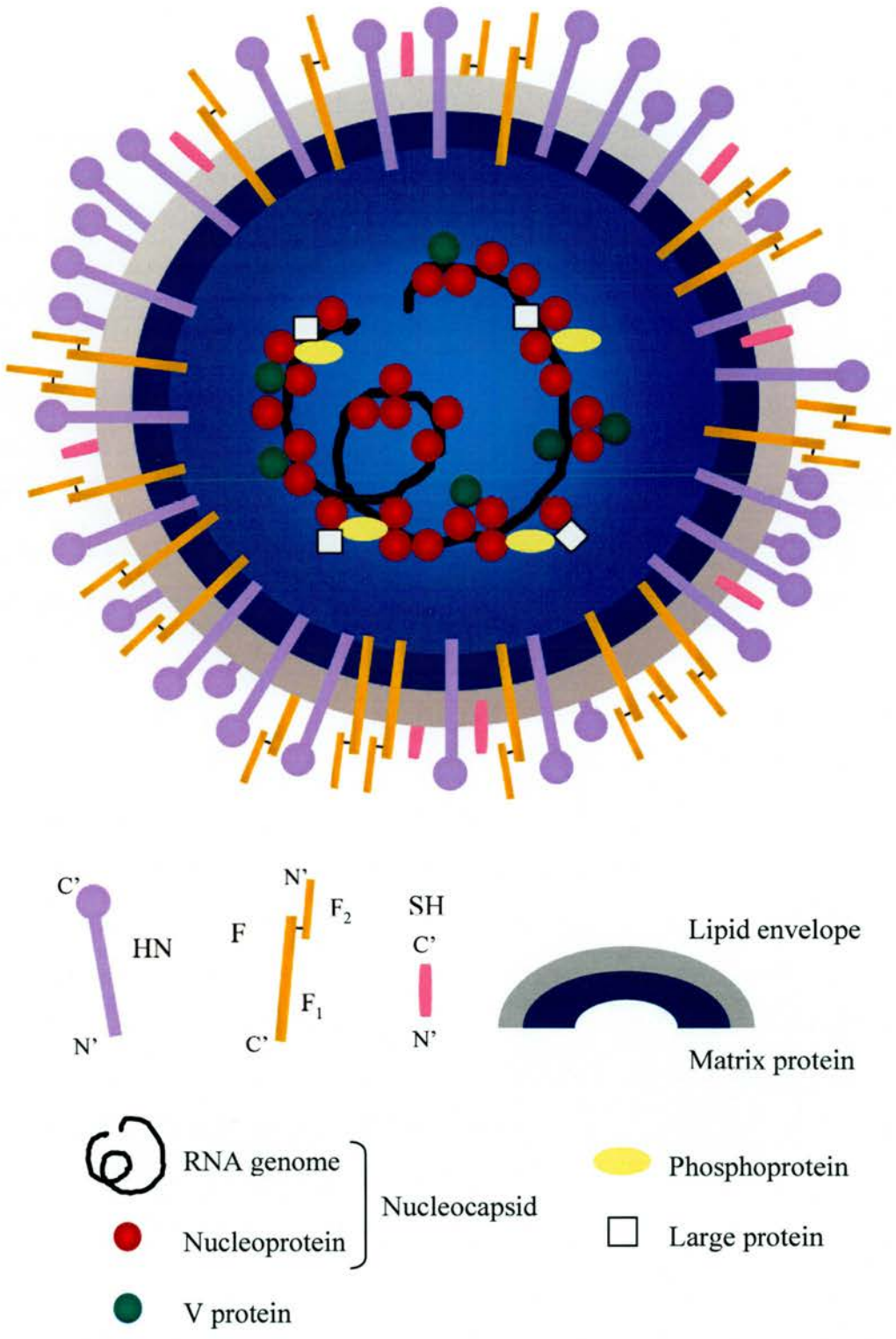


Figure 1.1 Electron micrograph of a human paramyxovirus virion, showing the "herring-bone" appearance of the helical ribonucleo-protein capsid structures (obtained from Linda Stannard's Virus Ultrastructure web site, Department of Medical Microbiology, University of Cape Town; http://web.uct.ac.za/depts/mmi/stannard/paramyx.html).

Figure 1.2 Schematic representation of the virion structure of simian virus type 5 (SV5), a typical paramyxovirus (not drawn to scale).

The virus is coated by a lipid envelope (shown as a grey circle), and underlying the lipid bilayer is the viral matrix protein (shown as a dark blue circle), which contacts with the nucleocapsid core and is essential for the architecture of the virion. Embedded in the lipid bilayer are the hemagglutinin-neuraminadase (HN) attachment glycoprotein and the fusion (F) glycoprotein, which project from the surface of the virus particle. The HN protein is thought to have a structure that comprises a stalk region and a globular head, whilst the F protein consists of two disulphide-linked chains (F_1 and F_2). The HN protein is tetrameric and the F protein trimeric, features not illustrated in the diagram neither is their relative abundance. The small hydrophobic (SH) protein is also shown, which is inserted in the lipid bilayer in some rubulaviruses, such as SV5. The orientation of the membrane-anchored proteins is indicated in the key of the diagram. The matrix protein is thought to hold the virus particle together, bridging the surface proteins with the nucleocapsid. The helical nucleocapsid is found in the core of the particle and comprises the single-stranded, non-segmented, negative-sense genomic RNA of the virus, which is encapsidated with the nucleocapsid protein (NP). Associated with the nucleocapsid are the phospho- (P) and large (L) proteins, and together this complex has RNA-dependent RNA transcriptase activity (P and L together form the virus RNA polymerase). The cysteine-rich V protein of SV5, as in all rubulaviruses, is also associated with the nucleocapsid, whereas for other members of the *Paramyxoviridae*, the V protein is only found in infected cells. Possible interactions between the cytoplasmic tails of the glycoproteins and the M protein, and the interactions between the M protein and the nucleocapsid are not clear, and thus no attempt has been made to show them in the diagram (reviewed in Lamb & Kolakofsky, 2001).

Figure 1.2


C'
HN
N'
N'
F
F_2
F_1
C'
SH
C'
N'
Lipid envelope
Matrix protein
RNA genome
Nucleoprotein
V protein
Nucleocapsid
Phosphoprotein
Large protein

(NSs). The different coding potentials of the P genes is another criterion used to distinguish members of the subfamily *Paramyxovirinae*.

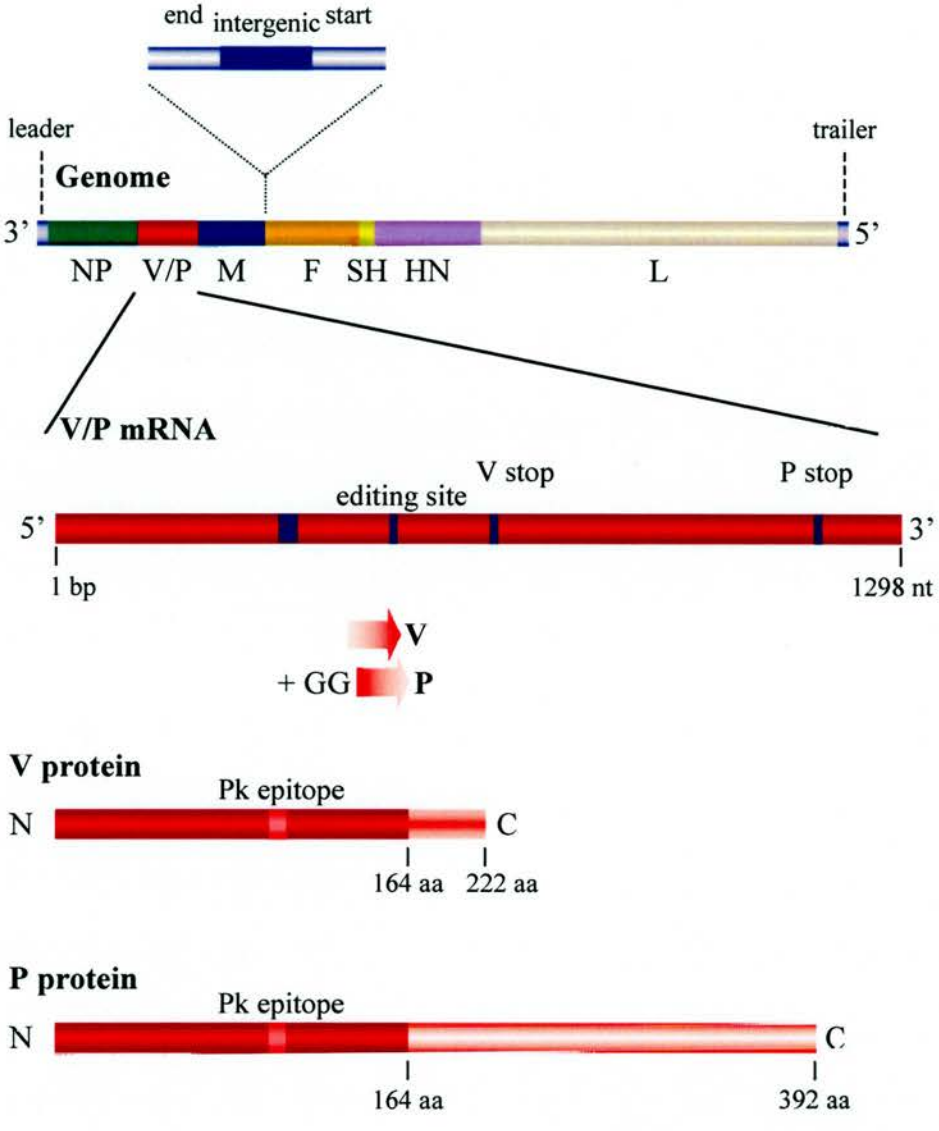
1.1.3 Virus genome

The genome of paramyxoviruses is single-stranded, non-segmented, negative-sense RNA and its length ranges from 15 kb to 19 kb. One of the smallest genomes in the *Paramyxovirinae* subfamily is that of SV5 (15246 bp; GenBank Accession number AF052755), whilst one of the longest genome is the one of the newly identified Nipah virus (NiV) (18246 bp; GenBank Accession number AF212302). The complete genome sequences for most of the known members of the *Paramyxoviridae* have been determined and they contain 6 to 10 tandemly linked genes, depending on the species. However, the number of proteins encoded by the genome of *Paramyxovirinae* is extended by the use of overlapping open reading frames (ORFs) in their P genes, which leads to distinct gene products, as described below. The organization of the SV5 genome, which includes seven genes, and the structure of the SV5 V/P gene are schematically represented in Figure 1.3. By convention, for paramyxoviruses, the term gene refers to a part of the genome (between two intergenic sequences) which encodes one or more mRNAs and/or mRNAs containing overlapping reading frames, thus allowing more than one gene product per gene. Each gene contains transcriptional control sequences at the beginning and end of the open reading frame that are copied into the mRNA, which are highly conserved in some genera and semiconserved in others. All genes are separated by non-transcribed intergenic regions, which are precisely three nucleotides long for the respiroviruses and morbilliviruses but are quite variable, differing both in sequence and length, for the rubulaviruses (1 to 47 nucleotides) and pneumoviruses (1 to 56 nucleotides). The paramyxovirus gene junctions are, by definition, divided into three regions: the gene end region at the 3' end of the upstream gene, the non-transcribed intergenic region between the two genes, and the gene start site of the 5' downstream gene. The nucleotide sequences located at the gene junctions of SV5 genome are listed in Table 1.2. The junction between the tandemly linked viral genes contains important *cis*-acting signals that direct

Figure 1.3 Schematic representation of the SV5 genome organization and its V/P gene coding strategy, as well as the structure of the encoded P and V proteins.

The SV5 genome contains seven genes that code for eight distinct proteins, namely NP, V/P (V and P proteins), M, F, SH, HN and L. The genome, which is single-stranded, non-segmented, 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 separated 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 non-templated 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 (reviewed in Lamb & Kolakofsky, 2001).

Figure 1.3



adapted from Chatziandreou, 2002

the viral polymerase to carry out sequential and polar stop-start transcription (described below), but these signals are ignored during the replication process.

Table 1.2 The nucleotide sequences at the gene end, intergenic and gene start regions for each SV5 gene (adapted from Rassa & Parks, 1998). The sequences are listed as genomic RNA (3' to 5').

Genes	Gene end	Intergenic	Gene start
NP-P	$CCGAAAUUUCU_7$	A	UCCGGGCCUG
P-M	$UCCCAAAAUCU_6$	GCUAAUUGCUAUUUA	UUCGGGCUUG
M-F	$ACUAAGUUUCU_4$	GUUUAGUAUAAUUCUGAUAGGA	UUCGUGCUUG
F-SH	$UUAAAAAUUCU_7$	GCUA	UCCUGGCUUG
SH-HN	$AUAAAAUUUCU_6$	A	UCCGGGCUUG
HN-L	$ACCAAAAUUCU_5$	GGUUCUCUUGUUA	UCCGGUCUUA
L-tr	$AAUCAAAUUCU_6$		

The genome of paramyxoviruses is flanked by control regions that play an important role in transcription and replication, the 3' extracistronic (leader) and the 5' extracistronic (trailer or the (-) leader) regions, which are about 50 nucleotides in length (reviewed in Whelan *et al.*, 2004). In the SV5 genome, the leader and trailer sequences are 55 and 30 nucleotides, respectively, and are essential for virus RNA synthesis. The paramyxovirus genomic and antigenomic RNAs contain 3' end *cis*-acting signals that are a major factor controlling viral RNA replication. The 3'-terminal 12 nucleotides of the genomes and antigenomes are almost identical in each genus of the *Paramyxoviridae*, suggesting their critical roles as the replication promoter. Genomic RNAs can be found at high(er) levels in infected cells, they serve essential functions in the viral growth cycle, namely acting as a template for producing mRNAs, acting as a template for synthesis of antigenomic RNA, and incorporation into progeny virions (reviewed in Lamb & Kolakofsky, 2001). In contrast, the antigenomic RNAs are found in lower amounts, and it appears to function only as a template to amplify genomic RNA.

1.1.4 Replication cycle

To initiate infection *in vivo*, paramyxoviruses have to infect mucosal surfaces of the respiratory tract of the host. The replication cycle of a typical paramyxovirus (SV5), which is schematically presented in Figure 1.4, begins with attachment of the virus particle to the receptor proteins on the outer surface of a suitable host cell (adsorption), followed by fusion of the virion envelope with the cell membrane (fusion). Adsorption and fusion are driven by the catalytic action of the attachment and fusion proteins, respectively. The next event involves entry, uncoating and release into the cytoplasm of the viral RNA genome, encapsidated with the nucleocapsid protein (ribonucleocapsid complex; RNP) and associated with the RNA-dependent-RNA polymerase. Like other paramyxoviruses, SV5 must generate three types of RNA during its infectious cycle, namely mRNAs, and full-length antigenomic and genomic RNAs. Following entry of the RNP complex, all the subsequent intracellular phases of replication take place, i.e. transcription and translation for synthesis of viral proteins, genome replication for synthesis of the new viral nucleic acid and, finally, assembly and release of the newly maturated virus particles (virions). The M protein, which lines the inner face of the cell membrane, is believed to be the driving force for the assembly of the new nucleocapsids and release of new virus particles into the extracellular environment. At this stage the newly synthesised glycoproteins have been also embedded into the cell membrane, and are believed to play a role in this final step, as the neuraminidase activity of the attachment glycoprotein (rubulaviruses and respiroviruses) prevents the aggregation of the newly formed virus particles (Lamb & Kolakofsky, 2001). The life cycle is completed in the cytoplasm of the host cell and generally extends from 14 to 20 hours.

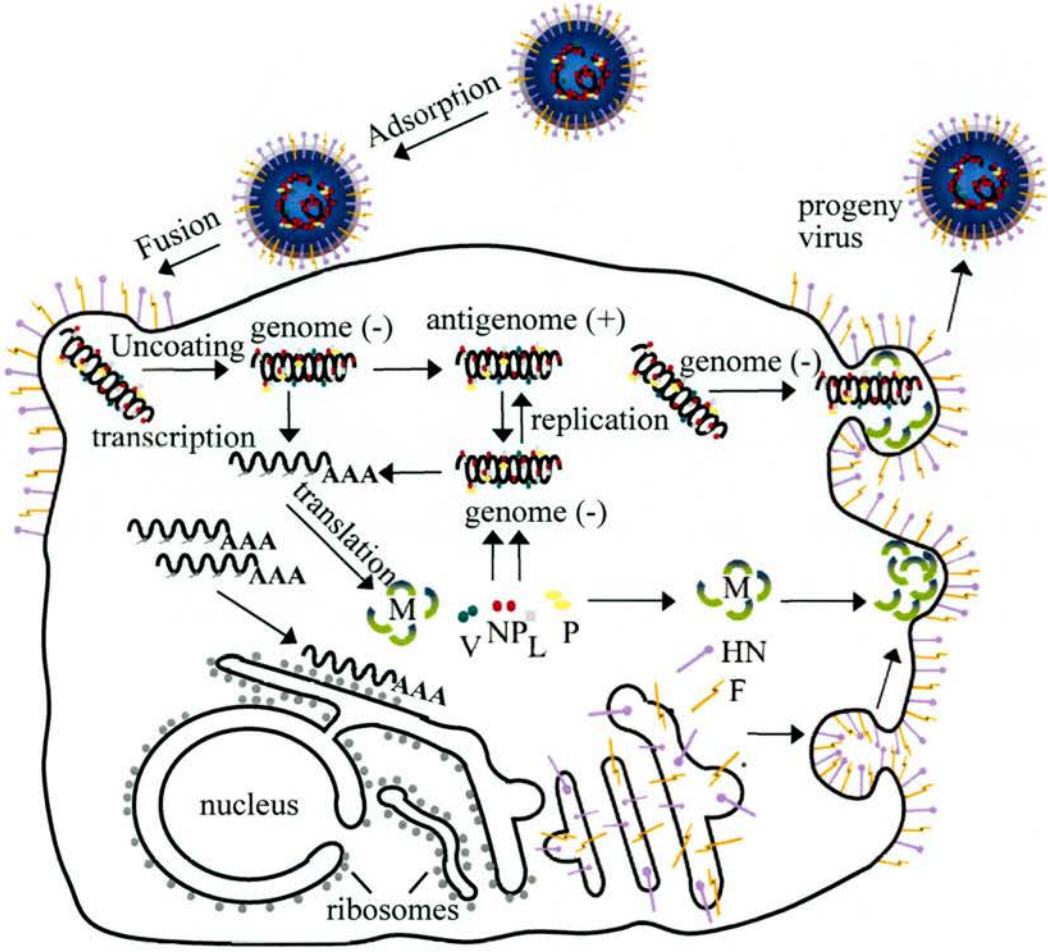
1.1.4.1 Primary transcription and translation

It is believed that all viral RNA synthesis begins at the very 3' end of the genome, at position 1, and the *cis*-acting promoter sequences serve the dual function of initiating a leader RNA and subsequently the viral mRNAs, as well as antigenomes. Early in infection (before the accumulation of primary translation

Figure 1.4 Schematic representation of the SV5 replication cycle.

The first step necessary for the virus to infect a cell is virion attachment, in which the hemagglutinin-neuraminadase (HN) protein plays a central role as this viral protein binds glycoprotein receptors on the surface of the host cell. Fusion of the virus envelope with the host cell membrane is mediated primarily by the fusion (F) protein, virus entry continues with uncoating and release of the genome (negative-sense, input RNA) into the cytoplasm. The entire replication cycle occurs in the cytoplasm and begins with transcription of the input viral (negative-sense) genomes by the viral RNA polymerase complex (primary transcription), the resulting capped, polyadenylated mRNAs are translated into viral proteins. The polymerase complex also replicates the entire (negative-sense) genome via a replicative (positive-sense) intermediate. These newly synthesised genomes, together with newly synthesised viral proteins, are packaged to make progeny virions, these genomes also act as templates for further synthesis of mRNAs (secondary transcription). The glycoproteins are glycosylated in the Golgi apparatus of the cell and exported to the cell membrane, where virus assembly and maturation are thought to take place. The M protein has been suggested to be the driving force for the assembly and budding of the newly formed virus particles (reviewed in Lamb & Kolakofsky, 2001).

Figure 1.4



adapted from Lamb & Kolakofsky, 2001

products) the viral polymerase, of which the major components are the L catalytic subunit and the P protein cofactor, is restricted to the production of leader RNAs and mRNAs. The input viral polymerase responsible for transcription enters the template at the 3' end of the non-coding leader sequence of the genomic template and must first produce a short, uncapped and non-polyadenlyalted (+) leader RNA. The polymerase then moves along the template, reinitiates at the gene start of the first gene (NP or N), and sequentially synthesizes the NP, V/P, M, F, SH, HN and L mRNAs, which are capped and polyadenylated, by terminating and restarting at each of the gene junctions. Gene expression from the non-segmented negative-sense genome of RNA viruses, including paramyxoviruses, has been accepted to follow the "stop-start" model for transcription mechanism, in which the access of polymerase to downstream genes is entirely dependent on termination of synthesis of the gene directly upstream (therefore, "stop-start"). The gene end region contains a signal that directs termination of transcription by the viral polymerase and a stretch of uridine residues (U-tract) which acts as a template for polyadenylation of the nascent mRNA by a stuttering mechanism. This process of reiterative transcription, involves correctly templated RNA synthesis followed by realignment of the template and nascent strand relative to the viral polymerase polymerisation site. Cycles of these steps allow the nucleotides of the U-tract to be repeatedly copied and thus generate a poly (A) tail many times the length of the U-tract template (reviewed in Whelan *et al.*, 2004). This process leads to termination and release of the mRNA, and the viral polymerase is then free to move on to the next gene start site and reinitiate the next mRNA. The next gene start sequence is separated from the gene end sequence by the intergenic region. It is thought that the viral polymerase remains attached to the nucleocapsid template as it moves across the intergenic nucleotides before reinitiating transcription at the downstream gene start site. The gene start region also contains signals that direct the polymerase to add a 5' cap and methylate the nascent mRNA (Abraham *et al.*, 1975). Distinct gradient effects on transcription have been observed, as the frequency with which the viral polymerases reinitiates at the downstream gene start is not perfect, thus NP mRNA is the most abundant species and L mRNA the least abundant. It is thought this occurs because the polymerase only binds to the genomic RNA at a

position within the 3' leader sequence and has an increasing chance of disengagement the further it proceeds along the genome during transcription. Reducing reinitiation at one gene junction leads to a direct reduction in the gene expression of all the downstream genes. Observations of transcription in the rhabdovirus vesicular stomatitis virus (VSV) included an altered termination signalling as a result of defective 5' mRNA cap methylation (Rose *et al.*, 1977) and altered polymerase processivity by changes to the gene start sequence (Stillman & Whitt, 1999). This, together with the demonstrated inability of the viral polymerase of VSV to terminate transcription at a wild-type gene end sequence within a transcriptional unit less than 51 nucleotides in length (Whelan *et al.*, 2000), led to the suggestion that the process of termination may be mechanistically linked to that of initiation.

Occasionally, the polymerase fails to terminate at the gene end sequence and proceeds to transcribe mRNA across the intergenic regions generating both di- and tri-cistronic mRNA species (Parks *et al.*, 2001). However, due to ribosomal scanning only the first cistron is translated into protein and hence transcriptional read-through results in a decreased expression of the gene immediately downstream of the failed termination signal. The polymerase is still able to terminate and reinitiate downstream of the second or third gene (if di- or tri-cistronic mRNA species were produced, respectively), so providing a mechanism whereby genes in the middle of the genome can be down-regulated (reviewed in Whelan *et al.*, 2004). Various paramyxoviruses specifically down-regulate the expression of particular genes, specially the F gene, by generating di-cistronic M-F mRNAs. SV5 generates abundant read-through RNAs, the M-F read-through transcript being predominant. In addition to failure of transcriptional reinitiation and poor termination of transcription at the end of the genes (transcriptional read-through), another method of transcriptional attenuation of gene expression has been observed in paramyxoviruses, which involves the existence of overlapping ORFs, such as for RSV. In the RSV genome, the L gene start sequence is located upstream of the gene end sequence of the upstream M2 gene (Collins *et al.*, 1987). Thus, 90% of the transcripts that initiate at the L gene start terminate at the M2 gene end sequence, generating aborted L mRNAs, and the L mRNA (which is

only required in small amounts), is only produced by relatively rare transcriptional read-through events.

The gene junctions of the non-segmented negative sense RNA viruses can be highly conserved or highly variable both in sequence and length. Examples of viruses with high degree of genetic conservation in their gene junctions include the paramyxoviruses Sendai virus (SeV), human parainfluenza virus type 1 (hPIV1), hPIV3, and the rhabdovirus VSV. For VSV, in which each gene end sequence is invariant, mutagenic analysis of the gene junction has shown that polyadenylation and transcription termination requires an intact U_7 tract, and can be signalled by a linear sequence comprising 11-nucleotide-long gene end sequence and the first nucleotide of the intergenic sequence (Barr *et al.*, 1997; Hwang *et al.*, 1998; Hinzman *et al.*, 2002). Examples of paramyxoviruses with highly variable gene junctions include the pneumovirus RSV, and the rubulaviruses hPIV2, mumps virus (MuV), simian virus 41 (SV41), and the prototype member SV5. Despite the considerable variation in each gene junction of these viruses, their gene ends conform to the shared common feature of the non-segmented negative-sense viruses, having a conserved U-tract and an upstream sequence that is adenosine (A)/U rich in nucleotide composition. The SV5 gene junctions, in contrast to VSV, are highly variable, exhibiting variations in the number of uridine residues (from four to seven residues) in the gene end U-tract and in the sequence and overall length of the intergenic sequence (see Table 1.2). It was shown that the variable U-tracts at the SV5 gene end regions are functionally equivalent in templating the addition of a poly (A) tail to viral mRNAs. Furthermore, the variability in SV5 gene end U-tracts has been suggested to have a role as a spacer to maintain a distance of at least six bases between a gene end and gene start site, and therefore it can be an important structural feature of the nucleocapsid template (Rassa *et al.*, 2000). As mentioned above, a striking feature of a number of paramyxoviruses with variable gene junctions is the elevated read-through transcription across the M-F junction. For SV5, this finding was correlated with unique features of the M-F gene junction. The M gene end sequence (3'-A<u>G</u>UUUCU$_4$) differs from that of the SV5 overall consensus sequence at one position (3'-A<u>A</u>NUUCU$_{4\text{-}7}$), and most strikingly in

that it contains the shortest U-tract. However, mutational analysis of the SV5 gene end sequence showed that lengthening the U tract did not affect transcription termination, except when the guanosine (G) five nucleotides upstream of the U-tract was converted to A, in which situation the viral polymerase response to gene end sequence was restored and read-through at this junction was prevented (Rassa & Parks, 1998). This increased read-through transcription across the M-F gene junction has been suggested to serve as a mechanism to fine-tune the level of polymerase that is optimal for virus growth (Parks *et al.*, 2001). The ratio of polymerase-associated proteins is an important factor in optimal mRNA synthesis for the non-segmented negative-strand viruses. Indeed, recombinant viruses that resulted in a higher L to HN mRNA ratio had slower growth kinetics and form smaller plaques (He & Lamb, 1999).

1.1.4.2 Replication

Once the primary transcripts have generated sufficient viral proteins and the levels of the nucleoprotein have risen sufficiently, unassembled N (as a P-NP complex and also as a V-NP complex for SV5) begins to associate with the nascent leader chain, and the coordinate assembly and synthesis of the RNA causing the polymerase to ignore the gene junctions (and editing sites), changing to a read-through mode, and yielding the antigenome nucleocapsid. The antigenome is a full-length complementary copy (+; positive sense) of the (-) genome, and like the genome is found only in helical complexes with the N protein. The newly synthesised (+) antigenome functions only as a template for further copies of full-length progeny genomes. These genomes can be then utilized as templates for secondary transcription, or assembled into infectious particles.

Regulation of transcription *versus* replication in paramyxoviruses is believed to follow the single entry and initiation site model of RNA synthesis, whereby the viral polymerase initiates all synthesis at position 1 of the 3' end of the genome. As described above, initially, in primary transcription, the viral polymerase responds to specific signals in the template and begins to synthesise a leader RNA (Le+), during which a critical regulatory signal determines whether to terminate

Le+ and initiate mRNA synthesis at the first gene start or to read-through the leader and first gene junction to synthesise a full-length antigenomic RNA. What determines whether the viral RNA polymerase will function as a transcriptase or a replicase is poorly understood, although the requirement for the nucleocapsid protein to drive the process of replication (encapsidation of the genomic and antigenomic RNA products) is thought to be a key factor. Thus, a source of soluble nucleocapsid protein is necessary for encapsidation of the nascent RNA strand, and therefore replication requires ongoing protein synthesis to provide N. This coupling of genome assembly and protein synthesis led to the suggestion of a self-regulatory system for controlling the relative levels of transcription and replication, in which the viral polymerase activity is regulated by the availability of N protein to encapsidate the nascent leader RNA. According to the proposed self-regulatory mechanism, when N protein concentration is low, the viral polymerase preferentially transcribes Le+ and viral mRNAs, raising the intracellular levels of all viral proteins, including unassembled N. When N protein concentration is high, termination of Le+ synthesis is suppressed and instead the viral polymerase switches to replication, thereby lowering the levels of unassembled N, as each initiation of encapsidation would require a high number of N monomers to finish assembly of the genome chain. Genome synthesis from the antigenome templates is believed to occur in a similar manner to that of antigenome synthesis, such that the promoter of the antigenome signals the viral polymerase to synthesise a short minus sense leader RNA (Le-, or trailer) independently of ongoing protein synthesis. When the levels of unassembled N are sufficient, the nascent trailer chain begins to be encapsidated and assembled, leading to the synthesis of the full-length progeny genome (reviewed in Lamb & Kolakofsky, 2001; Whelan *et al.*, 2004).

The promoters of paramyxoviruses RNA replication are major determinants of the level of RNA replication and are located at the 3' end of the genomic and antigenomic RNAs (reviewed in Lamb & Kolakofsky, 2001). Typically, paramyxoviruses RNA replication is asymmetric as the replication directed by the genomic promoter to produce antigenomes is much lower that that from the antigenomic promoter which produces genomes. It has been reported for the

paramyxovirus SV5 that the genomic promoter directs RNA replication about 14-fold lower than that seen from the antigenomic promoter. This was suggested to be due to a combination of positive acting signals in the antigenomic promoter and a negative-acting signal in the genomic promoter (Keller & Parks, 2003). Furthermore, the overall length of a paramyxovirus RNA genome template can be a major factor determining the level of RNA replication, and efficient replication for most paramyxoviruses was observed when the total number of nucleotides was a multiple of six (named the 'rule of six'). The rule of six seems to follow taxonomic grouping, being apparently specific to the *Rubulavirus*, *Respirovirus* and *Morbillivirus* genera, although it appears to apply with varying stringency. Very strict for SeV, softer for other members of the family such as SV5, and it does not apply to *Pneumovirus* genus. Genomes whose lengths deviate from 'the rule of six' do not replicate efficiently and it has been proposed that the templates for transcription and replication are nucleocapsids in which each nucleoprotein subunit is associated with six nucleotides of genomic RNA. Presumably, as the antigenome is synthesised, the nucleotides on the nascent strand are encapsidated, concomitantly with replication, in units of six, with 5'-to-3'-end polarity, beginning at the first nucleotide at the 5' end, and it continues until the 3' end is reached, by assembling six nucleotides at a time, maintaining a precise hexameric arrangement (reviewed by Kolakofsky *et al.*, 1998). At the 3' end, when the total number of replicated nucleotides is a multiple of 6, the last 6 nucleotides synthesized are perfectly covered by the last N subunit (3'-OH congruence), which may be required for this 3' end to be recognized as an active promoter (Vulliemoz & Roux, 2001).

Another feature conserved among the viruses of the subfamily *Paramyxovirinae* is that the genomic and antigenomic replication promoters of paramyxoviruses are found within the terminal 96 nucleotides or 16-N subunits of each RNA and are bipartite in nature, as the promoter consists of two distinct elements which are separated by a spacer region with unimportant sequence. However, this spacer has to be of defined length, as shown for SeV (Tapparel *et al.*, 1998) and other members of the *Paramyxovirinae*, such as SV5 (Murphy & Parks, 1999). The known bipartite promoters consist of a stretch of about 30 nucleotides located at

the 3' end of the genome (in which the first 12 nucleotides are conserved between genomes and antigenomes, and across each genus) and a second well-defined element within the nontranslated region of the first (genomic promoter) or the last gene (antigenomic promoter), respectively. For SV5 this has been identified as a 3'-GC dinucleotide repeated three times in the N subunits 13, 14 and 15 (Murphy *et al.*, 1998). Thus, the two promoter regions are adjacent to each other in the helical nucleocapsid, forming a common or contiguous surface on two turns of the helix. Thus, both elements of the bipartite replication promoter are found on the same face of the helix, and therefore both can interact simultaneously with the viral polymerase to initiate RNA synthesis at the 3' end of the nucleocapsid. Another noteworthy conserved feature among the *Paramyxovirinae*, is that the first gene (N) mRNA always starts precisely on the opposite face of the helix (at a U base, position 56).

1.1.4.3 Assembly and budding

The intracellular site of assembly of the nucleocapsids is, as all events in the cycle of replication of paramyxoviruses, the cytoplasm. After replication and encapsidation of the (-) genome with free N subunits to form the helical ribonucleocapsid structure, polymerase complexes formed by homotrimers of P protein associated with the L protein attach to the assembled helical nucleocapsids and relocate to the cell surface. The viral integral membrane proteins are synthesised in the endoplasmic reticulum, and only correctly folded and assembled proteins are transported to the Golgi, where the carbohydrate chains may be modified. Cleavage of the fusion proteins containing multiple basic residues at the cleavage site, such as those of MeV and virulent NDV strains, occurs intracellularly during transport through the *trans* Golgi network. Finally the glycoproteins are transported and embedded into the cell membrane. The mechanism by which infectious virus particles are formed is believed to be by a budding process, and buds emerge from sites in the plasma membrane of the cell, to where the viral glycoproteins, matrix proteins, and other viral components such as assembled ribonucleocapsid protein complexes have been transported. Although these components arrive at the plasma membrane by separate

mechanisms, coordination between the glycoproteins and other viral components must take place for selection of a budding site. Finally, interactions between viral components and the host machinery allow bud formation and membrane fission, resulting in the release of particles. The viral M proteins, which are thought to play a key role in virus assembly and budding, are positioned beneath the lipid envelope in virions such that they potentially make contact with both ribonucleocapsid cores and envelope glycoprotein cytoplasmic tails. Therefore, it is likely that M proteins are important in organization of virus assembly in that they induce separate viral components to concentrate together at defined budding sites on the plasma membranes of infected cells. The viral attachment and fusion glycoproteins of paramyxoviruses have a role in promoting efficient virus budding (Schmitt *et al.*, 2002). The neuraminidase activity of the attachment glycoprotein of some paramyxoviruses in the *Paramyxovirinae* (rubulaviruses and respiroviruses) prevents the aggregation of the newly formed virus particles and reattachment to the infected cell.

1.1.5 *Paramyxoviridae* genes and encoded proteins

As shown in Figure 1.3, SV5 genome contains seven genes that give rise to eight gene products. These include the nucleocapsid protein (NP), the V protein and the phosphoprotein (P), which are both encoded by the V/P gene, the large protein (L), the matrix protein (M), and the three membrane-anchored haemagglutinin-neuraminidase (HN), fusion (F) and small hydrophobic (SH) proteins. The structural characteristics and function of the SV5 proteins are discussed below.

1.1.5.1 Nucleocapsid protein

The nucleocapsid proteins of the paramyxoviruses can range from 489 to 553 amino acids, as predicted from the nucleotide sequence of the NP gene, and a predicted molecular weights range from 53167 to 57896 Da. The prototype member SV5 has a NP gene of 1787 nucleotides (GenBank accession number M81442, Parks *et al.*, 1992) that encodes a nucleocapsid protein with 509 amino

acids (accession number AAA47880) and a molecular mass of 56535 Da (SWISS PROT primary accession number Q88435).

The nucleocapsid protein of paramyxoviruses, which is associated with genomic RNA to form the helical nucleocapsid structure in the core of the virus particles, carries out various functions in virus replication. NP is the key protein in protection of the genomic RNA as it encapsidates the genome, forming an RNase-resistant nucleocapsid around it. This ribonucleocapsid structure is the template for RNA synthesis, and it is the NP protein that associates with the P-L viral polymerase complex during replication and transcription. Also, interactions of NP proteins with M proteins have been well established and suggested to be critical for efficient incorporation of genomes into budding viruses. Furthermore, as described above, the intracellular concentration of unassembled NP is believed to be the major factor controlling the relative rates of transcription and replication from the genome templates.

Surprisingly, given the interaction of NP with RNA, which is predicted to be through association of one monomer of NP protein with precisely six nucleotides, the protein appears not to be a classic RNA-binding protein, as NP does not contain any previously recognized RNA-binding motifs. It has been suggested, on basis of experimental data, that NP has two domains, the globular amino-terminal domain that represents 80% of the protein and the carboxy-terminal domain that represents 20% of the protein appears to be a tail that extends from the surface of the globular amino-terminal body. The amino-terminal domain of about 500 amino acids is relatively well conserved among related viruses and has been suggested to contain the RNA binding motifs and the determinants for the helical nature of the nucleocapsid (Curran *et al.*, 1993; Parks *et al.*, 1992). In contrast, the carboxy-terminal domain of the protein is poorly conserved, although it always contains a highly charged region that is mostly negative, and this domain of NP contains the majority of the phosphorylation and antigenic sites of the protein (Compans *et al.*, 1972; Heggeness *et al.*, 1980; Mountcastle *et al.*, 1974).

1.1.5.2 Phosphoprotein gene and its multiple encoded proteins

The V/P gene of paramyxoviruses extends significantly the coding capacity of the genome by the use of overlapping reading frames within the V/P gene, leading to the production of distinct gene products. The V/P gene of paramyxoviruses encodes more than one protein through a pseudo-templated transcription mechanism, in which the mRNA gene transcript is edited and G residues are inserted at specific sites. This process seems to occur by co-transcriptional RNA editing, whereby the viral polymerase is believed to stutter at a sequence containing a poly-pyrimidine (poly (U)) which is located upstream of the G residue-insertion site that consists of a poly-cytidine (poly (C)) stretch (Vidal *et al.*, 1990). The virus transcriptase is thought to pause at the conserved editing sequence, and on resuming some of the paused polymerases slip backwards along the template (one or two nucleotides), taking the nascent RNA molecule with them and when transcription restarts these one or two residues are copied into the nascent mRNA a second time, adding one or two extra G residues. As a consequence of the addition of pseudotemplated nucleotides to an mRNA, there is a shift in the reading frame downstream of the editing site, and translation of the edited mRNA gives rise to different gene products. In this way, members of the *Paramyxovirinae* produce proteins with a common amino-terminal domain, and as the ORFs that encode the different proteins differ past the editing site, the carboxy-terminal domain of these proteins are different. The paramyxovirus co-transcriptional editing site was first disclosed for SV5 in the *Rubulavirus* genus, and the vital transcriptase is believed to stutter and may or may not insert G residues at a sequence 3'-AAAAUUCUCCC-5' (Thomas *et al.*, 1988). The V/P gene of SV5 is 1298 nucleotides long (GenBank accession number J03142) and codes for two proteins. A faithful transcript of the V/P gene of SV5 generates the V protein, while the P protein results from an edited mRNA in which two Gs are inserted. Thus, the V and P proteins of SV5 are amino-coterminal, having the first 164 amino acids in common, and their carboxy-terminal domains are different, as the ORFs that encode the V and P proteins are different past the editing site (+2 G residues). The structure of the SV5 V/P gene is schematically represented in Figure 1.3.

All viruses in the *Paramyxovirinae* contain an editing site in their P gene, where G residues can be inserted in a programmed manner to produce additional proteins from the P gene. Different paramyxoviruses require a different number of G residues to be inserted in the gene transcript in order to synthesise distinct proteins. Thus, members of the *Respirovirus*, *Morbillivirus*, *Avulavirus*, and *Henipavirus* genera as illustrated by SeV, MeV, NDV and Hendra virus (HeV) require a single non-templated G residue insertion, whilst this number can vary from one to six residues for the bovine parainfluenza virus type 3 (bPIV3) and hPIV3.

Moreover, depending on the paramyxovirus, the faithful transcript of the V/P gene may or may not encode the P protein as the default product. In respiroviruses, morbilliviruses, avulaviruses and henipaviruses the P mRNA for the P protein is transcribed faithfully (unedited) from the viral genome, while transcriptional editing with the addition of one G nucleotide at the editing site produces an mRNA that encodes the V protein. Exceptions are the hPIV1, which undergoes no editing and possesses an interrupted V ORF (several stop codons), and hPIV3, which possesses a closed trans V frame by two stop codons present between the editing site an the V ORF. Therefore, the V ORF terminates shortly after the editing site, and the presence of these V residues suggest that hPIV1 and hPIV3 once expressed a V-like protein (reviewed in Nagai & Kato, 2004). All members of the *Rubulavirus* genus, including SV5, share the same P gene expression strategy, in which 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 (Thomas *et al.*, 1988).

In addition to the production of various proteins by RNA editing, in respiroviruses and morbilliviruses further P gene complexity is provided by the presence of two (overlapping) ORFs upstream of the editing site, only one of which extends to the editing site, which have different start sites that are accessed by ribosomal choice. Morbilliviruses, respiroviruses and henipaviruses, such as MeV, hPIV1, hPIV3 and HeV encode C proteins in a reading frame distinct from that of P and V,

accessed by alternative ribosomal initiation (Lamb & Kolakofsky, 2001). SeV and hPIV1 have the most complex C ORF (ORF that terminates before the editing site), as it contains four ribosomal initiation sites, encoding a carboxy-terminal nested set of C proteins: C', C, Y1 and Y2 (Curran & Kolakofsky, 1990). The P gene products of *Paramyxovirinae* are shown in Table 1.3.

Table 1.3 Products of the P gene in the subfamily *Paramyxovirinae* (reviewed in Nagai & Kato, 2004). There is no evidence for the presence of a V protein in hPIV3, but a relic is present.

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

Phosphoprotein (RNA polymerase α-subunit)

The phosphoprotein (P protein) of respiroviruses, morbilliviruses, henipaviruses and avulaviruses is translated from the unedited mRNA, whilst for the viruses in the *Rubulavirus* genus, including SV5, the P protein is produced from the addition of two G residues at the editing site of the V/P gene transcript (Figure 1.3). The P protein of SV5 contains 392 amino acids (GenBank accession number AAC95513) and has a molecular mass of 42106 Da (SWISS PROT primary accession number P11208; Thomas *et al.*, 1988). The P protein as its name

indicates is highly phosphorylated and phosphorylation of P proteins in the *Paramyxoviridae* is relatively well conserved, suggesting it to be functionally significant, such as facilitating P multimerization and protein complex formation. Additionally, the carboxy-terminal domain of P, which is also reasonably well conserved in predicted secondary structure for all viruses of the *Paramyxovirinae*, it is found as an essential part of all P proteins. The protein forms a homo-oligomer via a predicted coiled coil at the carboxy-terminal end, and the entire protein is expected to form a tetramer (Tarbouriech *et al.*, 2000).

The P proteins of paramyxoviruses play a central role in transcription and replication of the viruses, as it forms, together with the large protein, the viral RNA polymerase complex (L-P). It is estimated that approximately 50 copies of the L protein and 300 copies of the P protein are associated with each paramyxovirus nucleocapsid (Lamb *et al.*, 1976). The P protein has no enzymatic activity, but it acts as a transactivator of L, the catalytic subunit of the RNA polymerase. The L protein by itself cannot bind to the NP-RNA, so it is the P protein that mediates the binding of L to the NP-RNA template, by forming the L-P complex. The conserved carboxy-terminal domain of the P protein is believed to be involved in the interaction of P with L, as well as in binding to NP-RNA, and is predicted to form an α-helical bundle. The P-carboxy-terminal region represents the polymerase cofactor module of the protein. The P protein also has a vital function in encapsidation of the nascent RNA chains during genome replication by interacting with the NP protein, and keeping it in a suitable form for RNA encapsidation. The P protein, by forming the assembly complex with NP (NP-P), confers specificity of NP encapsidation of genomic RNA, preventing non-specific encapsidation of cellular RNAs by the NP protein. Independently of the complex formation with NP (assembly complex) and L (viral polymerase complex) proteins, P has an additional role to play in viral RNA synthesis. This function involves, at least in part, the binding of additional copies of P to the NP-RNA template. These supplemental P proteins may play a role during elongation, for instance by enhancing the processivity of the polymerase or by modulating the conformation of the coiled NP-RNA template and thereby facilitating reading of the bases (Curran *et al.*, 1995; Horikami *et al.*, 1992).

P is known to bind soluble and polymeric NP and it has been shown that P has two binding sites for NP, one mapping to the V/P common amino-terminal domain and the other being in the P-unique carboxy-terminal domain, the latter has been shown to be able to recognize P in its polymeric form (Randall & Bermingham, 1996). Immunofluorescence data demonstrated that, when expressed alone, the NP protein had a granular cytoplasmic distribution and the P protein had a diffuse cytoplasmic distribution. Co-expression of the NP and P proteins was shown to result in the accumulation of large cytoplasmic aggregates containing both proteins, similar to those visualised at late times in virus infected cells (Fearns *et al.*, 1994; Precious *et al.*, 1995). The P protein has also been shown to bind RNA, and the RNA binding region was determined to consist of five basic residues (K74, K76, K77, R79, and K81) which are in the V and P shared 164 residue amino-terminal region (Lin *et al.*, 1997).

V protein

The V ORF is located in the P gene in all viruses of the *Paramyxovirinae* subfamily, and the resulting V protein is the most widely distributed of the paramyxoviruses accessory proteins. The V ORF is the best conserved of the various P gene ORFs, even though the V ORF is absent in hPIV1 and may not be expressed in hPIV3. In SeV and bPIV3 respiroviruses, and in all morbilliviruses, avulaviruses and henipaviruses, the V protein is encoded by an edited mRNA, which has an insertion of a single non-templated G residue (+ 1G). The V protein of rubulaviruses, such as SV5, results from the translation of the faithful V/P gene transcript (Figure 1.3). The encoded V protein for SV5 has 222 amino acids (accession number AAA47882) and a molecular mass of 23935 Da (SWISS PROT primary accession number P11207, Thomas *et al.*, 1988).

Whereas the amino-terminal domain that is common to P and V is the least conserved in amino acid sequence among the paramyxoviruses proteins, the carboxy-terminal V-unique regions are the best conserved. The cysteine-rich carboxy-terminal domain of V (67 amino acids) is highly conserved in all

paramyxoviruses, and includes seven perfectly conserved, identically positioned cysteine residues. The identification of the cysteine residues in V suggested this protein to be a zinc-binding protein (Thomas *et al.*, 1988), and indeed several V proteins have been shown to bind zinc (Liston & Briedis, 1994). The carboxy-terminal domain of the SV5 V protein has been shown to bind two ions of Zn^{2+} (Paterson *et al.*, 1995). "Zinc-finger" domain of SV5 V may be involved in interactions of V with other proteins (Lin *et al.*, 1997).

The V protein of SV5 and other rubulaviruses as well as the avulavirus NDV is a structural protein associated with the nucleocapsid and is incorporated into virions (approximately 350 copies of V per virion), whereas it is a nonstructural protein and absent from the virions of respiroviruses and morbilliviruses. Although it has been shown that V interacts with the nucleocapsid structure and can bind to free NP, experimental evidence has shown that V does not interact with polymeric NP organized into nucleocapsids (Randall & Bermingham, 1996), this suggests the V probably interacts directly with viral RNA. Indeed, experimental work confirmed that the V protein can interact with RNA and that RNA binding maps to five basic residues (K74, K76, K77, R79, and K81) common to the P and V proteins (Lin *et al.*, 1997).

It is known that the amino-terminal domain of the V protein of SV5 interacts with soluble NP (Randall & Bermingham, 1996). V has a single binding site for NP, which maps to the V/P common amino-terminal domain (the same binding site as in the P protein). It has been suggested that V interacts with NP, keeping it soluble prior to encapsidation of viral RNA into nucleocapsids as viral genome is being replicated (Precious *et al.*, 1995). Immunofluorescence data has demonstrated that, when expressed alone, the V protein had a diffuse nuclear and cytoplasmic distribution, and the NP protein had a granular cytoplasmic distribution. However, co-expression of the V and NP proteins led to a redistribution of NP in that NP colocalized with V, having a diffuse distribution, which was both nuclear and cytoplasmic. Therefore, a suggested role for V is to keep NP soluble in the cell in the SV5 system, prior to viral RNA encapsidation, by preventing it from polymerising, as well as inhibiting the self-assembly of NP

(Precious *et al.*, 1995). Thus, V plays an important role in viral encapsidation (and therefore in viral transcription and replication) since encapsidation of the RNA genome is essential for recognition by the viral polymerase of the RNA genome as a template. Furthermore, it has also been suggested that V may compete with P for soluble NP, delaying encapsidation and thus being part of a controlling mechanism that switches from virus transcription to replication (Randall & Bermingham, 1996). More recently, SV5 V has been shown, using a reverse genetics system, to inhibit both SV5 RNA replication and transcription. The significance of the inhibition of SV5 RNA synthesis by V remains unclear, but it was suggested that the V protein prevents high levels of viral gene expression during early phases of infection to avoid activation of interferon-β (IFN-β) and/or induction of apoptosis of the infected cells (Lin *et al.*, 2005).

The V protein of SV5 is a multifunctional protein and plays important roles in viral pathogenesis. The V protein is essential to counter host IFN action (discussed in section 1.3). The carboxy terminal cystein-rich zinc finger domain of V is believed to be involved in protein-protein interactions. The V protein interacts with the 127-kDa subunit (DDB1) of the UV damage-specific DNA binding protein (DDB) that is involved in damaged DNA repair, and deletion of the carboxy-terminus from the V protein interrupts the V:DDB1 interaction (Lin *et al.*, 1998). Furthermore, substitutions of some of the conserved cysteines residues (positions 193, 207, and 214) in the V-unique region as well as deletions from both the amino- and carboxy- termini of V abolished the binding of V to DDB1 (Andrejeva *et al.*, 2002a). DDB1 is known to bind to various viral and cellular components including cullin 4A (Cul4a), which, as part of ubiquitin E3 ligases, plays an essential role in targeting proteins for degradation (discussed in section 1.3). Moreover, DDB1 interacts with the E2F transcription factor 1 (E2F1) (Hayes *et al.*, 1998), and this interaction may play a role in the delay of the cell cycle progression during the DNA repair process. SV5-infected cells proliferate slower than mock-infected cells, and over expression of the V in cells was shown to slow down the cell cycle. However, when excess DDB1 was co-expressed with V, the changes in cell cycle caused by V expression appeared to be restored by overexpression of DDB1. Thus, a normal progression through the cell

cycle was observed in this situation of co-expression of DDB1 and V proteins, suggesting a possible involvement of the V:DDB1 interaction in cell cycle changes. Interestingly, an interaction of V with DDB1 was also seen for the V proteins of MuV, hPIV2 and MeV, but not the V protein of SeV, which replicates faster than SV5, MuV, hPIV2 and MeV (Lin & Lamb, 2000).

1.1.5.3 Large polymerase protein (RNA polymerase β-subunit)

The large polymerase proteins of paramyxoviruses are the largest of the virus-encoded proteins, containing over 2000 amino acids. The L proteins are the least abundant component in the viral nucleocapsid, with approximately 50 copies per virion, correlating with the fact that it is encoded by the gene most distal from the viral genome promoter. The L gene of SV5 is 6859 nucleotides (GenBank accession number M81721, Parks *et al.*, 1992) and codes for a protein of 2255 amino acids (accession number AAA47879) and a molecular mass of 255923 Da (SWISS PROT primary accession number Q88434). The L genes of most paramyxoviruses have been sequenced, and a comparison of the predicted amino acid sequence has shown that although they are all very similar in length, there is little overall sequence homology outside the subfamily. However, comparison of the deduced amino acid sequences of five L proteins of rhabdoviruses (VSV and rabies virus) or paramyxoviruses (SeV, NDV and MeV) showed a high degree of homology along most of their length, with strongly invariant amino acids embedded in conserved blocks separated by variable regions, suggesting a structure of concatenated functional domains. The enzymatic functions of the polymerase are thought to be located within the six conserved domains of L, thereby resembling a "chain of enzymes". The most highly conserved central block contains the probable active site for RNA synthesis (Poch *et al.*, 1990). Characterization of the L proteins of SeV demonstrated that L forms a L-L complex in the RNA polymerase complex, and the L-L oligomerization domain resides in the amino-terminal 200 amino acids of the SeV L protein (Cevik *et al.*, 2003). Recent studies have extended these observations, demonstrating that hPIV3 (Smallwood & Moyer, 2004) and MeV (Cevik *et al.*, 2004) L proteins are an oligomer in the polymerase complex. Furthermore, an alignment of the

morbillivirus L proteins has demonstrated the presence of three highly conserved domains (D1, D2, and D3) separated by two variable "hinges" (H1 and H2), and the D3 domain suggested to be conformationally independent from the other domains in a limited way. Thus, the L proteins of all members of the *Mononegavirales* appear to function as multidomain proteins (Duprex *et al.*, 2002).

The large polymerase is part of the viral polymerase complex (RNA polymerase β-subunit), together with the phosphoprotein (RNA polymerase α-subunit). The P protein binding site on SV5 L protein was mapped to the amino-terminal 1247 amino acids and all internal deletions in this region lost P binding (Parks, 1994). The L protein is thought to function as a multifunctional enzyme, responsible for all catalytic activities necessary for viral RNA synthesis, including initiation, elongation, termination, capping, methylation, and polyadenylation. Thus, P-associated L is required for NP-RNA transcription and replication. Polyadenylation at the 3' end of the newly synthesised mRNA is thought to be the consequence of polymerase stuttering on a short sequence of U residues, but the capping step at the 5' end of the mRNA is believed to be performed by the L protein, which provides guanyl and methyl transferase activities that are required for capping. A 2'-O-ribose (cap 1) methyltransferase domain has recently been identified in the L protein of all mononegavirales (Bujnicki & Rychlewski, 2002; Ferron *et al.*, 2002). Moreover, L has been identified as the kinase that is associated with the core of the virus (Einberger *et al.*, 1990; Lamb, 1975; Roux & Kolakofsky, 1974).

1.1.5.4 Matrix protein

The matrix proteins in the *Paramyxoviridae* are the most abundant proteins in virions, and from the nucleotide sequence of the M genes and predicted amino acid sequences of the M proteins of many paramyxoviruses, these proteins contain 341 to 375 amino acids and predicted molecular mass of between 38500 and 42500 Da. The M gene of SV5 is 1382 nucleotides (GenBank accession number M32248, Sheshberadaran & Lamb, 1990) and codes for an M protein of 377

amino acid residues (accession number AAA46901) and a molecular mass of 42250 Da (SWISS PROT primary accession number P16629).

The M protein is basic and moderately hydrophobic, and has been shown to be only peripherally associated with membranes, as there are no domains on M that are sufficiently long to span the membrane. It is therefore likely that the M protein contains amphipathic α-helices that insert themselves into the inner leaflet of the lipid bilayer, (thus coating this surface) and organize its contacts with the helical nucleocapsid. Given its position, beneath the viral lipid envelope, contacting with the nucleocapsid core of the virus particle where the viral genome is located, it is believed that the M protein plays an important role in virus architecture and organization. As mentioned above, the interactions between M proteins and viral nucleocapsids are well established and are presumed to be critical for efficient incorporation of genomes into budding virions. For the paramyxovirus SeV it has been reported that the M protein binds independently to the cytoplasmic tails of the surface glycoproteins, HN and F proteins (Ali & Nayak, 2000). Moreover, M proteins have been also found to self-associate into ordered assemblies, which taken together with the multiple associations with other viral components (membrane, glycoproteins, and nucleocapsid), suggest that 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 (Schmitt & Lamb, 2004) (Figure 1.4).

1.1.5.5 Envelope glycoproteins

As described above, the virus envelope is a lipid bilayer, in which virus-encoded glycoproteins, the attachment (HN, H, or G) and fusion (F) proteins, are embedded on its outer surface. All paramyxoviruses have these two glycoproteins, and some rubulaviruses (including SV5) and all pneumoviruses encode an additional integral membrane protein (SH protein for SV5). The spike glycoproteins play a crucial role in the initial adsorption of the virus to the cell, as well as in virus budding and assembly at the host cell plasma membrane from which the lipid bilayer is derived. One glycoprotein (HN, H, or G) is involved in

attachment of the virus to a host-cell receptor, after which the virus envelope fuses with the host-cell membrane, the major viral protein involved in the latter process is the fusion glycoprotein (F). The respective roles of these proteins in paramyxovirus budding have been further investigated, and a virus-like particle (VLP) system was developed for SV5 (Schmitt *et al.*, 2002). In this study it was found that efficient budding of SV5 VLPs required expression not only of the M protein but also of NP protein and a glycoprotein (either HN or F protein). Furthermore, it was suggested that the cytoplasmic tails of the two SV5 glycoproteins have critical roles in budding.

Attachment protein (Haemagglutinin-neuraminidase)

The respirovirus and rubulavirus attachment protein is the haemagglutinin-neuraminidase (HN) surface glycoprotein that has both haemagglutinin and neuraminadase activities, while the morbilliviruses attachment proteins have haemagglutinin activity only (H), and the henipaviruses and pneumoviruses have an attachment protein with neither activity (G). The nucleotide sequence of the HN genes of most known *Paramyxoviridae* have been deduced, and the resulting HN proteins range from 565 to 582 amino acid residues. The HN gene of SV5 is 1789 nucleotides in length (GenBank accession number S76876, Baty *et al.*, 1991) and codes for the haemagglutinin-neuraminidase protein of 565 amino acids (accession number AAB21114) that has a molecular weight of 62204 Da (SWISS PROT primary accession number P04850, Hiebert *et al.*, 1985a).

HN is a type II integral membrane protein with an amino-terminal tail domain facing the cytoplasm, a single amino-terminal transmembrane domain, a membrane-proximal stalk domain, and a large carboxy-terminal globular head domain protruding from the outer surface of the lipid bilayer (Figure 1.2). The HN protein has a single hydrophobic domain near the amino-terminus by which the protein is anchored in the virus lipid bilayer, and the globular head domain contains the receptor binding and enzymatic activity. The HN glycoproteins of paramyxoviruses have four to six sites for the addition of amino-linked carbohydrate chains, and the SV5 HN protein has been shown to have four sites.

Comparison of the amino acid residues of the HN proteins of different paramyxoviruses show conserved amino acid residues (cysteine, proline, and glycine) between related members of the family, and this led to the suggestion of a similar protein structure. HN proteins are glycosylated and form non-covalently linked tetramers, which depending on the virus, can consist of two disulphide-linked homodimers (reviewed in Lamb & Kolakofsky, 2001).

In respiroviruses and rubulaviruses, HN is a multifunctional protein that has both haemagglutinin and neuraminadase activities. The determination of the crystal structure of NDV HN alone and in complex with an inhibitor, and comparison of these structures revealed differences in the active site, suggesting that the catalytic site is activated by a conformational switch (Crennell *et al.*, 2000). The X-ray structures of soluble NDV and hPIV3 HN globular neuraminidase regions showed that these domains are monomeric in solution, and both form similar dimer interactions in their respective crystals (Crennell *et al.*, 2000; Lawrence *et al.*, 2004). A potential tetrameric arrangement has been suggested for the NDV HN neuraminidase domains, and a second sialic acid binding site has been identified (Zaitsev *et al.*, 2004). More recently, the crystal structure of SV5 HN protein and its complexes with sialic acid, the inhibitor DANA (2,3-dehydro-2-deoxy-N-acetylneuraminic acid), and the substrate/ receptor trisaccharide sialyllactose have been reported. SV5 HN was shown to form dimers similar to those described for NDV and hPIV3 HN proteins, suggesting a well conserved oligomeric arrangement. However, differently from the previously HN determined structures, the SV5 HN forms a tetramer in solution (Yuan *et al.*, 2005).

The haemagglutinin activity of HN, which mediates virus cell attachment to sialic acid residues on cell surface proteins of target cells, brings the virus particle into contact with the host cell. Moreover, HN has neuraminidase activity, and therefore mediates cleavage of sialic acid from the surface of virions and from the surface of infected cells. Thus, HN prevents self-aggregation of viral particles during budding at the plasma membrane, such that assembled virions at the surface of infected cells are efficiently released. In SV5-infected cells, upon transportation of the synthesised copies of HN to the cell surface, HN is rapidly

internalised by the clathrin-mediated endocytosis pathway (Leser *et al.*, 1996), although the reason for this process, which appears to be in conflict with virus assembly, is not known (Lamb & Kolakofsky, 2001). In addition to the haemagglutinin and neuraminidase activities, HN of most paramyxoviruses also have a fusion-promoting activity, thereby allowing cell-cell fusion. Furthermore, it has been suggested that F and HN exist in a complex, and the importance of HN in fusogenic activity has been demonstrated by mutational analyses which showed that mutations of several domains of HN decrease or abolish fusogenic activity with no effect on receptor recognition (Colman & Lawrence, 2003).

Fusion glycoprotein

Paramyxoviruses fusion proteins are synthesised as an inactive precursor (F_0), and for the protein to function it has to be cleaved into two disulphide-linked subunits (F_1 and F_2). After cleavage of the inactive precursor by a host-cell protease, the new amino-terminus of the membrane-anchored F_1 subunit is released and F becomes biologically active. Paramyxoviruses F genes encode type I membrane proteins with 540 to 580 residues. The F gene of SV5 is 1873 nucleotides long (GenBank accession number K02253, Paterson *et al.*, 1984) and encodes a protein with 529 amino acids (accession number AAA47881), and the unprocessed precursor of the protein, F_0, has 510 amino acids residues and a molecular mass of 56597 Da (SWISS PROT primary accession number P04849). The N-linked glycosylation and proteolytic cleavage of the inactive precursor results in the fusogenically active form of the protein, which consists of two disulphide-linked subunits, the membrane-anchored F_1 subunit (427 residues) and membrane-distal F_2 subunit (83 residues). Cleavage activation of the F protein is crucial for viral infectivity, and can occur either within the cell by the furin cellular protease, during transport of the protein through the *trans* Golgi network, or at the surface of membranes (of infected cells or assembled virions) by secreted cellular proteases (Homma & Tamagawa, 1973; Scheid & Choppin, 1974). Respirovirus and rubulavirus (including SV5) 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 (Bagai & Lamb, 1995).

The F protein is anchored to the membrane by a hydrophobic domain near the carboxy-terminal end, leaving a cytoplasmic tail of 20 to 40 amino acid residues. The amino-terminal domain of the protein protrudes from the outer surface of the lipid bilayer (Figure 1.2). Studies on SV5 isolates with altered fusogenic properties have revealed that the length of the cytoplasmic tail of the protein can affect the fusogenic properties of the virus. It is the F_1 subunit that is thought to play a role in the fusogenic action of the protein. Analysis of the amino acid sequence of F proteins and of the nucleotide sequences of the F genes has indicated that, although there is a lack of sequence homology, all paramyxovirus F proteins have a similar structure, according to similar positions of certain amino acids (cysteine, glycine, and proline residues) and the overall hydrophobicity of the F proteins. The amino-terminal 25 residues of the fusion peptide (F_1) are highly hydrophobic and highly conserved among the *Paramyxovirinae*, showing up to 90% identity (Novick & Hoekstra, 1988). The crystal structure of the SV5 F_1 core trimer structure has been solved to a 1.4 Å resolution (Baker *et al.*, 1999).

The F glycoprotein mediates viral entry into cells by causing fusion of the virus envelope with the host cell plasma membrane, which occurs at neutral pH. As a consequence, the virus nucleocapsid is delivered into the host cytoplasm, where the paramyxovirus replication cycle occurs. Later in infection, syncytium formation is observed, which results from the fusion with neighbouring cells by the action of F that has been expressed on the membrane of infected cells. Syncytia are the most obvious aspect of the cytopathic effect typical of paramyxoviruses and their formation provides a direct means by which virus can spread from infected cells to neighbouring uninfected cells, syncytia can also lead to tissue necrosis *in vivo*. Furthermore, in addition of the function of F in membrane fusion, an attachment activity has been reported for RSV and SeV F proteins (Lamb & Kolakofsky, 2001).

It is believed, for the mechanism of action of F, that the hydrophobic fusion peptide (F_1) initiates the fusion process by intercalating into the target membrane. Thus, in addition to the activation of F by cleavage of the protein into its F_1 and F_2

subunits, during the attachment and fusion process, the F protein undergoes conformational changes that expose the fusion peptide in F_1 and results in the fusion peptide embedding in the host membrane, with or without the help of the HN protein. The activation of most paramyxoviruses F proteins has been shown to occur at neutral pH and is thought to be triggered by various steps including binding of the viral attachment protein (HN or H) to its cell surface receptor, followed by interaction of HN with F, which has been suggested to lead to conformational changes in F that mediate membrane fusion (Crennell *et al.*, 2000; Russell *et al.*, 2001). However, the SV5 isolate W3A F protein mediates fusion when expressed alone, although to a reduced level than when co-expressed with HN. Paramyxoviruses whose fusion does not absolutely require HN protein include MeV and RSV, in which F alone is sufficient to form syncytia. In other paramyxoviruses, such as NDV, hPIV2, hPIV3, MuV, and canine distemper virus (CDV), co-expression of HN and F proteins is essential for syncytia formation (Cattaneo & Rose, 1993; Ebata *et al.*, 1991; Horvath *et al.*, 1992; Hu *et al.*, 1992; Morrison *et al.*, 1991; Sakai & Shibuta, 1989; Taylor *et al.*, 1991; Wild *et al.*, 1991; Yao *et al.*, 1997). An additional requirement is that the HN and F proteins must come from the same virus and be co-expressed in the same cell. It is suggested that for those paramyxoviruses that require an interaction between HN and F proteins to observe fusion, once F binds HN, the latter undergoes conformational changes which in turn trigger a conformational change in F to release the fusion peptide to interact with the target membrane. This hypothesis was supported by coimmunoprecipitation assays, the data of which suggested that HN interacts with F only after binding its receptor (Deng *et al.*, 1999; Lamb, 1993).

Small hydrophobic protein

Two rubulaviruses, SV5 and MuV, have a small gene located between the F and HN genes (Figure 1.3) which is expressed as a separate mRNA encoding a small hydrophobic (SH) protein. A gene encoding a comparable SH protein is found in members of the *Pneumovirinae*. The SV5 SH gene is 292 nucleotides in length (GenBank accession number M11785, Hiebert *et al.*, 1985b) and encodes a 44

amino acid residue protein (accession number AAA47883) with a molecular mass of 5108 Da (SWISS PROT primary accession number P07577).

SH is a type II integral membrane protein expressed at the cell surface and incorporated into virions, with its carboxy-terminal projecting from the surface of the lipid membrane of virus particles and infected cells (Figure 1.2). SH probably does not play a role in virus assembly since this protein is dispensable for normal virus replication in tissue culture cells (He *et al.*, 1998). However, recent work has shown that one role of the SH protein is to block apoptosis in virus-infected cells (He *et al.*, 2001).

1.2 The interferon system

Interferon was discovered by Isaacs and Lindenmann in 1957 as a product secreted by Influenza virus-infected chick cells capable of preventing further infection of cells that were exposed to it (Isaacs & Lindenmann, 1957). When viruses infect their vertebrate hosts, they have to multiply in the face of a vigorous host immune response. One of the earliest obstacles virus encounter is the IFN system, an essential component of the defence system of many organisms, which forms part of the innate, non-specific arm of immunity. Virus-infected cells synthesize and secrete IFNs which warn the body of the dangerous intruders and cause susceptible cells to activate potent antiviral mechanisms, which limit further viral growth and spread of incoming virus (Stark *et al.*, 1998). This process is essential for the initial control of virus infection and buys time for the host to establish an adaptive immune response. IFNs form a large family of multifunctional secreted proteins, and play a key role in antiviral defence via their induction of enzymes that limit viral replication and/or interference with cellular processes such as protein synthesis which is necessary for viral replication. Furthermore, IFNs also have functions in cell growth regulation, by inducing the production of cell-cycle regulatory proteins, as well as in immune activation (reviewed in Goodbourn *et al.*, 2000). They can also induce apoptosis in infected cells, and have been associated with a wide range of immunomodulatory effects, as well as with the activation of cells of the adaptive immune response. Since IFN was discovered, much progress has been made in elucidating its mode of induction in response to virus infection, its mode of action on target cells by activating IFN-responsive genes, and the underlying mechanism of the resulting antiviral responses that mediate cell-autonomous resistance against viruses. In common with other cytokines, IFNs exert their effects by binding to specific receptors, which initiate intracellular signalling cascades that ultimately activate IFN-inducible genes.

IFNs are classified according to their amino acid sequence, mode of induction, receptor usage, and biological activity. The IFN superfamily includes two main

classes of related cytokines: type I and type II IFNs, which signal through distinct receptors.

Type I IFNs, all of which have considerable structural homology, are produced by cells in direct response to virus infection and are characterized by their ability to induce antiviral responses and cell growth inhibitory effects. The type I IFN family comprise a large number of IFN-α subspecies (at least 13, namely IFN-α1, IFN-α2, IFN-α4, IFN-α5, IFN-α6, I FN-α7, IFN-α8, IFN-α10, IFN-α13, IFN-α14, IFN-α16, IFN-α17 and IFN-α21) and a single IFN-β, as well as some additional family members, including IFN-δ, IFN-ε, IFN-κ, IFN-τ, and IFN-ω (Pestka, 1997; Pestka *et al.*, 2004). IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω exist in humans whereas IFN-δ and IFN-τ have been described only for pigs and cattle, respectively, and do not have human homologues. All type I IFNs bind a common cell-surface receptor, known as the type I IFN receptor. IFN-α and IFN-β (frequently designated as IFN-α/β or type I IFN) are the best known of these subfamilies, and their essential role in antiviral immunity and mechanisms of action is well established. Evidence from knockout mice that are unresponsive to IFN-α/β due to targeted deletions in the type I IFN receptor shows that IFN-α/β is important for protection against viral infection as these mice have little or no resistance to viral infections although they have a regular adaptive immune system (Knipe *et al.*, 2001; Weber *et al.*, 2004). Type I IFN induces an antiviral state in cells, preventing virus replication and spread, as well as slowing down cell growth, promoting apoptosis, and affecting other components of the immune system. IFN-α can be induced from cells of lymphoid origin upon viral infection whereas IFN-β can be induced from most cell types, particularly by fibroblasts (Stark *et al.*, 1998).

The type II IFN family consists of a single gene that codes for the cytokine IFN-γ, which is induced exclusively by immune cells, and plays an important role in immune regulation and viral clearance. In contrast to type I IFN, IFN-γ is made exclusively by cells of the immune system, being secreted by natural killer (NK) cells during innate immune responses, it is also involved in adaptive immune

responses, as it can be synthesised by activated T lymphocytes (Biron & Sen, 2001). IFN-γ binds to a receptor distinct from the IFN-α/β receptor, and is known as the type II IFN receptor. The type I and type II IFN molecules, which activate antiviral defence mechanisms by inducing genes that encode proteins with antiviral affects, have no apparent homology and show high specificity for their distinct cell surface receptors. However, they do display functional parallels, as both trigger IFN signalling pathways that partially overlap, thereby resulting in partial gene activation redundancy. Nevertheless, the two systems are not always redundant, and IFN-γ can mediate a number of unique functions, making it a very potent immune regulator. 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), as well as enhance the expression of other chemokines or immune-related cell surface receptors, including major histocompatibility complex (MHC) class I and II, interleukin (IL)-12 and tumor necrosis factor (TNF) receptors, which participate in inflammatory responses. Activation of monocytic cells to deliver antimicrobial defence mechanisms is another relevant function of IFN-γ, by stimulation of mononuclear phagocyte-produced enzymes with antimicrobial activity. Also, IFN-γ can induce nitric oxide synthase 2, NOS2 or iNOS, for the production of antiviral NO radicals (reviewed in Biron & Sen, 2001).

Novel IFNs consist of the recently discovered IFN-like molecules or IFN-λ molecules: IFN-λ1, IFN-λ2 and IFN-λ3, which are also known as IL-29, IL-28A and IL-28B, respectively. They are strikingly similar to the type I IFNs in being directly induced by virus infection and having antiviral activity, although they use distinct cell surface receptors. IFN-λ proteins, similarly to other IFNs, play a role in protection from virus infection, as they induce cellular genes that are known to encode antiviral proteins. Additionally, IFN-λ has been shown to be involved in enhancement of MHC class I antigen expression, an important immunoregulatory activity. According to this and the finding that virus infection induces IFN-λ expression it was suggested that the IFN-λ system might contribute to resistance to virus infection in the body. However, experimental data suggested that IFN-λ

may have a lower specific activity in some biological assays than IFN-α (Kotenko *et al.*, 2003; Sheppard *et al.*, 2003).

1.2.1 Induction of interferon genes

Type I IFNs are produced by basically all cells of the body, although those cells may differ in the subtypes of IFN they predominantly synthesise. A specialized subset of dendritic cells, plasmacytoid cells, and conventional dendritic cells secrete high levels of IFN-α. Fibroblasts secrete mainly IFN-β as an initial response to infection but switch to IFN-α during the subsequent amplification phase of the IFN response.

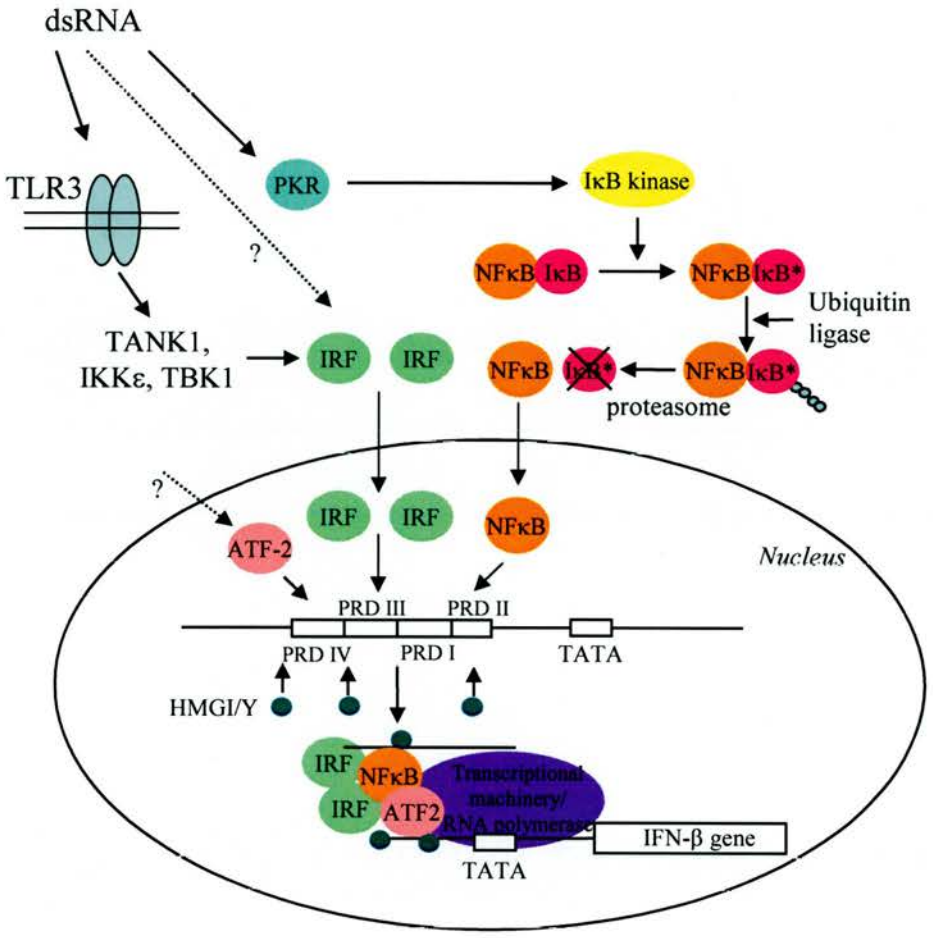
1.2.1.1 Interferon-β induction

Induction of type I IFN gene expression is believed to occur mainly at a transcriptional initiation level and production of IFN-β by virus infection was the first to be examined at the molecular level and remains the most intensely studied. Activation of the IFN-β promoter is initiated by the recognition of double-stranded RNA (dsRNA), which is generated during viral infection, either as an intermediate product during the virus life cycle or as the viral genome itself, and the events following stimulation by dsRNA are schematically represented in Figure 1.5. Interestingly, initial transcription from the IFN-β promoter relies on a relatively short *cis* regulatory element in the promoter that serves as a binding site for several cellular transcription factors that cooperate for maximal promoter activation. These include IFN-regulatory factor (IRF) family members IRF-3 and IRF-7 and the general transcription factors, nuclear factor κB (NF-κB) and activating transcription factor-2 (ATF-2)/c-jun, all of which need to be activated by serine phosphorylation in virus-infected cells, either directly or through phosphorylation of associated inhibitory proteins, such as IκB in the case of NF-κB (reviewed in Levy & Marie, 2005). Thus, the key initial biochemical event generated by viral infection is activation of kinase(s) which in turn activate the appropriate transcription factors.

Figure 1.5 Schematic representation of the activation of the IFN-β promoter by dsRNA.

dsRNA, a product of viral replication, activates PKR, toll-like receptor 3 (TLR3) and its associated kinases, and possibly other cellular kinases leading to activation of transcription factors. PKR is known to activate IκB kinase which targets IκB for proteasomal degradation, releasing active NFκB, which then translocates to the nucleus and binds to the IFN-β promoter with other transcription factors such as IRF-3 and IRF-7, ATF-2/c-jun and the accessory factor HMGI/Y, forming the enhanceosome complex. This complex also associates with the basal transcriptional machinery and recruits RNA polymerase II to the promoter, resulting in the induction of the transcription of the IFN-β gene.

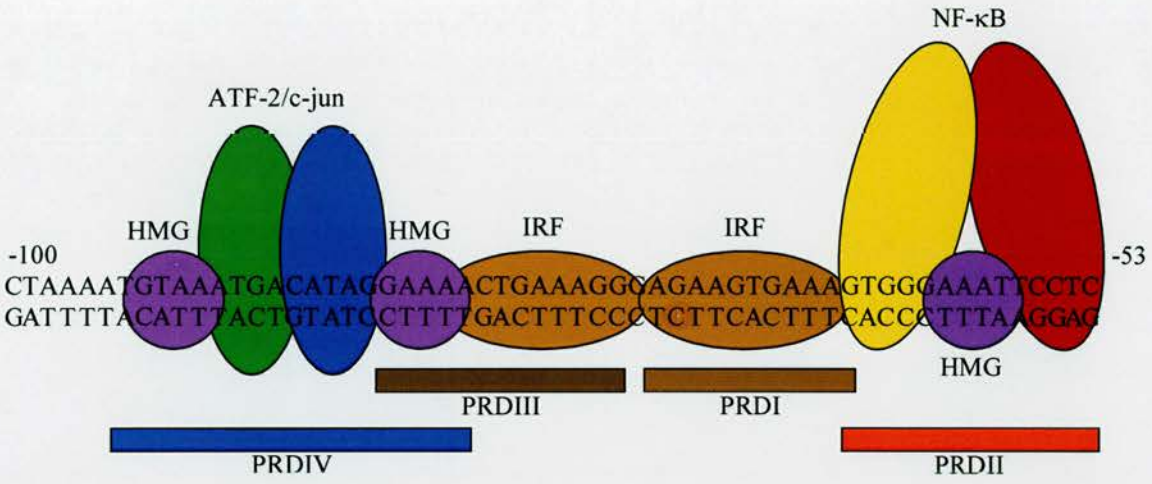
Figure 1.5



adapted from Goodbourn et al., 2000

Enhanceasome

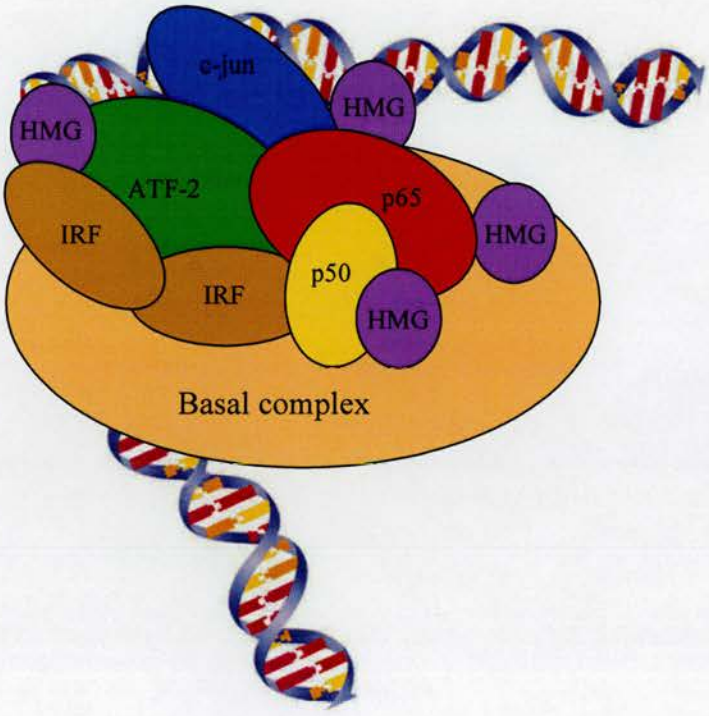
The IFN-β promoter is controlled by an enhancer element, located immediately upstream of the promoter, and contains binding sites for transcription factors. More precisely, the IFN-β enhancer contains an ATF-2/c-jun binding site, two tandem binding sites for IRF proteins and a NF-κB binding site. Once the cellular transcription factors are activated and transported to the nucleus, their co-operative interaction, concerted recruitment of coactivator proteins, and binding at the individual sites of the IFN-β enhancer leads to the formation of a multimeric structure, termed the enhanceosome complex. This structure is further stabilised by induced DNA bending, at least in part through the action of non-histone high mobility group (HMG)-I/Y chromatin proteins. The ATF-2/c-jun complex is a dimeric activator that binds to positive regulatory domain IV (PRD IV), along with two molecules of HMG-I/Y. The NF-κB complex is formed by p50/p65 dimers, and it binds to PRD II, along with a molecule of HMG-I/Y (reviewed in Thanos, 1996). The identity of the IRF protein(s) contributing to the enhanceosome complex activity is not completely clear, although IRF-3 and IRF-7 have been demonstrated to be essential (Sato *et al.*, 2000). The IRF transcription factors bind to PRD III and I (Figure 1.6; Du *et al.*, 1993). The IFN-β enhancer also contains binding sites for negative-acting proteins, that are possibly displaced by the binding of activators, including the IRF-2 protein that competes for binding at the IRF sites with activating IRF family members. The structure of the enhanceosome appears to be very important as simultaneous interaction of multiple transcription factors enhances the subsequent recruitment of coactivator proteins. Additionally, the bent structure of the enhanceosome structure favours cooperative binding and therefore the efficiency of enhanceosome assembly increases. Also, DNA bending is believed to favour the association of the enhanceosome with the basal transcriptional machinery to recruit RNA polymerase II to the IFN-β promoter (Figure 1.7; Thanos & Maniatis, 1995), and therefore transcription of the IFN-β gene occurs, which subsequently leads to production of IFN-β by activated cells.



adapted from Du et al., 1993

Figure 1.6 Enhanceosome assembly model at the IFN-β gene.

Binding of the high mobility group protein HMG I(Y), as well as the transcription factors ATF-2/c-jun dimeric complex, NF-κB complex (heterodimerised p50/p65), and the IRF in positive regulatory domains (PRD) of the human IFN-β gene promoter. The HMG-I/Y establishes a transcriptional synergy between PRDII and PRDIV by promoting the activities and/or binding of NF-κB and ATF-2 and by facilitating their interaction. The IRF transcription factors bind to PRD III and I.



adapted from Thanos & Maniatis, 1995

Figure 1.7 Enhanceosome assembly model at the IFN-β gene.

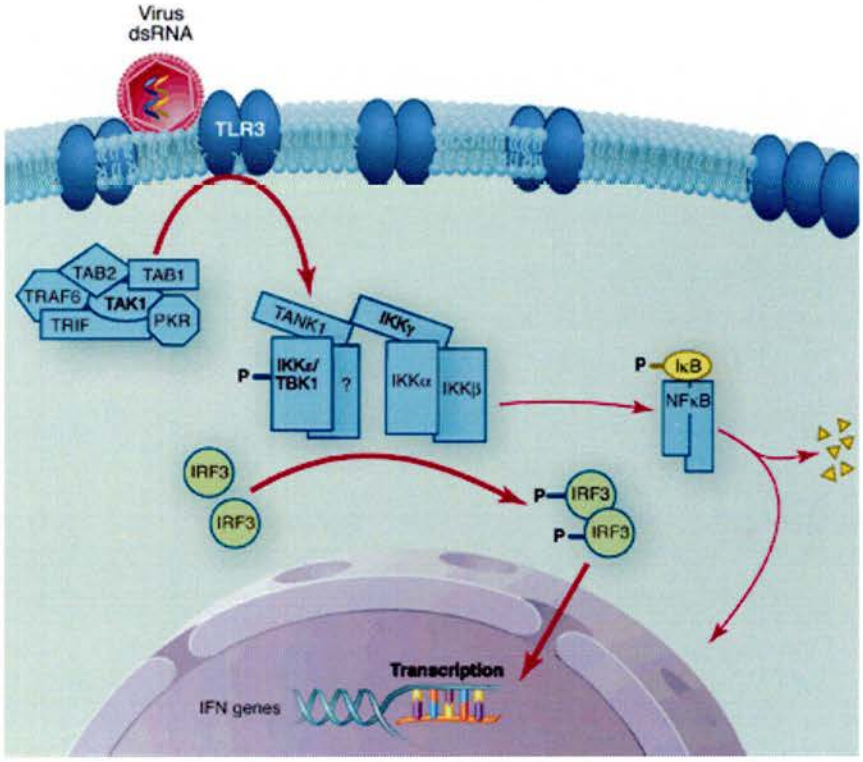
To be transcriptionally activated, the IFN-β gene requires the assembly of a higher order transcription enhancer complex, termed the enhanceosome. This complex includes the high mobility group protein HMG I(Y), as well as the ATF-2/c-jun dimeric complex, the NF-κB complex (p50/p65 heterodimers), and the IRF transcription factors.

Activation of transcription factors

NF-κB is a ubiquitous transcription factor in animal cells, which is critical for the induction of many immunomodulatory genes. In unstimulated cells, NF-κB is predominantly found in the cytoplasm, bound to its inhibitory protein IκB. However, dsRNA from some viruses has been shown to act through the serine-threonine protein kinase R (PKR) by converting it in an active form, which can then phosphorylate and activate the cellular IκB kinase complex, IKK (IKKα/IKKβ) (Zamanian-Daryoush *et al.*, 2000). Activated IKK, coupled with its structural partner IKKγ, also called NEMO (NF-κB essential modulator), phosphorylates IκB, which is then polyubiquitinated by an E3 ubiquitin ligase and thereby targeted for proteasomal degradation. The degradation of IκB releases the active NF-κB protein for complex formation and nuclear translocation (reviewed in Israel, 2000). The ATF-2/c-jun complex also needs to be activated, which requires phosphorylation of ATF-2 by c-jun amino-terminal kinases (JNK), leading to an enhanced DNA binding activity and to an increased activity of this heterodimeric transcription factor. The other transcription factors, IRF-3 and IRF-7, also need to be activated by direct phosphorylation by a cellular kinase complex on a regulatory domain, leading to dimerization and nuclear translocation. Among the transcription factors, IRF-3 is thought to play a central role for the primary activation of IFN genes. The virus activated protein kinase (VAK) complex responsible for phosphorylation of IRF-3 has been recently identified (described below). There are a number of intracellular and extracellular pathways which can lead to the activation of VAK. These include activation through toll-like receptors (TLRs).

TLRs are a family of type I trans-membrane receptors that recognise conserved, pathogen-associated molecular patterns, which respond by inducing IFN. Stimulation of TLRs initiates potent innate immune responses through toll-interleukin 1 receptor (TIR) domain-containing adaptors such as myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor-inducing IFN-β (TRIF).

TLR3 and TLR4 have been shown to induce the IFN-β promoter by activating the transcription factor IRF-3. TLR3- and TLR4-mediated IFN induction is triggered by the binding of virus-derived dsRNA and binding of lipopolysaccharide, respectively (reviewed in Smith *et al.*, 2005). Thus, TLR3 recognizes dsRNA produced during viral replication, and activation of TLR3 by dsRNA was shown to activate NF-κB and IRF-3, and as a consequence the production of IFN-α/β (Alexopoulou *et al.*, 2001). Engagement of TLR3 by dsRNA results in recruitment of the TRIF adaptor protein, also called TIR-containing adaptor molecule-1 (TICAM-1). TRIF then recruits the TANK binding kinase I (TBK1) and its relative IKKε. It has been suggested that IKKε and TBK1 form the virus activated protein kinase that is responsible for phosphorylation of IRF-3 and IRF-7 in response to viral infection (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). Phosphorylation of IRF-3 results in its dimerization and subsequent interaction with the transcriptional coactivators CREB-binding protein (CBP) and p300, and this complex then translocates to the nucleus. It has also been suggested that the VAK (IKKε/TBK1) complex associates with the IKKα/IKKβ/IKKγ complex through the adapter protein TANK (TRAF family member associated NF-κB activator), leading to the IKK complex activation that results in phosphorylation of IκB, and the release and translocation of NF-κB into the nucleus to switch on target genes involved in the immune response and cell survival (Figure 1.8; Williams & Sen, 2003). TRIF has also been shown to associate with TNF receptor-associated factor 6 (TRAF6) and receptor-interacting protein 1 (RIP-1) by direct interaction, which culminates in activation of the NF-κB family of transcription factors through IKK dependent phosphorylation and subsequent proteasome-mediated destruction of the IκB inhibitor of NF-κB (Meylan *et al.*, 2004). Although TLR3 appears to have an important role in induction of IFN-β promoter, by binding to extracellular dsRNA and consequent activation of kinases, leading to the activation of transcription factors, recent observations suggest that intracellular dsRNA, such as that generated during virus replication, does not require TLR3 to activate VAK (Diebold *et al.*, 2003; Fitzgerald *et al.*, 2003; Yoneyama *et al.*, 2004). Indeed, dendritic cells and fibroblast cells lacking TLR3 still secrete type I IFN after intracellular introduction of dsRNA, and this



from Williams & Sen, 2003

Figure 1.8 Induction of IFN synthesis by the TLR3-dependent pathway.

Virus infection or dsRNA induces the activation of a multicomponent protein complex, which activates the IKKε and TBK1 kinases that may be present in a larger complex, probably together with TANK1, IKKα, and IKKβ. This complex, once activated, phosphorylates IRF-3, which can then dimerise, translocate to the nucleus and activate the transcription of specific IFN genes.

induction was shown to require TBK1, IKKε and IRF-3, indicating the existence of a TLR3-independent pathway that converges with the TLR3-dependent pathway at TBK1. Thus, a pattern-recognition receptor which senses intracellular dsRNA that is involved in the TLR3-independent signalling pathway for activation of the IFN-β promoter must exist. Findings from the work presented in this thesis, in terms of IFN modulation by paramyxoviruses infections, have contributed to the demonstration of a signalling intermediate involved in activation of the IFN-β promoter by dsRNA, independently of TLR3, as discussed in Chapter 4.

In contrast to TLR3 that senses dsRNA leading to the production of IFN-β, TLR7 and TLR8 recognise guanosine- and uridine-rich viral single-strand RNA (ssRNA) in the acidic compartment of endosome, and have been demonstrated to induce IFN-α production in response to viral RNA. It has been suggested that TLR7 and TLR8 detect viral RNA at later times in infection when viral nucleic acids are released from infected cells and are taken up by intact neighbouring cells (Diebold *et al.*, 2004; Heil *et al.*, 2004). In addition, TLR9, a receptor for unmethylated DNA, has been also implicated as viral sensor at some stage of infection. Recently, the adaptors MyD88 and TRAF6, known mediators of TLR9 signalling were shown to directly associate with IRF-7 and stimulate its activity, leading to efficient IFN-α gene induction (Kawai *et al.*, 2004).

1.2.1.2 Interferon-α induction

The IFN-α genes are also transcriptionally induced by virus infection and this induction is known to occur in fibroblast cells and leukocytes through distinct mechanisms. In contrast to IFN-β promoters, IFN-α promoters do not contain binding sites for NF-κB but they do have similar sites to PRD I for IRF proteins and also ATF-2 binding elements. In fibroblasts, IFN-α production appears to require IFN-β-stimulated gene expression, as fibroblasts from mice lacking the IFN-β gene are unable to produce IFN-α (Erlandsson *et al.*, 1998). Members of the IFN-α gene family are activated by IRF-7, which is not constitutively

expressed in most cell types, but is an IFN-β stimulated gene product. Thus, IRF-7 is induced in response to early secretion of IFN-β through the action of IRF-3, and multiple IFN-α species are produced, resulting in potent antiviral activity (Levy *et al.*, 2002; Marie *et al.*, 1998). Induction of IFN-α by leukocytes does not require IFN-β stimulation, so there must be a different pathway for IFN-α gene expression in these cells.

1.2.1.3 Interferon-γ induction

The IFN-γ is produced by $CD4^+$ T helper 1 (Th1) cells, by nearly all $CD8^+$ cells, as well as by NK cells. Production of IFN-γ by NK cells occurs regardless of antigen presentation, while IFN-γ production by $CD4^+$ Th cells and $CD8^+$ cells depends on exposure to antigen-presenting cells (APCs) (Young, 1996). The IFN-γ promoter appears to differ depending on cell type. In $CD4^+$ Th1 cells, the promoter consists of a proximal element that is activated by transcription complexes that contain c-jun and ATF-2, and a distal element that is activated by GATA binding protein 3 (GATA-3) and ATF-1. In $CD8^+$ cells, only the distal regulatory element is activated, and consequently IFN-γ transcriptional activation is less in these cells. Production of the cytokines IL-12 and IL-18 by APCs enhances the expression of IFN-γ in response to antigen stimulation in activated T cells. Although neither of these cytokines alone can stimulate IFN-γ production significantly in unstimulated T cells, IL-12 and IL-18 together can stimulate T cells to produce IFN-γ in the absence of antigen (reviewed in Goodbourn *et al.*, 2000). Activated NK cells can also produce IFN-γ, which occurs in an antigen-independent manner, although this is also dependent on APC-produced IL-12 and is further stimulated by IL-18 (Singh *et al.*, 2000).

1.2.2 Mechanisms of interferon signalling

More is known about the molecular machinery involved in initiating transcription of the interferon stimulated genes (ISGs) than the IFN genes themselves, although they both possess similar downstream components. The biological activities of

IFNs are initiated by the binding of IFN-α/β, IFN-γ and IFN-λ to their cognate receptors on the surface of cells, which results in the activation of distinct but related signalling pathways (Figure 1.9). Thus the signalling pathways involve protein components that are specific for each type of IFN, although major components of the signalling machinery are common between the types I and II of IFN pathways, as they both use an intra-cellular signal transduction cascade involving protein tyrosine kinases of the Janus kinase (Jak) family and the signal transducer and activator of transcription (STAT) proteins. The Jak/STAT signalling in the transduction of the IFN-α/β and IFN-γ signal from the cell membrane receptors into the cell nucleus for activation of transcription is presented in Figure 1.10, and results in the expression of over 300 ISGs.

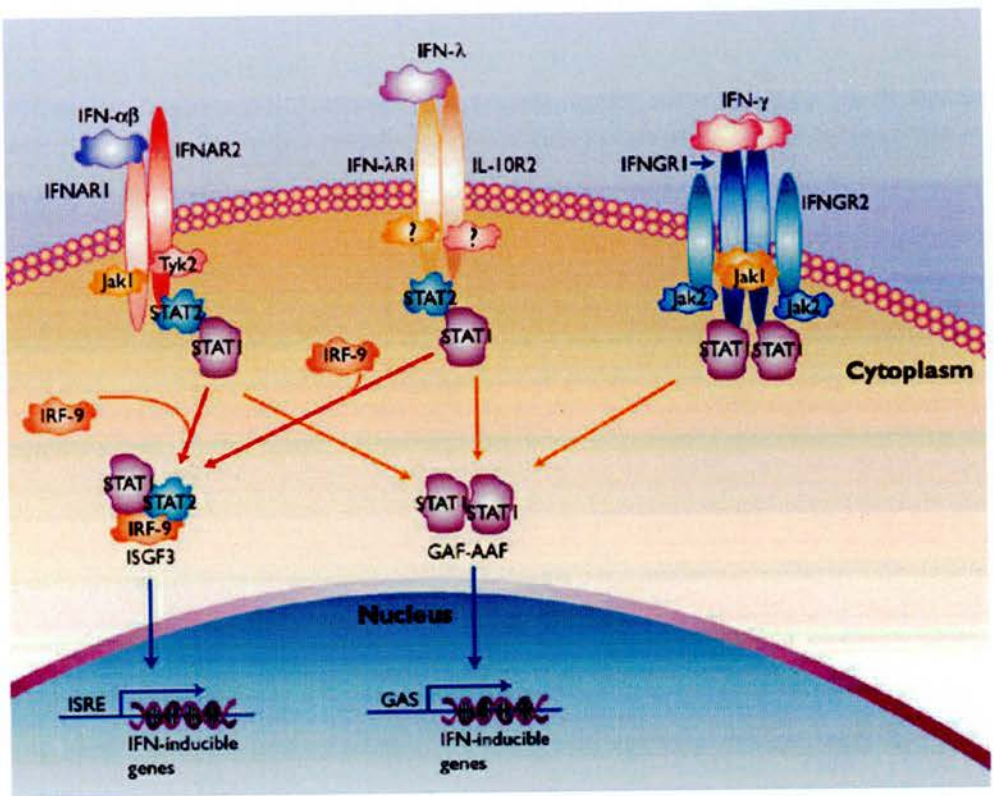
1.2.2.1 Interferon-α/β signalling

The large family of type I IFNs all bind a single type of receptor, a receptor complex that has a multichain structure, composed of at least two distinct subunits: IFNAR1 and IFNAR2 (reviewed in Mogensen *et al.*, 1999). In the absence of any stimulatory signal, the IFNAR1 subunit is constitutively associated with tyrosine kinase 2 (Tyk2) and the IFNAR2 subunit is associated with Jak1, as well as STAT2. The ligand-induced stimulation of IFNAR results in rearrangement and heterodimerisation of the receptor subunits, which results in cross activation by transphosphorylation of the two receptor associated Janus kinases, Jak1 and Tyk2. As a result, activated Tyk2 phosphorylates a tyrosine residue, Tyr^{466}, on IFNAR1 allowing STAT2 to bind, via its SH2 domain, to the phosphorylated receptor subunit. Subsequently, Tyk2 phosphorylates STAT2 at residue Tyr^{690}, which enables STAT1 to bind to the receptor complex. STAT1 binds to STAT2 via its SH2 domain and is phosphorylated at Tyr^{701}, which is necessary for its activation. Tyrosine phosphorylated STAT1 and STAT2 form a heterodimer which then dissociates from the receptor. The STAT1/STAT2 heterodimer then forms a heterotrimeric complex with a DNA binding protein of the IRF family, IRF-9 (p48). This transcriptional complex, now termed the ISG factor 3 (ISGF3) complex, translocates to the nucleus, and binds to specific elements known as IFN-stimulated response elements (ISREs) that are present in

Figure 1.9 A schematic representation of the signalling pathways activated by IFN-α/β, IFN-γ and IFN-λ in mammalian cells, showing the similarity of the IFN-λ signalling pathway to the IFN-α/β signalling pathway.

IFN-α/β and IFN-γ bind to specific and distinct heterodimeric receptors. IFN-α and IFN-β bind to their receptors, leading to the activation of the Jak1 and Tyk2 tyrosine kinases, and subsequent tyrosine phosphorylation of STAT1 and STAT2 proteins. Similarly, IFN-λ molecules bind to their cognate receptors on the cell surface causing dimerization, bringing currently unknown cellular kinases associated with the receptor subunits into contact. These kinases are activated and subsequently phosphorylate STAT proteins, which form heterodimers of STAT1 and STAT2. In both signalling pathways, phosphorylated STAT1 and STAT2, in association with p48 (IRF-9), form the trimeric ISGF-3 complex, which translocate to the nucleus, and binds to IFN-stimulated response elements (ISRE) in the promoters of IFN-α/β-stimulated genes thereby activating transcription. In contrast, IFN-γ binds to its receptors, leading to the activation of the Jak1 and Jak2 tyrosine kinases, which results in the phosphorylation of STAT1 but not STAT2. Molecules of phosphorylated STAT1 homodimerize, forming the gamma-activated factor (GAF) complex, which translocates to the nucleus and binds to the IFN-γ activation sequence (GAS) element present in IFN-γ-inducible genes. IFN-α and IFN-β signalling can also lead to the formation of the GAF complex and its binding to the GAS regulatory element.

Figure 1.9

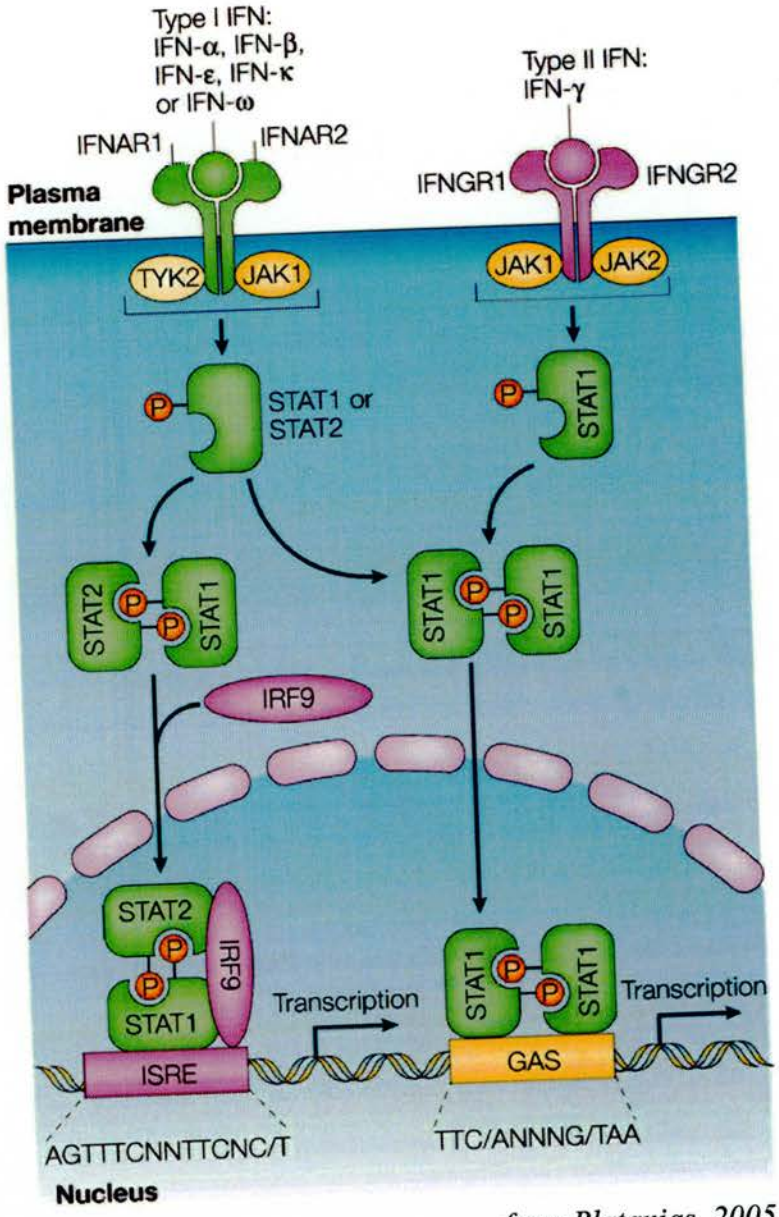


from Vilcek, 2003

Figure 1.10 Schematic representation of the interferon receptors and activation of classical Jak/STAT signalling pathways by type I and type II interferons.

All type I IFNs bind a common receptor at the surface of human cells, named the type I IFN receptor, which is composed of two subunits, IFNAR1 and IFNAR2. These are associated with the 'Janus' tyrosine kinases Tyk2 and Jak1, respectively. The receptor activation leads to phosphorylation of Tyk2 and Jak1 kinases, which phosphorylate signal transducers and activators of transcription (STATs) 1 and 2 on tyrosine residues, causing heterodimerisation of STAT1 and STAT2. Heterodimers of STAT1 and STAT2 associate with other protein, p48 (IRF-9), forming the trimeric IFN-stimulated gene factor 3 (ISGF3) complexes, which translocate to the nucleus. These complexes bind to the IFN-stimulated response element (ISRE) in DNA to initiate the transcription of many IFN-α/β-inducible-genes. IFN-γ binds to a distinct cell-surface receptor, which is composed of two subunits, IFNGR1 and IFNGR2, resulting in the phosphorylation of the receptor-associated kinases, Jak1 and Jak2. This leads to tyrosine-phosphorylation and homodimerisation of STAT1. Homodimers of STAT1, termed 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). The consensus GAS element and ISRE sequences are shown, and N is any nucleotide (reviewed in Platanias, 2005).

Figure 1.10



from Platanias, 2005

the promoters of certain ISGs, thereby initiating their transcription (reviewed in Goodbourn *et al.*, 2000 and Levy & Garcia-Sastre, 2001). It is also biologically relevant that the ISRE sequence can be bound by other members of the IRF family particularly IRF-1, IRF-2, and IRF-8. Indeed, both IFN-α/β and IFN-γ induce IRF-1 that can bind ISREs, thereby leading to complex patterns of gene expression and system activation redundancy, which is crucial to the host antiviral defence (reviewed in Goodbourn *et al.*, 2000). On the other hand, the direct binding of proteins, such as IRF-2 and IRF-8 to ISREs results in the suppression of transcription. IRF-2 and IRF-8 are therefore thought to be involved in the attenuation of IFN signalling by preventing ISG expression in the absence of IFN and in controlling the response to IFN.

In addition to STAT1 and STAT2, other STATs are activated in response to type I IFNs, including STAT3 and STAT5. In certain cell types, such as endothelial cells or cells of lymphoid origin, STAT4 and STAT6 can also be activated by IFN-α. Several STAT-containing complexes, in addition to the STAT1/STAT2/IRF-9 heterotrimer, are formed during engagement of the type I IFN receptor, resulting from association of distinct combinations of STAT proteins. These include STAT1/STAT1, STAT3/STAT3, STAT4/STAT4, STAT5/STAT5 and STAT6/STAT6 homodimers, as well as STAT1/STAT2, STAT1/STAT3, STAT1/STAT4, STAT1/STAT5, STAT2/STAT3 and STAT5/STAT6 heterodimers. These different type I IFN-inducible STAT complexes bind another type of element present in the promoter of ISGs, the gamma-activation sequence (GAS) element. IFNs induce the expression of hundreds of ISGs, of which some have only ISREs or only GAS elements in their promoters, while others have both elements. Thus, the optimal transcriptional activation of a particular ISG might require different STAT-containing complexes.

1.2.2.2 Interferon-γ signalling

The IFN-γ receptor is also a heterodimeric glycoprotein, comprising at least two species of major subunit, IFNGR1 and IFNGR2 (reviewed in Bach *et al.*, 1997),

which are pre-associated weakly in unstimulated cells. Similarly to IFN-α/β membrane receptors, the cytoplasmic domains of the IFNGR1 and IFNGR2 receptor subunits are associated with the cellular Janus family kinases Jak1 and Jak2, respectively. When dimeric IFN-γ binds to the receptor subunits the signalling pathway is initiated by triggering receptor dimerization, which brings Jak1 and Jak2 into close proximity, resulting in the activation of Jak2 which in turn transphosphorylates Jak1, thereby activating it. As with the IFN-α/β activation cascade, the activated Jaks subsequently phosphorylate a tyrosine-containing region in the carboxy-termini of IFNGR1 (Tyr^{440}-Tyr^{444}), creating a pair of binding sites for STAT1. Two STAT1 molecules subsequently interact through their SH2 domain with IFNGR1, and are phosphorylated at Tyr^{701}, which results in their activation and dissociation from the receptor. The activated STAT1 molecules dimerise through SH2 domain-tyrosine phosphate recognition, forming a STAT1/STAT1 homodimer. This homodimer, also referred to as gamma-activated factor (GAF), translocates to the nucleus and binds to the unique element of IFN-γ inducible genes, the gamma-activation sequence and stimulates transcription. As IFN-γ does not induce formation of ISGF3 complexes, in contrast to IFN-α/β, transcription of genes that have only the ISREs in their promoter are not induced by IFN-γ (reviewed Goodbourn *et al.*, 2000 and Platanias, 2005).

1.2.2.3 STAT1 transactivation in interferon-α/β and interferon-γ signalling

As described for the formation of the various complexes and their translocation to the nucleus, STATs need to be phosphorylated at tyrosine residues by activated Jaks, a crucial step in IFN-mediated signalling. However, for optimal IFN-regulated gene transcription, several other events involving biochemical modification of STATs or the interactions of STATs with other proteins (transcriptional coactivators) are required. For example, before STAT1 can bring about full transcriptional activation, it must also be phosphorylated on the serine residue at position 727 (Ser^{727}) (reviewed in Platanias, 2005). The serine kinase

(possibly more than one) that regulates the phosphorylation of Ser^{727} in response to either type I or type II IFNs has been suggested to be a member of the protein kinase C (PKC) family, particularly PKC-δ, which has been shown to interact with STAT1 (Deb *et al.*, 2003; Uddin *et al.*, 2002). Additional IFN-dependent serine kinases, which may be activated in a cell-type-restricted manner, have also been shown to be involved in the regulation of Ser^{727} phosphorylation of STAT1. For example, both PKC-ε (Choudhury, 2004) and calcium/calmodulin-dependent protein kinase II (Nair *et al.*, 2002) have been shown to regulate IFN-γ-dependent STAT1 Ser^{727} phosphorylation.

Ser^{727} phosphorylation is known to facilitate the interaction of STAT1 with the basal transcription machinery, as well as with the chromatin-associated protein, minichromosome maintenance deficient 5 (MCM5), and a protein called NMI (nMYC and STAT interactor). NMI acts to enhance the association between STAT1 and CBP/p300 transcription factors (Zhu *et al.*, 1999). The CBP/p300 family of transcription factors potentiate the activity of several groups of transcription factors (reviewed in Janknecht & Hunter, 1996). STAT2 is not known to be serine-phosphorylated in response to IFN, but it also binds CBP/p300 and facilitates interaction with the basal transcriptional machinery (Bhattacharya *et al.*, 1996).

1.2.2.4 Attenuation of interferon signalling

In contrast to the mechanism of IFN-α/β and IFN-γ activation, less is known about the mechanism by which IFN signal attenuation occurs. As mentioned above, the direct binding of IRF proteins, such as IRF-2 and IRF-8, to ISRE gene regulatory elements results in down-regulation of IFN signalling, particularly type I IFN signalling. Accordingly, in contrast to the ISGF3 complex and IRF-1, which bind to ISRE and induce gene expression, IRF-2 does not activate transcription and it competes for binding to the ISRE sequence, thereby suppressing gene activation. It is believed that this occurs as a signal attenuation mechanism in conditions where signalling has been triggered by IFN-α/β, but also as a mechanism to prevent expression of ISGs in the absence of IFN that would

have harmful effects to the cell. Furthermore, other IFN-induced proteins can act in a negative feedback loop, inhibiting further expression of IFN-induced genes. An example is the suppressors of cytokine signalling (SOCS) protein family, which are induced by IFN-γ and other cytokines, and which bind and inhibit activated Janus kinases, thus preventing STAT phosphorylation and activation, resulting in signal down-regulation. In addition, there is also evidence for the presence of a tyrosine phosphatase, such as SH2-containing tyrosine phosphatase-1 (SHP-1), that may dephosphorylate and thus inactivate Janus kinases and STATs, resulting in a transient activation of STAT1 and therefore a transient activation of the IFN signalling pathway. SHP-1 is known to associate with a subunit of the IFN-α/β receptor following IFN-α stimulation, and experimental data has shown higher levels of Jak1 and STAT1 phosphorylation in cells from mice lacking functional SHP-1, implying a role of SHP-1 in signal attenuation (reviewed in Goodbourn *et al.*, 2000). Members of the protein inhibitor of activated STAT (PIAS) family, such as PIAS1 and PIASy have also been implicated in negative regulation of IFN signalling. PIAS1 associates with STAT1 homodimers, disrupting their ability to bind DNA. PIASy does not block the binding of STAT1 to DNA but prevents it from activating transcription of ISGs.

1.2.2.5 Alternative mechanisms of interferon signalling

The Jak/STAT pathway was the first signalling pathway shown to be activated by IFNs and is required for the induction of many of the effects of IFNs. However, as the activation of this classical pathway alone does not seem to be sufficient for the generation of all of the biological activities of IFNs, it has now become clear that the Jak/STAT pathway is not the only way by which IFN stimulate the transcription of ISGs. Indeed, recent studies have uncovered some additional IFN-regulated signalling elements and cascades, which are required for the generation of many of the responses to IFNs. These are important for optimal activation and function of the classical Jak/STAT pathways, resulting in an optimized transcriptional regulation of target genes. Some of the various distinct signalling pathways include the mitogen-activated protein kinase (MAPK) p38

cascade, the phosphatidylinositol 3-kinase (PI3K) and the CRK family of adaptor proteins (reviewed in Platanias, 2005).

1.2.2.6 Interferon-λ signalling

As mentioned above, IFN-λ is a recently identified family of cytokines which include distinct but analogous proteins that were designated IFN-λ1, IFN-λ2 and IFN-λ3 (or IL29, IL-28A and IL-28B, respectively). The heterodimeric functional receptor complex (IFN-λR), which is utilized by all three IFN-λ proteins for signalling, is composed of the newly identified class II cytokine receptor subunit IFN-λR1 (also known as cytokine receptor family 2, member 12 [CRF2-12]) and a second subunit, IL-10R2 (also known as CRF2-4), which also serves as a subunit of the IL-10R and of the receptor for the IL-10 related cytokine IL-22. Despite binding to a unique receptor, IFN-λs share many functional characteristics with IFN-α/β. Both families of IFN are induced by virus infection or dsRNA, and up-regulates the transcription of antiviral genes (Kotenko *et al.*, 2003; Sheppard *et al.*, 2003). Furthermore, studies have shown that although IFN-λs bind to distinct receptors to those used by IFN-α/β, these cytokines activate similar Jak/STAT signal transduction pathways to those activated by IFN-α/β. Accordingly, like the IFN-α/β receptor, phosphorylation of specific tyrosine residues within the cytoplasmic domain of the IFN-λ receptor is necessary for triggering STAT activation and transduction (Dumoutier *et al.*, 2004). The kinases associated with the IFN-λR, which have yet to be identified but are likely to be Jak1 and Tyk2, induce tyrosine phosphorylation of STATs, including STAT2, which is a characteristic feature of a typical IFN-α/β response. Moreover, as with IFN-α/β signalling, IFN-λ signalling can also lead to the formation of STAT1 homodimers (GAF complex), which leads to the activation of GAS-regulated gene expression. Thus, IFN-λ and IFN-α/β signalling pathways overlap (as shown in Figure 1.9; Vilcek, 2003), and consequently some of their biological activities are similar, including the induction of antiviral protection and up-regulation of MHC class I antigen expression (Kotenko *et al.*, 2003). However, it has been shown that IFN-λs have a lower specific activity and a more limited range of target cell types, as

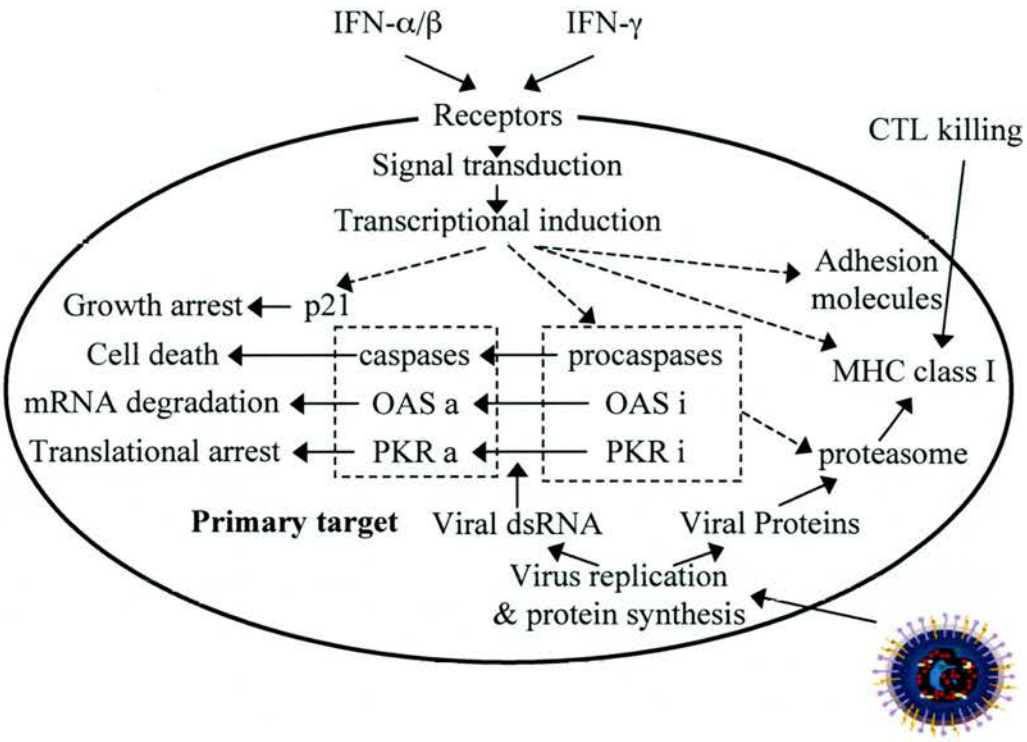
they induced antiviral activity in fewer cell lines and more weakly than IFN-α/β (Meager *et al.*, 2005). IFN-λ also activates STAT3 and STAT5, which is more characteristic of signalling by IL-10 and related cytokines.

1.2.3 Interferon antiviral response

Following the production of IFNs, binding of these cytokines to specific cell surface receptors initiates intracellular signalling cascades that ultimately activate the expression of hundreds of IFN-inducible genes (Figure 1.11). The genes up-regulated by IFN stimulation are involved in the establishment of an antiviral response within infected cells, in the neighbouring uninfected cells and cells of the adaptive immune system. Type I IFNs activate the expression of more than 300 ISGs which have antiviral, antiproliferative, and immunomodulatory activities (de Veer *et al.*, 2001; Der *et al.*, 1998). IFN-induced proteins include enzymes, transcription factors, cell surface glycoproteins, cytokines, chemokines and a large number of factors that are not yet fully characterized. Well characterized examples of these antiviral proteins include protein kinase R, the family of 2'-5' oligoadenylate synthetases (2-5 OAS)/endoribonuclease L (RNaseL), and the Mx GTPases. Others include ISG20, p56 protein, promyelocytic leukaemia protein (PML), dsRNA-specific adenosine deaminase (ADAR) and guanylate-binding protein-1 (GBP-1). The exact functions of many IFN-inducible proteins remain unknown but it is clear that a major role of these proteins is to dramatically reduce the activity of the host enzymatic machinery which viruses utilize in order to replicate. Therefore, the IFN-induced antiviral state limits virus replication in infected cells, as well as preventing infection of neighbouring uninfected cells, thereby gaining time for the host to activate the adaptive arm of the immune response.

1.2.3.1 dsRNA-dependent protein kinase R (PKR)

PKR is a ubiquitously expressed, IFN-inducible, serine/threonine kinase with multiple functions related to the control of transcription and translation. PKR contains two well characterized domains, the amino-terminal regulatory domain



adapted from Goodbourn et al., 2000

Figure 1.11 The biological properties of IFN-α/β and IFN-γ.

After binding of IFN molecules to their specific cell surface receptors, there is upregulation of a variety of genes, the products of which establish an anti-viral state in the cell. Many of these products, such as PKR and 2-5 OAS, are synthesised as inactive precursors (PKRi and 2-5 OASi), which are then activated by viral dsRNA (PKRa and 2-5 OASa, respectively). Activated PKR and 2-5 OAS shut down translation and up-regulate mRNA degradation, respectively. Other anti-viral products are involved in apoptosis, cell cycle arrest and the upregulation of innate immune responses. IFNs can also induce the synthesis of proteins that are involved in the processing and presentation of virus proteins to $CD8^+$ cytotoxic T lymphocytes (CTLs).

that contains two conserved dsRNA-binding motifs (the first mediates dsRNA-binding activity and includes residues critical for binding to dsRNA) and the carboxy-terminal catalytic domain that contains all of the conserved motifs for protein kinase activity. PKR is normally present in the cell in an inactive form, but is activated when dsRNA (or other polyanions) bind to its regulatory domain (George *et al.*, 1996; Katze *et al.*, 1991; Meurs *et al.*, 1992). dsRNA produced as a byproduct of viral replication is a strong activator of PKR, while another known PKR activator, the cellular protein PACT, is most likely to be a relevant activator in uninfected cells. However, efficient activation of PKR by PACT (PKR-activating protein) requires additional cellular stress signals, which induce phosphorylation of PACT at specific serine residues (reviewed in Sarkar *et al.*, 2005).

Upon binding viral dsRNA, PKR undergoes a conformational change that exposes its catalytic domain, causing autophosphorylation and activation. Once activated, PKR phosphorylates a limited set of cellular proteins, which interfere with transcription and translation. Regarding translation, it is known that PKR interferes with this process via its interaction with the α subunit of the translation eukaryotic initiation factor eIF2 (eIF-2α), part of the eIF2 complex that recruits the methionyl-tRNA (Met-tRNA, where tRNA denotes transfer RNA) initiator to the 40S ribosome subunit. Normally, eIF2 forms a complex with GTP, which upon binding of Met-tRNA forms a ternary complex Met-tRNA/GTP/eIF2 that binds to the 40S ribosomal particle forming another complex which then interacts with mRNA, other initiation factors and the large ribosomal subunit to form a pre-initiation complex. Hydrolysis of GTP to GDP allows the GDP/eIF2 complex to dissociate from the 40S ribosome, leaving Met-tRNA initiator attached. eIF2B, an initiation factor whose role is to activate eIF2 by replacing GDP with GTP, binds to GDP/eIF2 complex and once GDP has been exchanged for GTP, eIF2B dissociates from the complex. Now, eIF2/GTP and Met-tRNA can bind again to form a new complex and begin a new round of initiation. However, in the presence of activated PKR, eIF-2α is phosphorylated and binds more tightly than usual to eIF2B, preventing recycling of eIF2 between successive rounds of protein synthesis (Williams, 1999). Extensive phosphorylation of eIF-2α and strong

inhibition of eIF2B activity leads to no recycling of the eIF2 complex, which results in the down-regulation of the translation of cellular and viral mRNAs, thus contributing to PKR-mediated control of certain viral infections and cell growth (reviewed in Clemens & Elia, 1997).

As described earlier, PKR is also involved in the phosphorylation of IκB, the inhibitor of NF-κB, resulting in the degradation of IκB and release of active NF-κB, and therefore transcriptional activation of various promoters, including that of the IFN-β gene. PKR has been proposed to also be involved in the activation of other transcription factors that are involved in virus clearance, such as STAT1, IRF-1 and p53. Additionally, PKR has been shown to be an important element in the transcriptional signalling pathways activated by specific cytokines, growth factors, dsRNA, and extracellular stresses (reviewed in Williams, 2001). A large number of cellular processes, such as differentiation, cell growth, apoptosis, and oncogenic transformation have been also suggested to be induced by PKR. More specifically, apoptosis can be induced either directly by viral dsRNA through a PKR-mediated mechanism, or indirectly via PKR-dependent induction of Fas ligand and receptor. Bcl-2- and capase- dependent mechanisms have been suggested to be involved in PKR-mediated apoptosis. Indeed, the human proto-oncogene bcl-2 has been shown to block PKR-induced apoptosis (Lee *et al.*, 1997). However, apoptosis of cells infected with certain viruses has been observed to occur by pathways that do not require PKR, demonstrating that, although PKR plays a major role in antiviral mechanisms, there are cellular antiviral responses that depend on non-PKR-mediated processes (reviewed in Goodbourn *et al.*, 2000).

1.2.3.2 2'-5' oligoadenylate synthetases (2-5 OAS)/ endoribonuclease L (RNaseL) system

An important group of IFN-inducible proteins in mammalian cells are those of the 2'-5' oligoadenylate synthetase system, which consist of enzymes which, when activated by dsRNA, catalyse the synthesis of three- to five-unit oligoadenosine molecules from ATP. These oligoadenylates bind to and activate latent RNaseL,

which degrades ssRNA, including both viral and cellular mRNA, thereby blocking protein synthesis and leading to viral inhibition (Zhou *et al.*, 1997). RNaseL also cleaves 28S ribosomal RNA, thus disabling the ribosomal machinery, leading to translational inhibition (Iordanov *et al.*, 2000). The turnover of oligoadenylates is very rapid, and therefore active RNaseL is only found in the close proximity to activated 2-5 OAS, which is only activated by the presence of dsRNA during a viral infection. As a result, RNaseL activity in the intracellular environment is primarily focussed on viral RNA, and viral mRNAs appear to be preferentially cleaved compared to cellular mRNAs. RNaseL is constitutively expressed in most cell types, but its expression is up-regulated by IFN-α enhancing its activity in stimulated cells.

Similarly to PKR, OASs are synthesized as inactive precursors, which are activated during viral infection. The exact RNA activator has not been clearly defined, although it is believed that some single-stranded viral RNA with significant amount of double-stranded structure could activate 2-5 OAS. In addition, other viral sources of dsRNA include replicative intermediates of viral replication and the dsRNA genomes of viruses such as reoviruses. There is no structural homology between the dsRNA binding domain of 2-5 OAS and the binding domains of other dsRNA-binding proteins, such as PKR and ADAR. However, in common with these proteins, 2-5 OASs have separate domains for the RNA-binding site and the catalytic active region (reviewed in Sarkar *et al.*, 2005).

In addition to ribonuclease activity, RNaseL is believed to induce apoptosis of virally infected cells, given that experimental data *in vivo* demonstrated that RNaseL$^{-/-}$ mice are defective for apoptosis (Zhou *et al.*, 1997), and experimental data *in vitro* showed that RNaseL activation leads to direct induction of apoptosis in animal cells (Diaz-Guerra *et al.*, 1997). The mechanism by which RNaseL does so is unknown, although the cellular oncogene bcl-2 blocks the apoptotic effects of the RNaseL and the 2-5 OAS pathway (Diaz-Guerra *et al.*, 1997). Since bcl-2 blocks apoptosis mediated by either PKR or OAS, it was suggested that PKR and OAS may have a converging pathway of apoptotic induction in infected

cells, which is blocked by bcl-2 after the convergence point of those pathways (Lee *et al.*, 1997). Furthermore, apoptosis via the activation of RNaseL is independent of PKR, since the apoptotic effect of RNaseL is still observed in PKR-negative cell lines.

1.2.3.3 Mx proteins

Mx proteins are dynamin-like large GTPases that have antiviral activity, which inhibit the multiplication of several RNA viruses. Mx proteins are found in all vertebrates, including mammals, birds and fish (Arnheiter *et al.*, 1996; Staeheli *et al.*, 1993), and are induced upon virus infections in response to IFN. Different Mx proteins show different subcellular localizations and antiviral specificities. Interestingly, the subcellular compartment influences the antiviral spectrum to a great extent. The human MxA protein (76 kDa) is a cytoplasmic protein, which is rapidly induced in response to acute viral infections, and interferes with a number of RNA viruses known to replicate in the cytoplasm, including the *Paramyxoviridae* (Schneider-Schaulies *et al.*, 1994; Schnorr *et al.*, 1993; Zhao *et al.*, 1996), *Rhabdoviridae* (Pavlovic *et al.*, 1990; Schwemmle *et al.*, 1995) and *Bunyaviridae* (Frese *et al.*, 1995; Kanerva *et al.*, 1996), as well as members of the *Orthomyxoviridae* (Frese *et al.*, 1995; Frese *et al.*, 1997; Kochs & Haller, 1999; Pavlovic *et al.*, 1992; Pavlovic *et al.*, 1990) and *Togaviridae* (Landis *et al.*, 1998). In contrast, the nuclear mouse and rat Mx1 proteins are active exclusively against influenza and Thogoto viruses, which are known to have a nuclear replication phase (Haller *et al.*, 1995; Staeheli *et al.*, 1986). Mx proteins, in contrast to other ISGs, are not constitutively expressed in the cells, and their expression is not induced directly upon viral infection or the presence of dsRNA. The expression of Mx is stimulated exclusively by IFN-α/β via the Jak/STAT signalling pathway, thus Mx has been considered as a marker for IFN action in clinical settings (Antonelli *et al.*, 1999; Deisenhammer *et al.*, 2004; Roers *et al.*, 1994).

The importance of Mx proteins for host survival has been amply demonstrated (Arnheiter *et al.*, 1996; Hefti *et al.*, 1999; Pavlovic *et al.*, 1995), but their mechanism of action is still not completely understood. The viral target

recognition by MxA is virus- and cell type-specific, inhibiting transcription or mRNA translation, or targeting viral RNP complexes or transportation of nucleocapsids. Mx proteins have been suggested to interfere with virus replication by inhibiting the trafficking or activity of virus polymerases (Stranden *et al.*, 1993), thereby impairing the growth of various RNA viruses. Furthermore, MxA GTPases also appear to detect viral infection by sensing nucleocapsid-like structures, and as a consequence these viral components are trapped and sorted to locations where they become unavailable for the generation of new virus particles (Haller & Kochs, 2002). In the case of Thogoto virus, MxA prevents the incoming viral nucleocapsids from being transported into the nucleus, the site of viral transcription and replication (Kochs & Haller, 1999). More recently, MxA was shown to specifically inhibit replication but not transcription of the La Crosse virus (LACV) genome, a member of the *Bunyaviridae* family. In this case MxA specifically recognizes and sequesters the viral nucleocapsid protein into large perinuclear complexes, leading to relocalization of both components (viral nucleocapsid and cellular MxA proteins) in infected cells. Thus, MxA interferes with transport of the LACV nucleocapsid protein (N) to the Golgi compartment, where virus assembly takes place, and therefore this MxA-mediated sequestration of N protein accounts for the observed block in viral genome replication (Kochs *et al.*, 2002). It has been further demonstrated that in LACV-infected cells, MxA localizes to a subcompartment of the smooth endoplasmic reticulum where the viral N protein accumulates. In addition, oligomeric MxA/N complexes were shown to form in close association with COP-I-positive vesicular-tubular membranes, where MxA and N preferentially interact, leading to efficient sequestration and mis-sorting of an essential viral component (Reichelt *et al.*, 2004). Although it is known that MxA affects the *Paramyxoviridae*, such as MeV (Schneider-Schaulies *et al.*, 1994; Schnorr *et al.*, 1993) and hPIV3 (Zhao *et al.*, 1996), and the *Rhabdoviridae*, such as VSV (Schuster *et al.*, 1996), the mechanism by which it inhibits virus replication is not completely understood.

1.2.3.4 Other antiviral mechanisms

In addition to Mx GTPases, 2-5 OAS and PKR, studies involving fibroblasts derived from triple knock-out mice lacking Mx, RNaseL and PKR have shown that such cells still mount a limited IFN-induced antiviral state, suggesting the existence of additional IFN-induced antiviral pathways (Zhou *et al.*, 1999). Additional proteins with potentially important antiviral activities are ISG20, p56 protein, promyelocytic leukaemia protein, dsRNA-specific adenosine deaminase and guanylate-binding protein-1.

ISG20 is induced by IFN and is a 3' to 5' exonuclease that specifically degrades ssRNA. It has been shown, in the absence of IFN treatment, that stable and constitutive expression of ISG20 confers resistance to VSV, influenza viruses, and encephalomyocarditis virus (EMCV) infection in human cells (HeLa), providing an alternative antiviral pathway against RNA genomic viruses. Particularly, ISG20 specifically interfered with VSV mRNA synthesis and protein production, whereas the expression of cellular control genes was unaffected. It was demonstrated that the antiviral action of ISG20 is mediated by its 3' to 5' exonuclease activity, and thus it is likely that ISG20 affects viral development by degrading viral RNA (Espert *et al.*, 2003).

p56 is the product of mRNA 561 encoded by the ISG56 gene (also known as IFN-induced protein with tetratricopeptide repeats 1 [IFIT1]), a gene that is highly induced in response to IFN, dsRNA, and many viruses (Der *et al.*, 1998). Untreated cells do not express p56, but ISG56 gene transcription is rapidly and strongly induced upon viral infection and other stresses. The induction of p56 by IFN is transient, and both mRNA and protein have relatively short half-lives. Thus, it seems that the p56 protein is designed to be accumulated in IFN-treated cells to a high concentration but for a short time, indicating that p56 may have a potential regulatory role. p56 belongs to a family of structurally related viral stress-inducible proteins that includes p54, p56, p58, and p60. Structurally, the significant feature of the p56 family members is the presence of multiple tetratricopeptides repeat (TPR) motifs, which are shown in other proteins to

mediate association with multiprotein complexes involved in diverse functions, as well as association with large protein complexes modifying their function (reviewed in Sarkar *et al.*, 2005). One of the best characterized interactions with p56 identified so far has been with Int-6/p48 (also known as eIF-3e), a subunit of eukaryotic initiation factor 3 (eIF3) (Guo & Sen, 2000). Binding of p56 to the eIF-3e subunit selectively inhibits the ability of eIF-3e to stabilize the ternary complex of eIF2, GTP and Met-tRNA, and therefore p56 inhibits initiation of translation (Hui *et al.*, 2003).

Another IFN-induced factor, the PML protein, also contributes to antiviral activity, as has been shown for VSV, influenza A virus, lymphocytic choriomeningitis virus and human foamy virus (Chelbi-Alix *et al.*, 1998; Djavani *et al.*, 2001; Everett, 2001; Regad & Chelbi-Alix, 2001). PML concentrates in speckled subnuclear structures referred to as PML nuclear bodies (PML-NBs), ND10 or PML oncogenic domains (POD). Various proteins have been shown to co-localize with the PML protein in PML-NBs either transiently or constitutively, including Sp100, ISG20, Daxx and p53 (Maul *et al.*, 2000; Regad & Chelbi-Alix, 2001). PML-NBs regulate cellular proliferation, apoptosis, as well as having a role in controlling the assembly and function of various transcription factors. PML is essential for the proper formation and integrity of the PML-NBs, and its modification by the Small Ubiquitinrelated Modifier (SUMO-1) was shown to be required for the localization of PML in the PML-NBs. The number and intensity of PML-NBs increases in response to IFN, suggesting a role in intracellular immunity and antiviral defence (reviewed in Regad & Chelbi-Alix, 2001). However, the mechanism by which PML inhibits viral replication is not known, although it has been suggested that PML indirectly inhibits viral propagation by modifying other cellular proteins that mediate viral replication (de The & Chelbi-Alix, 2001).

GBP-1 belongs to the dynamin superfamily of large GTPases like Mx. However, it is predominantly induced by IFN-γ and its antiviral activity against VSV is comparatively weak (Anderson *et al.*, 1999). GBP-1 was the first large GTPase to

be crystallized and its 3-D structure provided a valuable model for other family members (Prakash *et al.*, 2000).

Other factors that clearly play an important role in the IFN-induced antiviral response are caspases, enzymes involved in apoptosis (described below), and the ADAR which has a direct mutagenic effect on dsRNA. ADAR catalyzes deamination of adenosine residues on target dsRNA to yield inosine (I) residues. As inosine is recognised as guanosine by ribosomes, this editing effectively changes the sequence of the mRNA transcript and is likely to have significant effects on the function of the encoded protein. Also, the editing process of the dsRNA results in destabilization of its secondary structure due to a change from an AU base pair to the less stable IU base pair and mutations accumulate within the viral genome. Furthermore, it has also been suggested that an RNase with specificity for RNA containing inosine residues, I-RNase, may have antiviral activity by cleaving edited transcripts.

1.2.3.5 Antiproliferative action of interferon

IFNs can slow down cell growth and arrest cellular processes that are necessary for virus replication, thereby inhibiting the proliferation of certain viruses. The sensitivity of cells to the antiproliferative effects of IFNs is very cell-type dependent, and because of its great clinical importance, it has been extensively studied. There is evidence, from experimental data, that PKR and RNaseL are involved in the antiproliferative action of IFN, as overexpression of these proteins was shown to suppress or reduce the growth of transfected cells, and/or to be toxic to those cells. Moreover, IFN stimulates p21, a cyclin-dependent kinase inhibitor involved in cell cycle progression, specifically in the progression from G_1 into S phase, and therefore IFN exerts negative regulation of the cell cycle (Chin *et al.*, 1996; Subramaniam *et al.*, 1998; Subramaniam & Johnson, 1997). Transcription of c-myc, which is required for cell growth, has also been also shown to be down-regulated directly by IFNs, providing another direct link between IFNs and their antiproliferative properties (reviewed in Goodbourn *et al.*, 2000). Additionally, members of the P200 family of structurally related proteins are strongly induced

by IFN, and the P202 protein has been shown to impair cell proliferation by inhibiting the functions of many cellular factors, including NF-κB, E2F, p53, c-fos, c-jun, MyoD, myogenic, c-myc, and Rb (reviewed in Sarkar & Sen, 2004).

1.2.3.6 Control of apoptosis by interferon

IFNs, like other cytokines, can influence cellular apoptotic responses, by inducing or inhibiting apoptosis, depending on various factors such as the state of cell differentiation. However, upon viral infection, IFN has the major role of inducing apoptosis. Interestingly, 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 non-permissive for viral replication. As described above, IFNs can affect indirectly the apoptotic state of cells through PKR and the 2'-5' oligoadenylate system. However, IFNs are known to induce caspases (Balachandran *et al.*, 2000; Chin *et al.*, 1997; Subramaniam *et al.*, 1998), 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).

1.2.3.7 Effects of interferon on the immune system

The immunomodulatory effects of IFNs have been extensively studied, and IFNs, in particular IFN-γ, have been demonstrated to be very potent immune regulators, as they are known to profoundly affect nearly all phases of innate and adaptive immune responses. The effect of IFNs on the adaptive immune response is wide ranging, extending to the humoural and cellular branches of the system, as well as to other critical immune processes, such as antigen processing and presentation, which in turn promote downstream immune responses.

Regarding innate immune responses, a major immunomodulatory function of IFN is performed by IFN-α/β by enhancing the cytotoxicity of NK cells by up-regulating the perforin levels (Kaser *et al.*, 1999; Mori *et al.*, 1998), and by enhancing NK cell cytotoxicity, which is of extreme importance for antiviral measures. 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 through different mechanisms. IFN-γ also plays an important role in the activation of macrophages which, in turn, use a variety of IFN-γ-induced mechanisms to destroy microbial targets (reviewed in Goodbourn *et al.*, 2000).

As mentioned above, IFNs act to enhance cell-mediated immunity, and thus the antiviral response, by interacting with various components of the adaptive immune system. Indeed, both IFN-α/β and IFN-γ are known to stimulate $CD8^+$ T cell responses, by up-regulating MHC I protein expression, leading to an enhanced display of intracellular antigens on the surface of infected cells and therefore an improved $CD8^+$ response. In contrast, only IFN-γ up-regulates MHC II-dependent antigen presentation, which promotes $CD4^+$ T cell mechanisms. IFN-γ also up-regulates the expression of cellular proteins involved in antigen processing including specific subunits of proteasomes, which degrade protein antigens to generate antigenic peptides for presentation on class I MHC. The additional proteasome subunits expressed in response to IFN-γ, including latent membrane protein 1 (LMP1), LMP7 and MECL1, have different substrate specificities compared to the normal subunits, resulting in different peptides that are presented to the immune system. Furthermore, IFN-γ also promotes trafficking of processed antigen to the endoplasmic reticulum through the up-regulation of transporter 1 (TAP1) and transporter 2 (TAP2), proteins involved in peptide transfer, thereby increasing the amount of peptides loaded onto and displayed by MHC I. It is well known that viral infections generate intracellular antigens that are subject to the above processing mechanisms, and therefore IFNs strongly facilitate the ability of the cell to present viral antigens, thereby affecting virus survival (Goodbourn *et al.*, 2000). The humoural arm of the adaptive immune system is also affected by IFNs, as they influence the development of

specific subsets of 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. Moreover, IFN-α/β regulates the production of IL-15, which stimulates memory T cell division and it is believed that IFN-α/β itself supports directly the survival of activated T cells. IFN-γ is additionally known to regulate the balance between the two types of T helper cells, Th1 and Th2 cells, which determine whether the immune response to an antigenic challenge is predominantly cell-mediated or antibody-mediated (Goodbourn *et al.*, 2000).

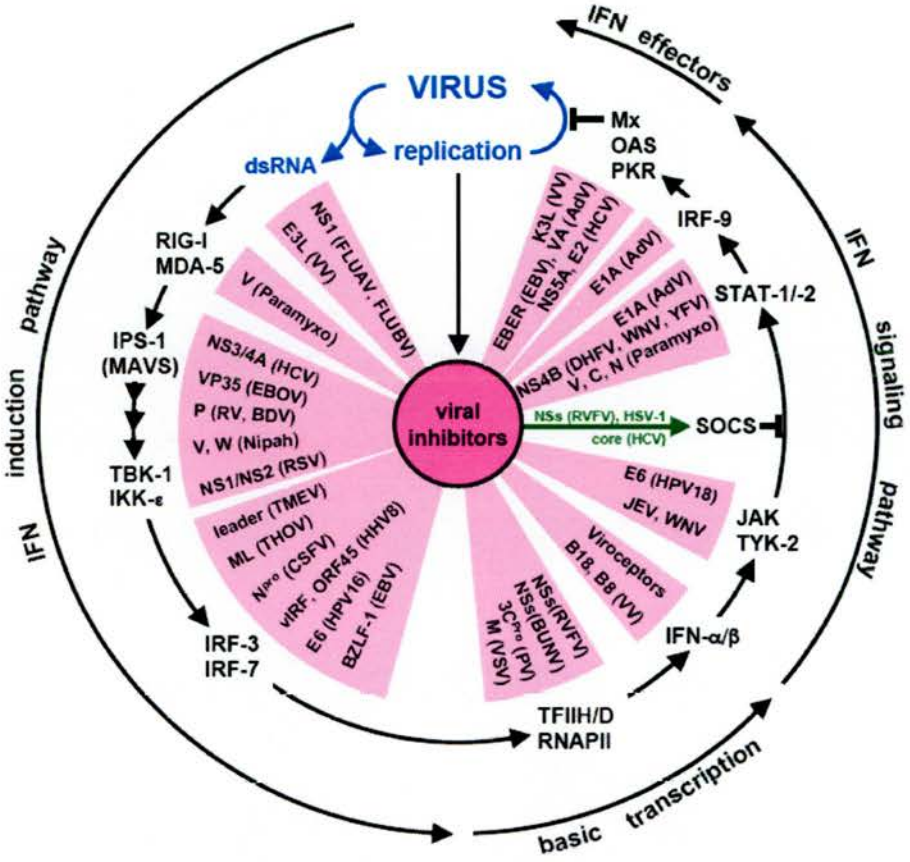
1.3 Viruses and Interferon Evasion

Since IFN was discovered by Isaacs and in 1957 as a cytokine interfering with virus replication (Isaacs & Lindenmann, 1957), IFNs have been extensively studied and progress has been made to demonstrate how IFNs are induced and how they work by activating IFN-responsive genes that mediate cell-autonomous resistance against viruses. However, an early observation by Lindenmann, who described for the first time a viral IFN-suppressive function, is only now being fully appreciated. In that study, an infection of cells with a live virus inhibited the subsequent induction of IFN by an inactivated virus, and this phenomenon was called "inverse interference" (Lindenmann, 1960). IFN expresses antiviral, anti-proliferative and immunoregulatory properties and its powerful contribution to the immune response is proven by the many infectious agents who expend significant portions of their limited coding capacity in inhibiting, at least to some extent, the host IFN system. Interestingly, it appears that different viruses antagonise IFN-mediated defence responses by either inhibiting IFN synthesis, binding and inactivating secreted IFN molecules, blocking IFN-activated signalling, or disturbing the action of IFN-induced antiviral proteins. In Figure 1.12 the range of activities mediated by IFN antagonists of various viruses is schematically presented. Remarkably, viral proteins or functions have been discovered that cover the whole range of the IFN response in infected cells. Moreover, different components of the IFN induction and signalling cascade can be inhibited by a single viral protein. Another important point that can be seen from Figure 1.12 is that a certain virus may have more than one IFN antagonistic activity targeting different pathways (reviewed in Haller *et al.*, in press). However, virus countermeasures to the IFN response are rarely absolute and the IFN response limits virus spread to buy time for the generation of an acquired immune response to the invading virus. Therefore, the viruses must evade the IFN response efficiently and rapidly, as without such evasion mechanisms virus infections would be rapidly cleared from the host. Viruses in which evasion mechanisms have been discovered include DNA viruses such as herpes simplex virus, human cytomegalovirus, adenoviruses and poxviruses, and RNA viruses such as influenza virus, Ebola virus, and Bunyamwera virus. Paramyxoviruses are not an

Figure 1.12 Viral inhibitors of the virus-induced IFN-α/β response loop.

Viral gene products interfere with the type I IFN system at all levels. The following viral IFN antagonists are shown in order: NS1 of influenza A virus (FLUAV), NS1 of influenza B virus (FLUBV), E3L of Vaccinia virus (VV), V of paramyxoviruses, NS3/4A of Hepatitis C virus (HCV), VP35 of Ebola virus (EBOV), P of Rabies virus (RV), P of Borna disease virus (BDV), V and W of Nipah virus, NS1/NS2 of respiratory syncytial virus (RSV), leader protein of Theiler's meningoencephalitis virus (TMEV), ML of Thogoto virus (THOV), N^{Pro} of classical swine fever virus (CSFV), vIRF and ORF45 of human herpes virus 8 (HHV-8), E6 of human papilloma virus 16 (HPV16), BZLF-1 of Epstein-Barr virus (EBV), M of vesicular stomatitis virus (VSV), $3C^{Pro}$ of Polio virus (PV), NSs of Bunyamwera virus (BUNV), NSs of Rift valley fever virus (RVFV), B18 and B8 of VV, Japanese encephalitis virus (JEV), West Nile virus (WNV), E6 of HPV18, core protein of HCV, herpes simplex virus 1 (HSV-1), NSs of RVFV, V, C and N of paramyxoviruses, NS4B of Dengue hemorrhagic fever virus (DHFV), WNV, and Yellow fever virus (YFV), E1A of adenovirus (AdV), NS5A and E2 of HCV, EBER of EBV, VA of AdV, K3L of VV (reviewed in Haller *et al.*, in press).

Figure 1.12



from Haller et al., in press

exception, and many members of the *Paramyxoviridae* have been shown to circumvent the IFN response.

1.3.1 Viral shut-off of host cell protein synthesis

Many viruses take over the protein machinery of the cell, resulting in a selective expression of viral proteins, whereas the host mRNA production or protein synthesis is down-regulated (Table 1.4).

Table 1.4 Factors produced by viruses that target general host cell gene expression (adapted from Weber *et al.*, 2004).

Virus	Viral gene product	Cellular target
Rift Valley fever Virus	NSs protein	TFIIH
Bunyamwera virus	NSs protein	RNA polymerase II
Poliovirus	$3C^{Pro}$ protein	transcription (TFIIH)
Influenza A virus	NS1 protein	host cell mRNA processing, nucleo-cytoplasmatic mRNA transport
Herpes simplex virus	ICP27 protein	host cell mRNA synthesis and splicing
	vhs protein	host cell mRNA stability
	unknown	translation
Vesicular stomatitis virus	M protein	transcription (TFIIH), nucleo-cytoplasmatic transport and translation
Foot-and-mouth disease virus	Leader protein	translation

Shutting-off cell protein synthesis results in higher availability of energy and cellular reagents to translate viral proteins. Additionally, the induction of IFN-mediated antiviral responses depends on translation of host factors, e.g. IFN itself, and therefore virus-induced shut-off of cellular protein synthesis can contribute to the inhibition of the IFN responses in infected cells. A typical example of this situation is the very well established role of the matrix (M) protein of VSV, which causes a general inhibition of host-cell transcription, including transcription from the IFN-β promoter (Ferran & Lucas-Lenard, 1997). The ability of the VSV M protein to repress host gene expression is achieved by inhibition of host transcription, as well as the nuclear-cytoplasmic transport of

host mRNAs and proteins (Ahmed & Lyles, 1998; Black & Lyles, 1992; Her *et al.*, 1997; Petersen *et al.*, 2000); M also plays a role in the inhibition of host translation (Black *et al.*, 1994). Similarly, the L proteinase gene of the foot-and-mouth disease virus encodes a protein that shuts off host cell protein synthesis, and mutation of this gene was shown to be sufficient to generate an attenuated strain that induces elevated levels of IFN-α/β (Chinsangaram *et al.*, 1999). The general blockage of host cell transcription and translation is an IFN-antagonistic activity also used by other viruses. Accordingly, the nonstructural proteins of Rift Valley fever Virus (RVFV, a phlebovirus of the *Bunyaviridae* family) and Bunyamwera virus (BUNV, an orthobunyavirus of the *Bunyaviridae* family) target the RNA polymerase II, although by different mechanisms, resulting in a general block of mRNA transcription, and thus suppression of IFN gene expression (Billecocq *et al.*, 2004; Thomas *et al.*, 2004). The NSs protein of RVFV was demonstrated to target the p44 subunit of the essential general transcription factor IIH (TFIIH), thereby preventing proper assembly of the cellular RNA polymerase II (Le May *et al.*, 2004), whereas the NSs of BUNV appears to inhibit phosphorylation of the large subunit of the RNA polymerase II (Thomas *et al.*, 2004). Similarly, other viruses including poliovirus, influenza A virus (FLUAV), and herpes simplex virus-1 (HSV-1) evade the IFN system by generally shutting off transcription and translation, thereby suppressing IFN gene expression (Table 1.4). However, many viruses depend on a functional environment and the entire cellular metabolism can not be disturbed, and thus these viruses target specific components of the IFN signalling cascade.

1.3.2 Viral interference with interferon induction pathway

A prominent target for viruses that block IFN production is the signal dsRNA molecule. As described above (section 1.2.1), dsRNA is presumed to be the signal for the activation of cellular kinases that lead to the stimulation of the several transcription factors that participate in the activation of the IFN-β promoter, and thus lead to IFN expression. Several viruses encode dsRNA-binding proteins as part of their life-cycle, and therefore sequestering of dsRNA by these proteins not only inhibits IFN production, but also minimizes the

antiviral action of IFN by preventing the activation of the dsRNA activated enzymes like PKR, 2-5 OAS and ADAR, as well as dsRNA-dependent apoptosis. The NS1 protein of FLUAV was the first IFN antagonist of a negative-strand RNA virus to be identified. NS1 is pleotropic since it not only interferes with host mRNA synthesis and enhances translational initiation of viral mRNAs, but also interferes with IFN production. NS1 is a dsRNA-binding protein which may thus prevent the recognition of viral RNAs and the activation of the antiviral and antiproliferative protein PKR (Garcia-Sastre *et al.*, 1998), therefore inhibiting IFN production, as well as of induction of immunomodulatory genes. Similarly, the NS1 protein of influenza B virus (FLUBV) (Wang & Krug, 1996) and the E3L protein of poxviruses (Xiang *et al.*, 2002) bind and sequester dsRNA, thereby inhibiting activation of the transcription factors IRF-3, NF-κB and activator protein-1 (AP-1) (Dauber *et al.*, 2004; Garcia-Sastre, 2001; Ludwig *et al.*, 2002; Talon *et al.*, 2000; Wang *et al.*, 2000). Additionally, NS1 of FLUBV binds ISG15, an IFN-induced protein that is an ubiquitin-like modifier, thereby preventing conjugation of ISG15 to cellular target proteins (Yuan & Krug, 2001). Modification of proteins by ISG15 occurs in a manner similar to that of ubiquitin and other ubiquitin-like modifiers, and ISG15 has been shown to affect signal transduction via the Jak/STAT pathway, which is required for the amplification of the IFN response (Kim & Zhang, 2003).

Another attractive target, for viruses to interfere with IFN production, is the IFN-specific transcription factor IRF-3, and proteins of many viruses inhibit activation of IRF-3 (Table 1.5). Examples include the VP35 protein of Ebola virus (Basler *et al.*, 2003) and the NS3/4A protease of hepatitis C virus (HCV) (Foy *et al.*, 2003). The NS1/NS2 protein complex of RSV (Bossert *et al.*, 2003; Spann *et al.*, 2004) prevents phosphorylation of IRF-3, and consequently IRF-3 dimerization, nuclear transport, and thus also results in inhibition of IFN production. In the case of HSV-1, the early protein ICP0 inhibits nuclear accumulation of activated IRF-3, but it does not affect the phosphorylation step (Melroe *et al.*, 2004). The ML protein of Thogoto virus (THOV) does not inhibit phosphorylation or nuclear transport of IRF-3, but it has been shown to target activated IRF-3 at a nuclear step, specifically by interfering with IRF-3 dimerization and recruitment of the

transcriptional coactivator CBP by activated IRF-3 (Jennings *et al.*, 2005). Likewise, the E1A gene product of adenoviruses sequesters the coactivator CBP rather than IRF-3. In cells infected with bovine viral diarrhea virus (BVDV), the nuclear steps of IRF-3 function were also disturbed (Baigent *et al.*, 2002). The leader protein of Theiler's virus has been shown to inhibit IFN induction by interfering with nucleo-cytoplasmic trafficking of IRF-3 (Delhaye *et al.*, 2004). Other viruses, such as rotavirus and human papilloma virus 16 (HPV-16) encode proteins (NSP1 and E6, respectively) that directly bind and inactivate IRF-3 (Graff *et al.*, 2002; Ronco *et al.*, 1998). SV5 uses its V protein to inhibit the activation of IRF-3 (He *et al.*, 2002; Poole *et al.*, 2002).

Table 1.5 Viral factors that interfere with specific components of the IFN induction pathway (adapted from Weber *et al.*, 2004).

Virus	**Viral gene product**	**Cellular target**
Influenza A and B virus	NS1	intracellular dsRNA, IRF-3, NF-κB, AP-1
Thogoto virus	ML	IRF-3
Simian virus 5	V	IRF-3
Sendai virus	C, Y1, Y2, V	IRF-3
Measles virus	not known	not known
Respiratory syncytial virus	NS1 and NS2 proteins	IRF-3
Ebola virus	VP35	IRF-3
Herpes simplex virus 1	ICP0	IRF-3
Human herpes virus 8	vIRF-1, -2, and -3	IRF-1, IRF-3, IRF-7, and CBP/p300
Human papilloma virus 16	E6	IRF-3
Adenovirus	E1A	CBP
Theiler's virus	Leader protein	IRF-3
Bovine diarrhea virus	E^{rns}	extracellular dsRNA
	not known	IRF-3
Classical swine fever virus	N^{Pro} protein	intracellular dsRNA
Hepatitis C virus	NS3/4A protein	IRF-3
Reovirus	core proteins M1, S2, L2	IFN induction
Vaccinia virus	E3L protein	intracellular dsRNA
Sindbis virus	nsP2 protein	IFN production

Many negative-strand RNA viruses use the phosphoprotein, an essential protein of the viral polymerase complex, as an IFN antagonist. Examples are the P protein of Rabies virus, which prevent IRF-3 phosphorylation by TBK-1 (Brzozka *et al.*, 2005) and the P protein of Borna disease virus, which binds directly to TBK-1

reducing its activity (Unterstab *et al.*, 2005). Some viruses use a different strategy to block cellular IRF-3, such as certain herpesviruses. Human herpes virus 8 (HHV-8), the causative agent of Kaposi's sarcoma, encodes IRF homologues (vIRFs), which seem to inhibit cellular IRFs, such as IRF-1, IRF-3 and IRF-7 through a dominant negative effect (reviewed in Haller *et al.*, in press). Several other viruses, including classical swine fever virus, MeV, and Sindbis virus have been shown to interfere with IFN production by unknown mechanisms (reviewed in Weber *et al.*, 2004).

1.3.3 Inhibition of interferon signalling

Several viruses target IFN signal transduction for inhibition, therefore preventing ISG expression (Table 1.6).

Table 1.6 Viral factors that interfere with IFN signalling (adapted from Weber *et al.*, 2004).

Virus	**Viral gene product**	**Cellular target**
Paramyxoviruses (Henipahviruses, measles virus, mumps virus, Sendai virus, Simian virus 5)	V protein	STATs
	W protein	STAT1
	C protein	STAT1
	V and C proteins	Jak1
Hepatitis C virus	viral proteins	Jak/STAT pathway SOCS3 induction
Herpes simplex virus	not known	Jak1, STAT2 SOCS3 induction
Murine cytomegalovirus	M27 protein	STAT2
Murine polyoma virus	Large T antigen	Jak1
Influenza A virus	NS1 protein	not known
Influenza B virus	NS1 protein	ISG15
Adenovirus	E1A protein	STAT1, IRF-9
Vaccinia virus	B18R protein	secreted IFN-α/β
	B8R protein	secreted IFN-γ
Human papilloma virus 18	E6	Tyk2, STATs
Dengue virus	NS4B (NS2A, NS4A)	Jak/STAT pathway
Japanese encephalitis virus	not known	Tyk2, STATs

Since there are components in common between signalling pathways (described above), it is possible for a virus to block IFN-α/β or IFN-γ signalling or both. Using such strategies, viruses can disrupt the establishment of an antiviral state by

inhibiting the induction of cellular antiviral enzymes, such as PKR, 2-5 OAS and Mx, as well as preventing up-regulation of class I MHC molecules within infected cells, and as a consequence these cells are poor targets for cytotoxic T cells. In addition, given the positive feed-back loop (as some of the proteins involved in IFN production are themselves IFN-inducible) provided by IFN signalling for an enhanced IFN gene expression, its disruption also affects the overall amount of IFN produced. Both DNA and RNA viruses are known to have molecular mechanisms that specifically target IFN signal transduction pathways in their entire length. Interestingly, the mechanisms by which the different viruses achieve this are very different.

At the level of IFN receptor activation, several poxviruses inhibit initiation of signalling by producing soluble IFN receptor homologues ("viroreceptors") that essentially bind IFN and thus prevent cell membrane receptor activation. As a consequence, the autocrine IFN amplification loop is disrupted in the IFN-producing cells, thereby preventing the establishment of an antiviral state in those cells, as well as in surrounding uninfected cells (reviewed in Goodbourn *et al.*, 2000; Weber *et al.*, 2004).

Several viruses (amongst them *Paramyxoviridae*) inhibit IFN signal transduction by targeting cellular protein components of the Jak/STAT pathways, and thereby circumvent the IFN defence system to establish infections. For example, the V protein of SV5 blocks IFN signalling by targeting STAT1 for proteasome-mediated degradation, thereby blocking both IFN-α/β and IFN-γ signalling within infected cells (Didcock *et al.*, 1999b), while the V protein of human parainfluenza virus type 2 (hPIV2) primarily targets STAT2 (Young *et al.*, 2000) and thus blocks only IFN-α/β signalling. MuV V protein targets both STAT1 and STAT3 (Ulane *et al.*, 2003), and thus both IFN-α/β and IFN-γ signalling are disrupted. A requirement for the degradation of STATs by the V proteins of rubulaviruses is their interaction with DDB1. In addition, evidence has been presented which demonstrates that STAT2 is required for the degradation of STAT1 by SV5, and it was suggested that DDB1, STAT1, STAT2, and V may form part of a large multiprotein complex which leads to the targeted degradation of STAT1 by the

proteasome (Andrejeva *et al.*, 2002a). DDB1 is known to interact with cullin 4a in normal cells. Cullins are a family of proteins that form part of E3 ligases that target substrates for ubiquitin-dependent degradation by the proteasome (reviewed in Pintard *et al.*, 2004). Cul4a has been shown to be required for the ubiquitination and subsequent degradation of STAT1 in a complex reaction (Precious *et al.*, 2005; Ulane & Horvath, 2002; Ulane *et al.*, 2005).

MeV does not induce STAT ubiquitination and degradation but instead its V protein associates with cellular STAT1, STAT2, STAT3, and IRF9, as well as several unidentified partners. As a consequence, the V protein of MeV prevents IFN-induced ISGF3 assembly and STAT protein nuclear translocation, thus blocking both IFN-α/β and IFN-γ signalling (Palosaari *et al.*, 2003). At the same time, a different study showed that MeV V protein blocks the IFN-α/β-induced antiviral state, but not the IFN-γ-induced state. In this study the MeV V protein was shown not to induce the degradation of STATs, but instead to inhibit phosphorylation of both STAT1 and STAT2 (Takeuchi *et al.*, 2003). In addition, another study revealed that the C protein of MeV inhibits the cellular response to IFN-α/β signalling (Shaffer *et al.*, 2003). Also unlike infection by rubulaviruses, infection by the respirovirus SeV appears not to lead to STAT degradation. The C proteins of SeV block IFN-α/β and IFN-γ signalling by interfering with STAT1 phosphorylation or stability (reviewed in Stock *et al.*, 2005 and Nagai & Kato, 2004). Initial studies revealed that SeV infection and the transient expression of C proteins block both IFN-α/β and IFN-γ signalling pathways (Didcock *et al.*, 1999a; Young *et al.*, 2000). The mechanism of the C protein-mediated antagonism of the IFN response remains to be fully elucidated and it appears very complicated, with at least three distinct mechanisms operating, i) an early event in which IFN signalling is blocked by sequestration STAT1 into a complex, ii) a late event in which STAT1 becomes hyper-phosphorylated, and iii) the targeted degradation of STAT1 (reviewed in Stock *et al.*, 2005). The V proteins of the newly emergent HeV and NiV have been demonstrated to subvert IFN responses by sequestering STAT1 and STAT2 into high-molecular-weight cytoplasmic complexes. This sequestration of STAT1 and STAT2 prevents STAT activation and blocks antiviral IFN signalling (Rodriguez *et al.*, 2002; Rodriguez *et al.*,

2003). Similar strategies are used by other viruses, which also interfere with the IFN signalling pathway and cause persistent infections, such as HCV, HSV-1, polyomaviruses or cytomegalovirus (CMV). The adenovirus E1A protein both disrupts transcriptional responses to IFN-α/β and IFN-γ by sequestering the transcriptional coactivator CBP/p300, which binds STAT1 and STAT2 and is involved in transcription responses mediated by these proteins (Bhattacharya *et al.*, 1996) and by targeting STAT1 and IRF-9, which prevents formation of the ISGF-3 complex (Leonard & Sen, 1996). Dengue virus and Japanese encephalitis virus target the Jak/STAT pathway by interfering with STAT1 function and blocking Tyk2 activation, respectively (Lin *et al.*, 2004; Munoz-Jordan *et al.*, 2003). Likewise, the E6 protein of HPV-18 interacts with Tyk2, impairs its activation, and hence blocks the IFN-α/β signalling pathway (Li *et al.*, 1999). In the case of HPV-16, it is the multifunctional E7 protein which, by interacting directly with IRF-9, prevents the formation of ISGF3 and thus the activation of IFN-α/β inducible genes (Barnard & McMillan, 1999). A different way to interfere with IFN signalling is used by HSV-1, which induces the normal cellular suppressor of cytokine signalling, SOCS3, to down-regulate STAT and Jak phosphorylation. The core protein of HCV has also been shown to induce the expression of SOCS3. Similarly, the NSs protein of RVFV also activates the cellular suppressor of STAT activation, SOCS1, to impede IFN signalling (reviewed in Haller *et al.*, in press).

1.3.4 Inhibition of interferon-induced antiviral enzymes

IFN-induced antiviral proteins that mediate the antiviral state are also direct targets of viral counter-IFN strategies, which provide an obvious advantage to the proliferation potential of such viruses (Table 1.7).

Table 1.7 Viral factors that interfere with IFN-induced antiviral proteins (adapted from Weber *et al.*, 2004).

Virus	Viral gene product	Cellular target
Vaccinia virus	E3L protein	intracellular dsRNA, PKR
	K3L protein	PKR
Influenza A virus	NS1 protein	intracellular dsRNA, PKR
	not known	up-regulation of $p58^{IPK}$
Reoviruses	σ3 protein	intracellular dsRNA, PKR
Rotavirus	NSP3	intracellular dsRNA, PKR
Herpes simplex virus	γ34.5 protein	phospho-eIF-2α
	U_S11	intracellular dsRNA, PKR
	not known	RNaseL
Human herpes virus 8	vIRF-2 protein	PKR
Hepatitis C virus	NS5A protein	PKR
	internal ribosome entry site	PKR
	E2 protein	PKR
HIV	Tat protein	PKR
	TAR protein	PKR
	not known	RNaseL
Adenoviruses	VA_IRNA	PKR
Epstein-Barr virus	EBER RNA	PKR
Poliovirus	not known	PKR
Encephalomyocarditis virus	not known	RNaseL
Respiratory syncytial virus	NS1/NS2 proteins	not known

Many IFN effector proteins, such as PKR and the 2-5 OAS, are dependent on dsRNA for their enzymatic activation, and therefore viruses which produce factors that sequester dsRNA are able to block activation, and thus the activity of such effector proteins. Viruses that produce such dsRNA binding proteins also have the ability to interfere with IFN production and action, given the central role of dsRNA in IFN induction. An example of this is the dsRNA binding protein NS1 of influenza A virus, which inhibits the IFN system not only at the level of IFN synthesis (as described above) but also at the level of IFN action. As synthesis of IFN is most likely not completely blocked during FLUAV infection, the ability of NS1 to counteract the antiviral action of IFN should also result in increased viral replication in the host. Indeed, it has been shown that the NS1 protein inhibits dsRNA-mediated activation of PKR (Bergmann *et al.*, 2000; Lu *et al.*, 1995). Moreover, the E3L and K3L proteins of vaccinia virus are also known

to inhibit activation of the IFN-induced protein PKR, although by different mechanisms. E3L is a dsRNA-binding protein, and by sequestering dsRNA, E3L eliminates the cofactor of both PKR and OAS, preventing their activation (Chang *et al.*, 1992). Additionally, E3L has been shown to interact directly with and inhibit PKR (Romano *et al.*, 1998; Sharp *et al.*, 1998). Moreover, E3L is also involved in inhibiting IRF-dependent pathways involved in the triggering of IFN-α/β synthesis. In contrast to E3L, K3L is an IFN-α/β antagonist, the function of which appears to be focused on inhibiting PKR. K3L is a PKR substrate analogue, and competes with eIF-2α for PKR binding, thus preventing PKR-mediated phosphorylation of eIF-2α and PKR-induced inhibition of protein translation (Davies *et al.*, 1992). Likewise, other viruses such as HCV, HSV-1, and HHV-8 express proteins that either bind directly to or inactivate PKR. Similarly to the K3L protein, the E2 protein of HCV (Taylor *et al.*, 1999) and the Tat protein of human immunodeficiency virus-1 (HIV-1) (Brand *et al.*, 1997; Roy *et al.*, 1990) act as pseudotemplates for PKR, and as a consequence the kinase activity of PKR is inhibited and its inhibitory effect on protein synthesis and cell growth is blocked. In the case of HSV-1, the $\gamma_1$34.5 protein triggers dephosphorylation of eIF-2α, and thus enables continued protein synthesis despite the presence of activated PKR (He *et al.*, 1997). Interestingly, HSV-1 also encodes U_S11 (a γ_2 protein) which can block PKR activation by PACT (Peters *et al.*, 2002).

Some viruses produce abundant short RNA molecules that compete with dsRNA for binding to PKR (but are too short to cause activation), thereby inhibiting PKR activation and activity. Examples of viruses that use such a strategy include adenoviruses, HCV, Epstein-Barr virus (EBV) and HIV-1. The adenovirus VA_I RNA transcript is an mRNA molecule that can form a highly ordered secondary structure that binds strongly to the dsRNA-binding site on PKR, acting as a competitive inhibitor (reviewed in Mathews, 1995; Mathews & Shenk, 1991). EBV encodes two small RNA molecules (EBER-1 and EBER-2), similar to the VA_I RNAs of adenovirus, which bind to PKR and interfere with its activity, thereby protecting protein synthesis from inhibition by dsRNA (Sharp *et al.*, 1993). In the case of HCV, the NS5A protein has been shown to bind PKR and

inhibit its antiviral function (Pawlotsky & Germanidis, 1999). In addition, a region of the HCV viral RNA comprising part of the internal ribosome entry site (IRES) is able to bind to PKR, in competition with double-stranded RNA, and can prevent its autophosphorylation and activation *in vitro* (Vyas *et al.*, 2003). In the case of HIV-1, it is the short Tat-responsive region (HIV-TAR) RNA that inhibits PKR activity (Gunnery *et al.*, 1990). Influenza virus also employs a novel mechanism to repress PKR activity, more precisely by activating the cellular inhibitor $P58^{IPK}$, which binds to and possibly prevents PKR from dimerising, although the factor responsible is not known (Lee *et al.*, 1990).

Since dsRNA is also required for the activation of 2-5 OAS, viruses that express dsRNA-binding proteins, such as the E3L protein of vaccinia virus that sequesters dsRNA, (Rivas *et al.*, 1998), inhibit both PKR and the 2-5 OAS/RNaseL system. Several viruses have evolved strategies that specifically counteract the antiviral activity of the 2-5 OAS/RNaseL pathway. Viruses such as HIV-1 (Martinand *et al.*, 1999) and encephalomyocarditis virus (Martinand *et al.*, 1998) induce the expression of a cellular RNaseL inhibitor (RLI), which antagonizes binding of 2-5 oligoadenylates to RNaseL and hence prevents its activation. Infection with HSV-1 and HSV-2 has been shown to activate the synthesis of 2-5 oligoadenylate derivatives that behave as 2-5 oligoadenylates, thereby binding to and inhibiting activation of RNaseL (Cayley *et al.*, 1984). In cells infected with RSV the IFN antiviral effect is inhibited, and the NS1 and NS2 proteins have been shown to cooperate to counteract antiviral effects, although the mechanism by which these two viral proteins overcome the IFN response is still unknown (Schlender *et al.*, 2000). Other viruses interfere with ND10, by inducing its disruption by proteasome-dependent degradation of PML and Sp100 (reviewed in Weber *et al.*, 2004).

1.4 SV5 persistence

1.4.1 Host species and isolates of SV5

SV5 was originally isolated from rhesus monkey kidney cell cultures, and two of the original monkey isolates are referred as to WR and W3A (or commonly W3) (Choppin, 1964; Hull *et al.*, 1956). Thus, it was initially believed that monkeys were the natural host for SV5. However, subsequently epidemiological studies revealed that wild monkeys do not have antibodies against the virus, which together with experimental evidence showing that SV5 can also infect humans (Goswami *et al.*, 1984b; Hsiung, 1972) suggested that infection of monkeys occurs either in transit or shortly after contact with humans (Tribe, 1966). Furthermore, investigations on several SV5 strains isolated from bone marrow cells of human patients with multiple sclerosis (human isolates LN, RQ, DEN, MEL and MIL) suggested that there might be a link between SV5 and this disease (Goswami *et al.*, 1984a). Subsequent serological studies revealed that antibodies against SV5 could be identified in the cerebrospinal fluid of multiple sclerosis patients, providing further support for that hypothesis (Goswami *et al.*, 1987; Russell *et al.*, 1989). However, this is a subject of debate and controversy and findings from other studies dissociated SV5 from multiple sclerosis (McLean & Thompson, 1989; Vandvik & Norrby, 1989). Furthermore, no acute human disease has been linked reproducibly to infection with SV5.

SV5 (or canine parainfluenza virus, as it is also referred to in veterinary medicine) is known to cause tracheobronchitis in dogs and is often associated with kennel cough, and can have neurotropic character in dogs (Cornwell *et al.*, 1976; McCandlish *et al.*, 1978). It has been suggested that the virus may be able to establish persistent infections in canine hosts. A strain of SV5, termed CPI+, was isolated from the cerebrospinal fluid of a dog with temporary posterior paralysis (Evermann *et al.*, 1981; Evermann *et al.*, 1980), further studies of CPI+ as a neurotropic isolate showed it to cause acute encephalitis as a result of intracerebral infection of gnotobiotic dogs (Baumgartner *et al.*, 1981). From one such experimentally infected dog another SV5 variant, termed CPI-, was re-

isolated 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). Furthermore, 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). Further characterisation of CPI- and CPI+ showed that of a panel of 50 monoclonal antibodies, only one (the anti-Pk antibody) could distinguish between these two closely related isolates, and subsequently sequence analysis of the CPI- V/P gene revealed that the V (and P) protein of CPI- has three amino acid differences to that of CPI+, one of which maps to the Pk epitope (Southern *et al.*, 1991). The Pk epitope, which maps to the V/P common amino-terminal domain of V and P proteins, comprises eight amino acids and containing the asparagine (Asn) amino acid residue (N_{100}) is important for recognition by the anti-Pk mAb (see Figure 1.3; Dunn *et al.*, 1999). This epitope has been of great value in distinguishing between closely related isolates (Southern *et al.*, 1991). Interestingly, work by Chatziandreou and colleagues (2002) revealed that whilst CPI+ targets STAT1 for degradation, thereby blocking IFN signalling, CPI- fails to target STAT1 for degradation, and this is due to three amino acid differences in the V/P amino-terminal common domain of the V proteins.

Additional canine variants of SV5 include 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. In addition, an isolate of SV5, termed SER, has been recently isolated from the lung of a fetus of a breeding sow with porcine respiratory and reproductive syndrome (Heinen *et al.*, 1998; Tong *et al.*, 2002). Similar to T1, the F protein of SER was found not to induce cell fusion *in vitro*. There is also evidence that cats, hamsters and guinea pigs may naturally be infected by SV5 or a very closely related virus (Hsiung, 1972). Alignment of F and V/P genes

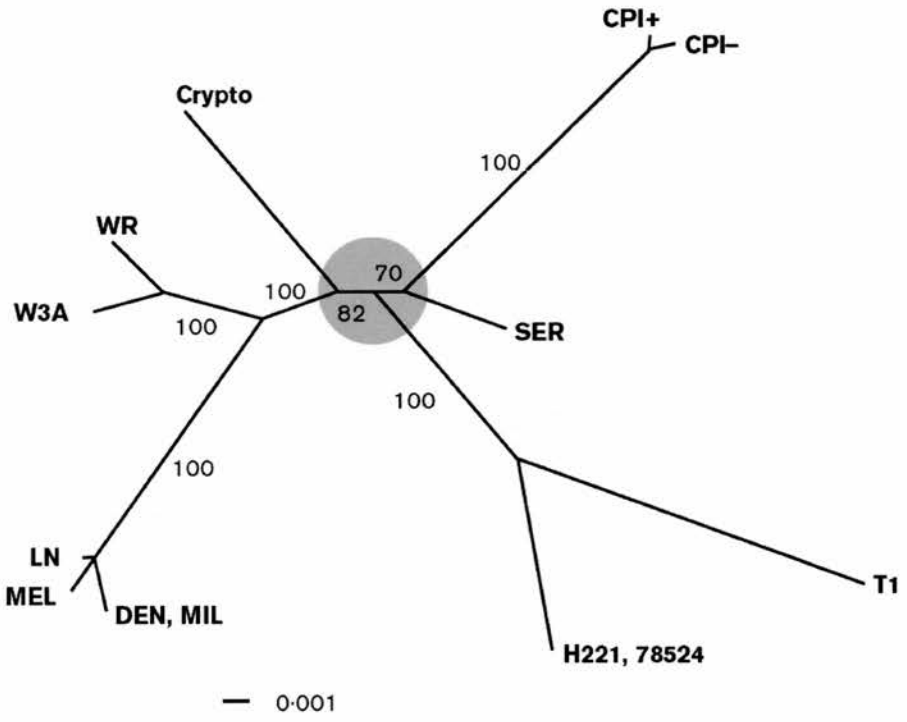
sequences of human, canine, porcine and simian isolates of SV5 has revealed a surprising lack of sequence variation at both the nucleotide and amino acid levels. Moreover, among the isolates that correlated completely with the species from which they were isolated, there were no clear distinguishing amino acid or nucleotide differences (Chatziandreou *et al.*, 2004). In that study, a gene tree was obtained on basis of the alignment of F gene sequences (Figure 1.13; Chatziandreou *et al.*, 2004)

1.4.2 SV5 persistent infections

Previous studies, using SV5 as a model for paramyxovirus persistence, have highlighted important features of SV5 regarding the mode by which this virus establishes persistent infections. The belief that SV5 can readily establish persistent infections was initially demonstrated by experimental evidence of SV5 recovery from simian kidney cell cultures long after infection (Atoynatan & Hsiung, 1969; Hsiung, 1972; Tribe, 1966). Since then, the ability of certain 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 within the host without causing any symptoms of harmful pathology. This property may influence the epidemiology of some viruses, and persistent paramyxovirus infections have been linked to paramyxovirus-induced chronic diseases, such as subacute sclerosing panencephalitis and Paget's bone disease (reviewed in Randall & Russell, 1991).

1.4.2.1 SV5 persistence and replication in murine cells

Experimental evidence revealed that although SV5 naturally infects simian, human and canine hosts it only causes a self-limiting infection in mice (Randall *et al.*, 1988; Young *et al.*, 1990). It is now known that SV5 fails to target STAT1 for degradation and thus to block IFN signalling in mouse cells (Didcock *et al.*, 1999a), and is non-pathogenic in normal and severe combined immunodeficient (SCID) mice (Didcock *et al.*, 1999a; Young *et al.*, 1990). However, SV5 is lethal in STAT1-knockout mice, i.e. mice that cannot respond to IFN (He *et al.*, 2001;



from Chatziandreou et al., 2004

Figure 1.13 Gene tree based on F gene sequences of SV5 isolates.

The MrBayes 3 program was used to analyze the alignment of F gene sequences of various isolates of SV5 from which resulted the consensus tree shown. Credibility values (%) for partitions of the isolates into two groups are indicated at appropriate branches. The two low values in the central part of the tree indicate that branching order in the region marked with a shaded circle could not be inferred with confidence. Bar, 0·001 substitutions per site (Chatziandreou *et al.*, 2004).

He *et al.*, 2002). Although it is difficult for the virus to establish infections in murine cells *in vitro*, Young and colleagues (1997) showed that SV5 can establish a persistent infection in murine cells. In this study, the isolation of a new SV5 strain from persistently infected murine cells, following prolonged passaging (over 40 passages) was reported. 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 W3-f remained as sensitive to IFN as the parental W3 isolate in murine cells. Remarkably, although W3-f spread much more rapidly than the parental W3 strain through BF cell monolayers, sequence analysis revealed no deduced amino acid differences between the F proteins of W3 and W3-f.

Murine cells passaged after high multiplicity of infection with SV5 showed no obvious cytopathic effect or signs of infection, immunofluorescence analysis of these cells suggested the virus was transcriptionally inactive although these aggregates of viral proteins, referred to as inclusion bodies, were readily detectable 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 fully reactivate under permissive conditions (Fearns *et al.*, 1994). In the study of Young *et al* (1997), murine cells were infected with SV5, and although the majority of cells cleared virus infection (8 to 15 days p.i.), some cells remained infected and became persistently infected. Virus fluxing between active and repressed states was observed upon passaging of the persistently infected cells presumably as a result of IFN production and action, thus providing a link between inhibition of SV5 replication and IFN production in murine cells. Noteworthy, in the persistently infected cultures, were areas in which the cells were positive for both NP and HN proteins, presumably revealing areas of ongoing virus protein synthesis. Adjacent to these, there were groups of cells which were positive for NP protein but negative for HN protein, and thus the virus appeared to be repressed in those cells. Such observations suggested that the production of IFN by a local group of cells may be sufficient to inhibit virus

replication in a restricted area but not necessarily throughout the whole culture, and that the virus presumably fluxes between active and repressed states in response to local production of IFN (Young *et al.*, 1997).

1.4.2.2 SV5 persistence in canine cells

More recently, the phenomenon of persistence has been demonstrated in canine cells infected with an IFN-sensitive SV5 strain (CPI-). Thus, following IFN treatment of canine cells infected with CPI-, whilst most the cells cleared the virus infection, about 60% of the cells in the population remained infected and in these persistently infected cells almost all of the viral P and NP proteins were found in cytoplasmic inclusion bodies. These inclusion bodies were also observed in canine cells infected with CPI- after long periods of infection, while virus glycoproteins were lost from the surface of infected cells, suggesting that in cells persistently infected with CPI- the virus may become repressed in response to IFN (Chatziandreou *et al.*, 2002). From this it was suggested that inclusion bodies may be the basis of SV5 persistence, as cells with inclusion bodies had very low levels of both surface glycoproteins and general reduction in virus protein expression which could enable such cells to escape destruction by the immune surveillance mechanisms of the adaptive immune system. Furthermore, IFN-sensitive paramyxoviruses such as CPI- (and W3 in murine cells) have been suggested to establish persistent infections by residing in inclusion bodies and sporadically reactivating to an IFN-resistant form by the way of point mutations in the V gene (Chatziandreou *et al.*, 2002; Young *et al.*, 2001). Indeed, 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. A model of virus persistence was proposed in which there is selection of IFN-resistant and IFN-sensitive viruses according to the state of the adaptive immune response. Thus, in the presence of IFN, although CPI+ (the IFN-resistant isolate) would be able to sustain protein synthesis it would always remain a target for acquired immune responses (mediated by CTL cytotoxicity or antibody-dependent inactivation). In contrast, in canine cells infected with CPI-, in response to IFN, nucleocapsids would aggregate in cytoplasmic inclusion bodies and the viral

genome would become unavailable for viral processes (Chatziandreou *et al.*, 2002; Fearns *et al.*, 1994). As a result of the repressed state of CPI-, the generation of CTL epitopes by CPI- in the cell would be reduced, and the expression and display of virus glycoproteins on the cell surface would be lost. Consequently, the effect of IFN on CPI- would essentially hide the virus from the adaptive immune system, thereby saving it from clearance. Moreover, given the ability of SV5 to flux between repressed and active states in response to local IFN production (described above; Young *et al.*, 1997), it was suggested by Chatziandreou and colleagues (2002) that the virus may be able to resume active replication once the acute immune response and inflammatory restraints were withdrawn from the area of the infected cells. Furthermore, point mutations in the V protein during phase of active replication may give rise to revertant IFN-resistant variants that would be able to maintain productive infections in the absence of acute immune responses. As a consequence, the virus would be able to further spread and probably be released in the environment to infect other individuals (Chatziandreou, 2002). It therefore appears that the ability of paramyxoviruses to establish persistent infections *in vivo* may be linked to their ability, or not, to block the IFN response.

1.5 Aims

Although SV5 blocks IFN signalling and reduces the production of IFN, its ability to counteract the IFN response is not absolute. Thus, SV5 forms larger plaques on cells that have been engineered to be non-responsive to IFN (Young *et al.*, 2003). Furthermore, it has been published that pre-treatment of cells with IFN inhibits virus replication. However, the IFN-induced mechanism(s) that interfere with SV5 (and other paramyxoviruses) replication have not been well characterized. It is also known that SV5, like other paramyxoviruses, can establish persistent infections *in vivo*, but little is known about the molecular mechanisms by which they achieve this. The primary aims of this thesis were two fold. Firstly, to examine in detail the molecular basis by which IFN inhibits the replication of SV5, and secondly to begin to investigate how the IFN response may modulate the establishment and maintenance of persistent SV5 paramyxovirus infections.

2. MATERIALS AND METHODS

2.1 Cells and Cell Culture

2.1.1 Cell lines

The main cell line used in the work presented in this thesis was monkey Vero cells; fibroblast-like cell line originating from kidney cells of the African Green monkey (cell line obtained from ICN Pharmaceuticals Ltd., United Kingdom).

Cells of human origin were also used:

- Human MRC-5 cells; human foetal lung fibroblasts.

- Hep-2 cells; human larynx carcinoma, epithelial.

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

- MRC-5/SV5-V; MRC-5 cells that express the V protein of SV5 constitutively (produced by D. Young; Young *et al.*, 2003).

- Hep-2/E3L, Hep-2/NS1 and Hep-2/V; Hep-2 cells that constitutively express the E3L protein of vaccinia virus, the NS1 protein of Influenza virus and the V protein of SV5, respectively (produced by Y-H Chen; unpublished).

- Vero/MxA and Vero/control; Vero cells that express the MxA protein constitutively and control Vero-SV2neo cells, respectively (produced by U. Sester; Frese *et al.*, 1995).

2.1.2 Cell culture

Cell cultures were maintained in 25 cm^2 or 75 cm^2 tissue culture flasks (Greiner, United Kingdom) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Ltd., United Kingdom) supplemented with 10% newborn calf serum (NCS) (Invitrogen Ltd., United Kingdom) at 37°C under 5% CO_2. Vero/MxA and Vero/control cells were maintained in DMEM/10%NCS supplemented with 1.5mg of the antibiotic G418 (Melford, United Kingdom). Cells were routinely passaged, trypsinised (trypsin, EDTA; Becton Dickinson Ltd., United Kingdom) and diluted every three to five days, depending on the growth rate of the cell line.

2.2 Viruses and virus infection of cells

2.2.1 Virus isolates and virus infections

The viruses used to infect cells in this study were mainly two closely related canine isolates of SV5, namely CPI+ (formerly called 78-238) and CPI- (Baumgartner *et al.*, 1987a; Baumgartner *et al.*, 1991; Baumgartner *et al.*, 1982; Baumgartner *et al.*, 1981). As described in Chapter 1, CPI+ was isolated from the cerebrospinal fluid of a dog with temporary posterior paralysis, and CPI- was isolated from the brain of a gnotobiotic dog that had been infected experimentally with CPI+. However, other paramyxoviruses were also used in experiments presented in this thesis:

- Respiratory syncytial virus, RSV (strain A2; subfamily *Pneumovirinae*, genus *Pneumovirus*).

- Mumps virus, MuV (Jeryl Lynn vaccine strain of MuV; subfamily *Paramyxovirinae*, genus *Rubulavirus*).

- Human parainfluenza virus type 2, hPIV2 (Colindale "prototype" strain; subfamily *Paramyxovirinae*, genus *Rubulavirus*).

- Human parainfluenza virus type 3, hPIV3 (Colindale "prototype" strain; subfamily *Paramyxovirinae*, genus *Respirovirus*).

Cells were infected as monolayers at a multiplicity of infection (m.o.i.) of 50 to 100 plaque-forming-units (pfu) per cell (pfu/cell), unless a lower m.o.i. was required (<0.1 pfu/cell), in DMEM containing 2% NCS (maintenance medium). After an adsorption period of 1 to 2 hours (h) on a rocking platform at 37°C, the virus inoculum (or plain growth medium, where mock infections were required as control treatments) was removed and replaced with fresh maintenance medium. To study the effect of IFN on infections, media were supplemented with recombinant human interferon-αA/D (rHuIFN-αA/D) (Rehberg *et al.*, 1982; PBL Biomedical Labs, New Brunswick, catalogue number 11200) or recombinant human interferon-γ (rhIFN-γ) (Gray *et al.*, 1982; R & D Systems, Minneapolis, catalogue number 285-IF), or left untreated as a control. The IFN was added to maintenance medium at 1000 units/ml. Cells were either harvested immediately (0 h) or further incubated for different times (as indicated in each experiment presented in Chapter 3).

2.2.2 Preparation of virus stocks

When required, virus stocks were prepared to obtain sufficient amounts of virus. Monolayers of Vero cells (25cm^2 flasks) were initially infected with the virus of interest using the appropriate master stock (prepared by D. Young) and then incubated at 37°C until plaques begun to form in the monolayer. The supernatant was subsequently collected and centrifuged at 4000 rpm for 5 minutes (min) to precipitate cell debris. A portion of the purified supernatant was then used to infect cells at a larger scale (cells grown as monolayers in roller bottles; Scientific Laboratory Supplies Ltd., United Kingdom). Cells were incubated with the inoculum on a rolling platform for 1 to 2 h at 37°C to allow virus adsorption onto the cells and the medium was then replaced with fresh maintenance medium and cells reincubated on rolling platforms at 37°C. At 10 to 12 h post-infection (p.i.) the medium was removed and replaced with fresh maintenance medium. When fusion was evident in cell monolayers (usually after two days), the supernatant

was harvested, centrifuged for 5 min to remove cell debris, and virus stock was aliquoted and stored at -70°C. Virus titre was subsequently determined by plaque assay method, as described below.

2.2.3 Titration of virus preparations

Monolayers of Vero cells were grown in 6-well plates (Greiner, United Kingdom) until they reached 70 to 80% confluency. The virus preparation was diluted in a series of ten-fold dilutions in DMEM supplemented with 2% NCS. Duplicated wells of cells were set up for each dilution, and cells were inoculated with each virus stock dilution (1ml/well). The cells with the virus inoculum were incubated for 2 h (37°C, 5% CO_2) on a rocking platform to allow adsorption of virus onto the cells. The virus inoculum was then removed and 10 ml of overlay, which consists of 0.5% of carboxy methyl cellulose (methocel MC; Sigma-Aldrich Co Ltd., United Kingdom) and 2% NCS in DMEM, were added to each well. The cells were incubated (at 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 in 5% formaldehyde and 2% sucrose in phosphate-buffered saline (PBS) for 30 min, after which the fixative solution was washed away with water. Areas of cell lysis and therefore sites of virus infection or plaques were visualised by Coomassie blue staining, by incubating the fixed cells with a solution of 0.1% Coomassie brilliant blue R250, 20% methanol and 20% acetic acid in distilled water for 10 to 20 min, on a rocking platform at room temperature. After washing briefly the monolayers with water, plaques were observed as "holes" or unstained areas of the monolayer. Plaques were counted and the titre was estimated (plaque-forming-units per ml of virus preparation; pfu/ml), taking into account the dilutions made.

2.2.4 Virus yield assay

Confluent-cell monolayers were infected at a m.o.i. of 10 pfu/cell in DMEM supplemented with 2% serum for 1 h on a rocking platform at 37°C. After the adsorption period, the virus inoculum was removed by two washings with PBS,

and the cultures were incubated in maintenance medium at 37°C under 5% CO_2. Samples of the culture supernatants were removed at 12 h, 24 h, 36 h, 48 h, 3 days and 6 days p.i. The virus titres were determined on Vero cells by virus plaque assay as described above.

2.3 Protein analysis

2.3.1 Antibodies

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

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 domain)	
SV5-P-k (mAb)	SV5 V and P (common N domain)	
SV5-polyclonal	SV5 V and P (common N domain and V unique C domain)	Andrejeva *et al.*, 2002a
V5 tag antibody	SV5 V and P V5 epitope tag	commercial source (1)
14E8 (mAb)	SV5 V (unique C domain)	Lamb, R.A. (2)
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 & Young, 1988
hPIV2-NP-a (mAb)	hPIV2 NP	
hPIV2-NP-b (mAb)	hPIV2 NP	
hPIV3-NP (mAb)	hPIV3 NP	Rydbeck, R. (3)
RSV-P (mAb)	RSV P	Taylor, G. (4)
Mumps-NP (mAb)	Mumps NP	Rima, B.K. (5)

Provided by: (1) Abcam (Cambridge, United Kingdom) (catalogue number ab15829), (2) R.A. Lamb (Northwestern University, Evanston, USA), (3) R. Rydbeck (Psykiatrigeriatriska kliniken, University of Lund, Sweeden), (4) G. Taylor (Institute for Animal Health, Compton, Berks), and (5) B. K. Rima (Queen's University, Belfast).

Other antibodies, specific for cellular proteins, were used in this study (Table 2.2).

Table 2.2- Antibodies and target proteins (the antibodies were provided by G. Kochs, Freiburg University, Freiburg, Germany).

Antibody	Target Protein
MxA (mAb)	MxA protein
MxA (pAb)	MxA protein
Anti-Actin (mAb)	Actin protein
Anti-Vimentin (mAb)	Vimentin protein
Anti-β-Tubulin (mAb)	Tubulin protein
Anti-p53 (mAb)	p53 protein
Anti-63 (mAb)	p63 protein
Anti-Giantin (mAb)	Giantin protein
Anti-Gal-T (pAb)	Galactosyltransferase protein
Anti-Syntaxin17 (pAb)	Syntaxin17 protein
Anti-Lamp1 (mAb)	Lysosome associated membrane protein 1
Anti-PMP-70 (pAb)	70-kDa peroxisomal membrane protein
Anti-Caveolin (pAb)	Caveolin protein
Anti-Sec13 (pAb)	Sec13 protein
Anti-M6PR (pAb)	Mannose 6-Phosphate-Receptor

2.3.2 Immunofluorescence

For immunofluorescence analysis, cells were grown on 13 mm-diameter coverslips (General Scientific Co. Ltd., Redhill, United Kingdom) in individual wells of a 6 well or 24 well plates. Cells were infected with CPI-, CPI+, MuV, hPIV2, hPIV3, or RSV, and the inoculum was adsorbed for 1 h. Treatment with exogenous IFN was as described above and as mentioned in each experiment presented in Chapter 3. A detailed account of the staining procedure has been previously described (Randall & Dinwoodie, 1986). At various times p.i., monolayers were first incubated in fixing solution (5% formaldehyde and 2% sucrose in PBS) for 15 minutes at room temperature, then permeabilized (5% Nonidet-P40 and 10% sucrose in PBS) for 5 min, and washed three times in PBS containing 1% foetal calf serum (FCS) and 0.1% azide. To detect the proteins of

interest, cell monolayers were incubated with 10 to 15 µl of appropriately diluted antibody for 1 h (primary antibody). Cells were subsequently washed in PBS containing 1% FCS and 0.1% azide several times, and the antibody-antigen interactions were detected by indirect immunofluorescence (1 h incubation) with a secondary goat anti-mouse or anti-rabbit immunoglobulin FITC-conjugated antibody or Texas Red-conjugated antibodies (Seralab, Oxford United Kingdom), depending on the primary antibody used. In a set of experiments antigen-bound primary antibodies were detected with preabsorbed fluorophore (Cy2, Cy3, Cy5)-conjugated donkey antibodies (DIANOVA, Hamburg, Germany). In addition, cells were stained with the DNA-binding fluorochrome 4', 6'- diamidino-2-phenylindole (DAPI, 0.5 µg/ml; Sigma-Aldrich Co Ltd., United Kingdom) for nuclear staining. All reactions were performed at room temperature and antibody treatments were allowed to progress in a humidified chamber in order to prevent desiccation of the monolayers. Following staining, monolayers were washed with PBS, mounted with coverslips using Citifluor AF-1 mounting solution (Citifluor Ltd., United Kingdom) and examined under a Nikon Microphot-FXA immunofluorescence microscope. More recently, immunofluorescence samples were also viewed with an Olympus IX70 inverted microscope using a 60x oil immersion lens. Images were captured with a 12-bit CCD camera using Delta Vision Software (Applied Precision). Some experiments presented in this thesis were performed in the University of Freiburg (Germany), where cells were analysed with a Leica (Deerfield, IL) TCS SP2 confocal laser scanning microscope.

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

Cells were grown, infected and treated, or not treated, with IFN as described above. At various times after the addition, or no addition, of IFN, cells were metabolically labelled for 1 h with L-[^{35}S] methionine (500 Ci/mmol; Amersham International Ltd., United Kingdom) in methionine-free tissue culture medium 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 immunoprecipitation, as described below.

2.3.4 Immune precipitation

At the end of the labelling interval, cells were washed twice in ice-cold PBS and lysed in immunoprecipitation buffer (10 mM TRIS-HCl, pH 7.8, 5 mM EDTA, 0.3% Nonidet P-40, and 0.65 M NaCl; 4×10^6 to 6×10^6 cells per ml buffer) by sonication with an ultrasonic probe. At this stage, 50 µl of total cell antigen extract sample was kept as a control to examine total cellular protein synthesis. Soluble antigen extract was prepared by subjecting the remainder of the cell lysate to centrifugation at 12,000 × g for 1 h to remove particulate material. Immune complexes were formed by incubating 1 ml samples of the soluble antigen extracts with a pool of monoclonal antibodies to the NP, P, M, and HN proteins of SV5 and a polyclonal antiserum to the V/P proteins of SV5 (1 µl of concentrated tissue culture fluid of relevant mAbs; see Table 2.1) for 2 h at 4°C. The immune complexes were isolated by incubation with protein G-Sepharose 4B Fast Flow (Sigma-Aldrich Co Ltd., United Kingdom) for 1 h at 4°C. The proteins in the immune complexes were dissociated by heating in gel electrophoresis sample buffer at 100°C for 5 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described below. After electrophoresis, gels were fixed, stained, and dried. The resolved labelled polypeptide bands were visualized by autoradiography and quantitated by phosphorimager analysis (Image Gauge Version 3.45, FUJI).

2.3.5 SDS Polyacrylamide Gel Electrophoresis

Protein samples were prepared in gel electrophoresis sample buffer (0.05 M TRIS-HCl pH 7.0, 0.2% SDS, 5% 2-mercaptoethanol, and 5% glycerol) and heated at 100°C for 5 min prior to electrophoresis analysis. Polypeptides were separated through SDS-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. Polypeptides were also separated through 4 to 12% Novex NuPAGE (Invitrogen, United Kingdom) polyacrylamide gradient gels (160 V), using 3-(N-Morpholino)-propanesulfonic acid (MOPS) buffer (Invitrogen, United Kingdom).

2.3.6 Immunoblotting

Cells were washed twice in PBS prior to harvesting, and subsequently disrupted in SDS gel electrophoresis loading buffer. Cell lysates were then sonicated and heated at 100°C for 5 min. Polypeptides in samples were separated by SDS-PAGE as described above, and transferred to polyvinylidene difluoride (PVDF) membrane (BDH, Merck) using a Trans-Blot Cell (Bio-Rad, United Kingdom), assembled according to the manufacturer's instructions, in standard transfer buffer (39 mM glycine, 48 mM TRIS base and 20% (v/v) methanol) for 2 hours at constant current of 200 mAmp (or 30 mAmp overnight). Following electroblotting, any unoccupied protein binding sites on the membrane were blocked by incubation of the membrane in 10% (w/v) skimmed milk powder (Marvel) and 0.1% polyoxyethylene sorbitan monolaurate (Tween 20) in PBS (blocking buffer) for 1 hour. Proteins were detected by incubating the membrane with the appropriate monoclonal antibodies (see Table 2.1 and Table 2.2) in antibody suspension (1/100 to 1/1000 dilution in blocking buffer) for 1 h or longer, depending on the antibody. The membrane was then washed carefully (three washes, each involving 15 to 30 min incubation on a rocking platform) to remove unbounded primary antibody. The protein-antibody interactions were detected by incubation of the membrane with a horse-radish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich Co Ltd., United Kingdom) suspension (1/1000 dilution in blocking buffer) for a maximum of 1 h on a rocking platform. The membrane was then washed as described above, and the protein bands were visualised by enhanced chemiluminescence (ECL) according to the manufacturer's recommendations (Amersham Biosciences Ltd., United Kingdom).

2.4 RNA analysis

2.4.1 RNA extraction from cells

Cells were washed twice in ice-cold PBS and lysed in Trizol reagent (Invitrogen), and total intracellular RNA was extracted from cell pellets according to the Trizol reagent supplier's protocol, except that the RNAs were extracted with phenol-chloroform and ethanol precipitated after the isopropanol precipitation.

2.4.2 Northern blot hybridization

The RNA samples were prepared by mixing the purified RNA with MOPS buffer to a final concentration of 0.5× (10×MOPS is 0.2 M MOPS, 20 mM sodium acetate and 10 mM EDTA pH 8.0), formaldehyde (final concentration of 6.5%), deionised formamide (final concentration of 0.5%) and RNase free water to a final volume of 40 µl. Prior to electrophoresis analysis, the samples were heated at 65°C for 12 min and mixed with 5 µl gel electrophoresis loading buffer (50% glycerol, 1 mM EDTA- bromophenol blue tracking dye). Approximately 4 µg of each RNA sample were analyzed by electrophoresis in small 1.5% agarose gels containing 0.44 M formaldehyde, and electrophoresed at 90 V for 2 h. The isolated RNA was then transferred to nitrocellulose membranes with a Turbo-Blot apparatus (Schleicher & Schuell), assembled according to the manufacturer's instructions, with a transfer buffer of 3 M NaCl and 8 mM NaOH. Following transfer overnight, RNAs were fixed by UV crosslinking (Stratagene). The nitrocellulose filters were prehybridized for 1 h at 65°C in 6×sodium chloride-sodium citrate (SSC) buffer (1×SSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% SDS, 5×Denhardt's solution, and 0.5 mg of sheared DNA per ml. Hybridization was performed overnight under the same conditions with the addition of the [^{32}P]dCTP-labeled negative-sense DNA probes specific for HN, NP, P, M, or actin mRNAs, which were prepared by run-off polymerization, as described below. Typically, a quarter of the probe preparation was used in each hybridization reaction; however, to ensure that the probe was saturating the blot, some blots were rehybridized with a two fold-higher concentration of probe. The

RNA pattern was similar regardless of the probe concentration in the hybridization mix, indicating that the probe was in excess in these experiments. The blots were washed once in 2×SSC-0.1% SDS at room temperature and three times in 2×SSC-0.1% SDS at 65°C for 30 min. The labelled RNA bands were visualized by autoradiography and quantitated by phosphorimager analysis.

2.4.3 Preparation of single-stranded DNA hybridization probes

2.4.3.1 Preparation of RNA from virus-infected cells for reverse transcription

Vero cells were used for viral RNA isolation for reverse transcription. Cells were grown as monolayers and infected with CPI- at a high m.o.i. (10 pfu/cell) to ensure high efficiency of infection. The monolayers were washed in PBS twice and lysed in Trizol reagent (Invitrogen) at 20 h p.i, and total intracellular RNA was extracted as described above. The RNA isolated was ressuspended in 50 μl RNase free water after isopropanol precipitation.

2.4.3.2 cDNA synthesis by reverse transcription

Total intracellular RNA from CPI- infected cells was used as a template in a reverse transcription (RT) reaction for synthesis of cDNAs representing specific segments of the HN, NP, P, and M viral genes. For the actin probe plasmid containing human cytoplasmic β-actin gene was used as a template for amplification of an actin DNA fragment. Virus RNA (an estimated amount of 2 μg) was mixed with the appropriate reverse orientation primer (0.5 μg), and this mix was heated at 70°C for 5 minutes 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 10,000 × g to collect condensation. Following primer annealing to RNA, RNasin (25 units; Promega Ltd., United Kingdom), dNTPs (0.2 mM of A, T, G and C; Amersham

International Ltd., United Kingdom), Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (200 units) and supplied M-MLV buffer (Promega Ltd., United Kingdom) were added to the mix, to a final volume of 25 µl in RNase free water. The reaction was incubated for 75 minutes at 42°C and the cDNA product was subsequently used in polymerase chain reactions, as described below.

2.4.3.3 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify the cDNA fragments obtained from reverse transcription. Each DNA fragment was approximately 400 to 500 bp in length. The 100-µl PCR reaction mixture (made up in sterile water) consisted of PCR enzyme buffer (at an appropriate dilution), 0.2 mM of each dNTP, 50 to 200 ng of DNA template (the whole 25-µl RT reaction mixture was used), 0.1 to 0.5 µM of each primer (reverse and forward primers, specific to each viral cDNA fragment and to the actin cDNA fragment; the details of primer sequences are presented in Appendix 1), and 3 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Promega Ltd., United Kingdom). PCR condition parameters were normally as follows: melting at 95°C for 45 seconds (sec), annealing at 55°C (or other, depending on the temperature of melting of the primers in use) 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.

2.4.3.4 Agarose gel electrophoresis

Amplified DNA was analysed by gel electrophoresis in horizontal mini-gels of 1% (w/v) agarose (Sigma-Aldrich Co Ltd., United Kingdom) in TBE buffer (0.045 M TRIS-borate, 0.001 M EDTA). DNA samples were mixed with the appropriate volume of DNA loading buffer (Promega Ltd., United Kingdom), prior to electrophoresis. Samples were electrophoresed at 45 to 80 V in TBE buffer containing 1 µg/ml ethidium bromide, until bands were clearly resolved. Along with the samples, known DNA molecular weight markers were run (1kb ladder; Promega Ltd., United Kingdom.). Electrophoresed DNA was exposed to

UV light and photographed (Gel Doc 2000 UV transilluminator/photography system; Bio-Rad, United Kingdom).

2.4.3.5 Purification of DNA fragments from agarose gels

Following analysis of DNA by agarose gel electrophoresis, as described above, the DNA fragments of interest were subsequently purified by excision of the resolved bands from the gel and recovery on Qiaquick gel extraction columns (Thermo Hybaid Limited, United Kingdom) according to the manufacturer's instructions. Typically, DNA was eluted in 50 µl and a fraction of the purified samples (usually 1/10 of the total volume of the eluate) was usually re-run on agarose gels, in order to check the purification of DNA.

2.4.3.6 Run-off polymerisation for synthesis of single-stranded DNA hybridization probes

The run-off polymerisation, consisting of repeated cycles of denaturation, annealing, and extension, was used to produce radioactively labelled single-stranded negative-sense DNA probes. The purified DNA fragments were used as templates in the run-off polymerization reaction together with the appropriate reverse primer to synthesise negative-sense DNA probes specific for each viral HN, NP, P, and M mRNAs, as well as for cellular actin mRNA. Each run-off reaction mixture contained 100 to 200 ng of DNA template; 2 µM primer; 200 µM (each) unlabeled dATP, dTTP, and dGTP; 6.25 µM unlabeled dCTP; 25 µCi of [α-^{32}P]dCTP (3,000 Ci/mmol); and 2.5 U of Thermus aquaticus (*Taq*) DNA polymerase (Promega Ltd., United Kingdom) in 1×thermoscript buffer (NEB). The "run-off" reaction parameters were as follow: melting at 94°C for 30 sec, annealing at 55°C for 30 sec, and strand extension at 72°C for 30 sec (1min/1kb). The PCR set (40 cycles) was preceded by an initial step of melting at 94°C for 5 min, followed by a final step of strand extension at 72°C for 5 min. Each probe was extracted with phenol-chloroform and heated to 95°C prior to hybridization.

2.4.4 Analysis of viral mRNA poly (A) tail length

The method used to analyze the length of the poly (A) tail of viral mRNA was adapted from that described by Rassa and colleagues (Rassa *et al.*, 2000), and is schematically presented in Chapter 3, Figure 3.8. Cell monolayers were washed once with PBS, trypsinized, and collected by centrifugation at 300 × g for 5 min. Poly $(A)^+$ RNA was isolated directly from the cells using an Oligotex Direct mRNA kit (QIAGEN Ltd., United Kingdom) according to the protocol for direct isolation of mRNA from animal cells, supplied in the manufacturer's instructions. Poly $(A)^+$ RNA representing ~10^5 cells was heated to 75°C for 5 min and ligated to 50 pmol of phosphorylated oligonucleotide A (5'-P-GGTCACCTTGATCTGAAGC-NH_2-3'), which contains an amino modification to block ligation at its 3' end, using T4 RNA ligase (New England Biolabs). The ligation was performed in a total volume of 10 µl at 37°C for 60 min. The reaction product was heated to 75°C for 5 min and cooled on ice, and the nucleic acid was extracted with phenol and chloroform, precipitated with ethanol, and resuspended in 12 µl of water. The purified ligations products were then used as a template in a reverse transcription reaction with 50 pmol of oligonucleotide A' (5'-GCTTCAGATCAAGGTGACCTTTTT), which is complementary to oligonucleotide A and part of the poly (A) tail, using Sensiscript reverse transcriptase (QIAGEN Ltd., United Kingdom). Reverse transcription was carried out at 37°C for 1 h in a 20 µl reaction volume. The resulting cDNA products were then digested with RNase A/T1 mix and used as a template in a PCR with an mRNA sense SV5 M gene-specific primer (5'-TAACACTACTATTCCAATAACTGG, which anneals 41 to 65 nucleotides from the site of polyadenylation), oligonucleotide A', and 1 µCi of [α-^{32}P]dATP. The PCR consisted of 25 cycles under the following parameters: 95°C for 30 sec, 52°C for 60 sec, 68°C for 60 sec. One-tenth of the resulting PCR products was mixed with loading buffer (5% glycerol, 1 mM TRIS, 0.1 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and subjected to electrophoresis on a nondenaturing 5% polyacrylamide gel. The gel was dried and analyzed by autoradiography and phosphorimaging.

3. RESULTS

3.1 Interferon induced changes to the replication cycle of an interferon-sensitive strain of SV5 (CPI-)

3.1.1 Interferon-α/β alters the pattern of CPI- virus protein synthesis

Previous work has shown that SV5 blocks both IFN-α/β and IFN-γ signalling by targeting STAT1 for proteosome-mediated degradation (Didcock *et al.*, 1999b). As well as blocking IFN signalling, most paramyxoviruses, including SV5, reduce the amount of IFN produced by infected cells (He *et al.*, 2002; Poole *et al.*, 2002; Wansley & Parks, 2002). However, the ability of SV5 to circumvent the IFN response is not absolute since infected cells still release small amounts of IFN that can induce an antiviral state in surrounding uninfected cells prior to infection, thereby limiting the replication of SV5 in these cells. Indeed, SV5 forms larger plaques in MRC-5 (human diploid) cells that have been engineered to be non-responsive to IFN (Young *et al.*, 2003). Nevertheless, the effect(s) on SV5 replication when IFN induces an antiviral state within a cell are not known. To address this question and further define how SV5 interacts with the antiviral factors of the IFN response a model system in which the effect of IFN on SV5 replication could be examined in the absence of virus countermeasures was developed. Vero cells were used because they do not produce IFN due to spontaneous gene deletion (IFN-α/β genes; Desmyter *et al.*, 1968; Mosca & Pitha, 1986), but they do respond to IFN once exogenous IFN is supplemented to the culture medium. As described in Chapter 1, CPI- and CPI+ are two closely related canine strains of SV5 that have been previously shown to differ in their ability to block IFN signalling; CPI+ blocks signalling by targeting STAT1 for degradation, whilst CPI- fails to block signalling due to three amino acid substitutions in the N terminal domain of the V protein (Chatziandreou *et al.*, 2002). Therefore, by infecting Vero cells with CPI- it was possible to examine

what effects the addition of IFN had on the replication of SV5, without any virus countermeasures to IFN signalling.

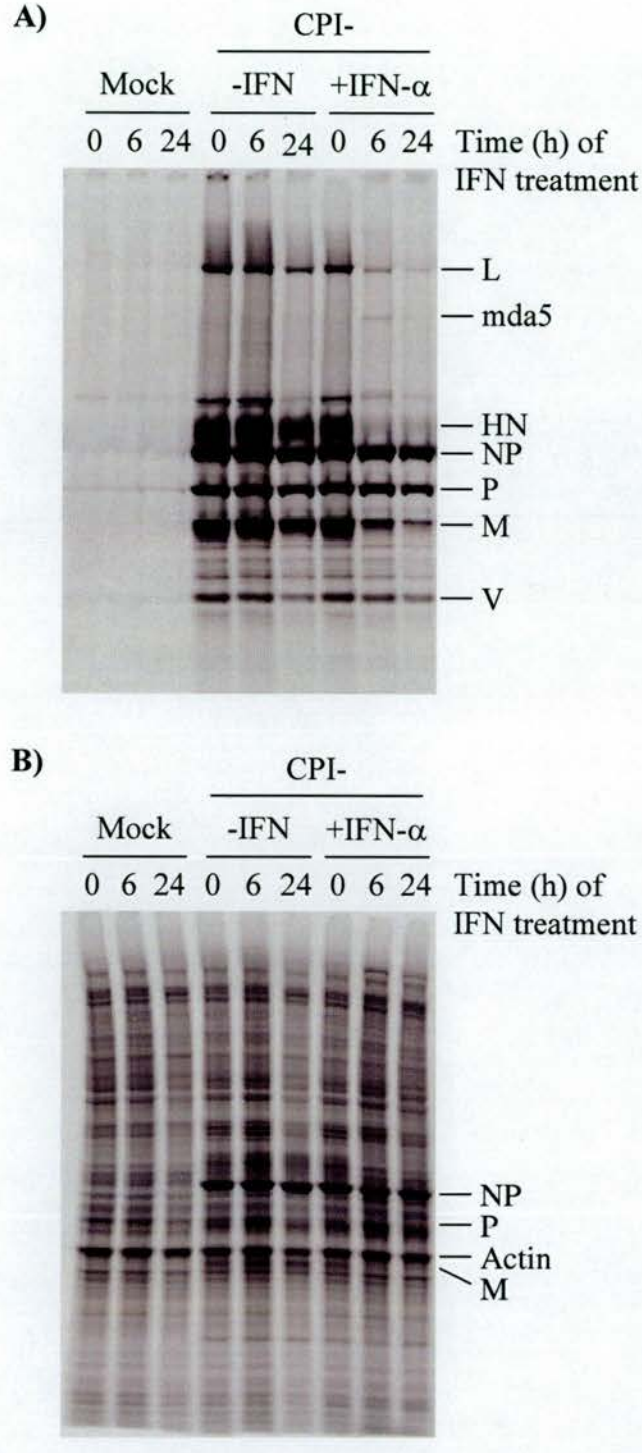
The initial aim was to investigate the effect of IFN under conditions where virus replication had already been established and where IFN-induced effects on early events, such as virus entry, could be ruled out. For this reason, Vero cells were infected with CPI- for 12 hours prior to addition of IFN, so that the consequences of IFN treatment could be examined in circumstances where virus protein synthesis was already taking place. At different times after IFN treatment, cells were metabolically labelled with [^{35}S]-methionine for 1 h and immunoprecipitation of virus proteins was carried out with a pool of antibodies specific for the NP, P, V, M, and HN proteins (note the L protein is co-immunoprecipitated due to its association with P protein). The precipitated proteins and the proteins present in the total cell extracts were subsequently separated on 4-12% polyacrylamide (PA) gradient gels and visualized by phosphorimage analysis (Figure 3.1).

The results from this analysis revealed a selective effect of IFN on synthesis of the different virus proteins (Panel A). The relative expression levels of NP, P and V proteins were not notably different in IFN treated compared to non-treated cells. However, there was an obvious reduction in the relative levels of M and other virus proteins synthesised from genes downstream of the V/P gene on the virus genome, including HN and L. This difference in protein expression was not an artefact of the immunoprecipitation step as confirmed by analysis of total cell extracts (Panel B). From this analysis was clear that IFN treatment drastically inhibited expression of the M protein but had little effect on synthesis of NP, P or V proteins, or cellular proteins (Panel B). Additionally, by 36 h p.i., although infected cells in the absence of IFN were still making larger amounts of viral proteins than infected cells in presence of IFN, the relative levels of virus protein synthesis in untreated cells were significantly less than at 18 h p.i. This was not due to cell death or a general reduction in the overall levels of cellular protein synthesis since the relative levels of total cell protein synthesis was similar at all of the time points examined (Panel B). It is likely that as a result of accumulation

Figure 3.1 The relative levels of CPI- protein synthesis is affected by IFN-α treatment in Vero cells, which do not produce IFN but respond to IFN.

Vero cells were infected with CPI- at an m.o.i. of 50 pfu/cell and treated or not treated with exogenous IFN-α at 12 h p.i. Cells were metabolically labelled with [^{35}S]-methionine for 1 hour at 0, 6 and 24 hours after addition or no addition of IFN. 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 B). Virus proteins were precipitated from soluble antigen extracts of these cells with a pool of monoclonal antibodies to the NP, P, M, and HN proteins and a polyclonal antiserum to the P/V proteins. The precipitated proteins from CPI- infected cells were subsequently separated on a 4-12% PAG gradient (Invitrogen) and visualized by phosphorimage analysis (Panel A).

Figure 3.1


A)
CPI-
Mock
-IFN
+IFN-α
0 6 24 0 6 24 0 6 24
Time (h) of IFN treatment
L
mda5
HN
NP
P
M
V
B)
CPI-
Mock
-IFN
+IFN-α
0 6 24 0 6 24 0 6 24
Time (h) of IFN treatment
NP
P
Actin
M

of virus proteins at later times p.i., the viral polymerase had switched to the replicative mode and a full-length genomic RNA was synthesised instead of synthesis of mRNAs for production of viral proteins.

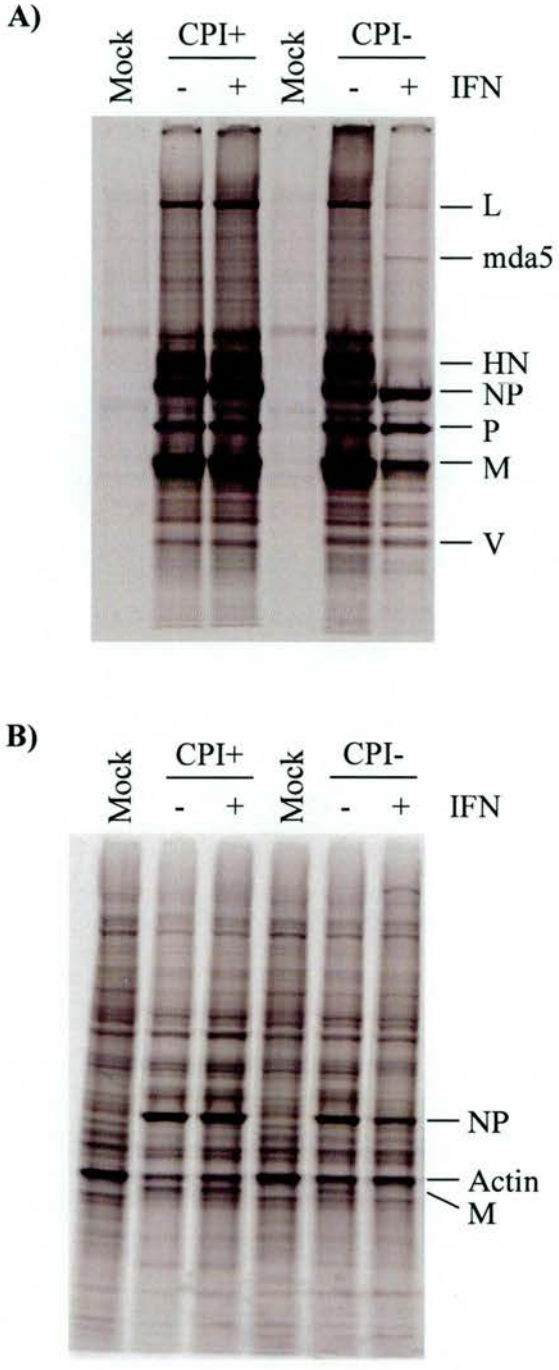
In order to confirm that the selective effect of IFN on the relative expression levels of virus proteins was specific for the situation in which a virus (CPI-) fails to block IFN signalling, a comparison between the effect of IFN on the protein synthesis profiles of CPI- and a strain of SV5 (CPI+) that blocks IFN signalling was subsequently performed. Vero cells were infected with CPI- or CPI+ at a high m.o.i. and at 12 h p.i. exogenous IFN-α was or was not added to the culture medium. At 6 h after treatment with IFN cells were metabolically labelled with [^{35}S]-methionine for 1 h. At the end of the radiolabelling incubation period, the cells were lysed and the NP, P, V, M and HN proteins were immune precipitated with a mixture of appropriate antibodies. The labelled proteins in the immune-precipitates and in the total cell extracts were analysed by SDS-PAGE and visualized by phosphorimage analysis (Figure 3.2). In contrast to the marked effect that IFN had on the expression of CPI- viral proteins (specifically, M, HN and L proteins), the addition of IFN to cells infected with CPI+ had no effect on the relative levels of virus protein synthesis (Panel A).

Whilst examining the effect of IFN on the protein synthesis of CPI- that fails to block IFN signalling, it was noted a considerable increase (compared with CPI+ that blocks IFN signalling) in the levels of a 150 kDa unidentified protein coimmunoprecipitated with virus proteins from cells treated with exogenous IFN. Follow up studies from this initial observation confirmed that this 150 kDa was induced by IFN and specifically associated with the V protein of SV5. It was then subsequently identified by Dr. J. Andrejeva as the melanoma differentiation associated gene-5 (mda-5) product. Further studies showed that the interaction of the V protein of SV5, and other paramyxoviruses, with mda-5 plays a critical role in the ability of these viruses to interfere with IFN production (Andrejeva *et al.*, 2004).

Figure 3.2 IFN treatment has an effect on CPI- viral protein synthesis but not on CPI+ protein synthesis.

Vero cells were infected with CPI- or CPI+ at an m.o.i. of 50 pfu/cell and at 12 h p.i. exogenous IFN-α was, or was not, supplemented to the culture medium. 6 hours later, cells were radioactively labelled with [^{35}S]-methionine for 1 hour and immunoprecipitation of virus proteins was performed from soluble antigen extracts of these cells using monoclonal antibodies specific for the SV5 NP, P, V, M, and HN proteins. The labelled precipitated proteins (Panel A) and the labelled polypeptides present in the total cell extracts (Panel B) were analysed by SDS-PAGE and visualized by phosphorimage analysis.

Figure 3.2


A)
Mock
CPI+
- +
Mock
CPI-
- +
IFN
L
mda5
HN
NP
P
M
V
B)
Mock
CPI+
- +
Mock
CPI-
- +
IFN
NP
Actin
M

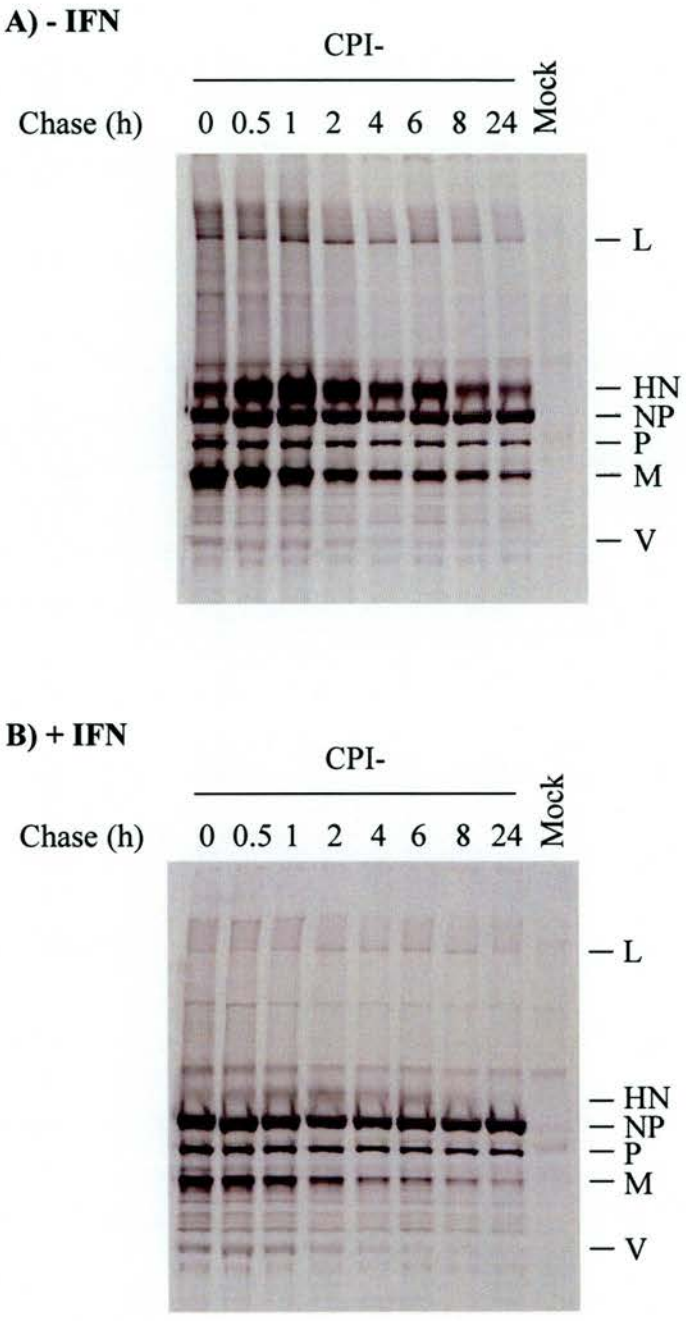
3.1.2 Interferon-α does not alter the half-life of viral proteins

Given the remarkable change in the pattern of CPI- protein synthesis in the presence of IFN, it was important to ascertain whether IFN had an effect on the stability of viral proteins. To address this question, the half-life of CPI- viral proteins was monitored in CPI- infected cells in the absence and presence of IFN. Vero cells were infected with CPI- at a high m.o.i., and 12 hours later exogenous IFN-α was, or was not, added to the culture medium. At 6 hours post-treatment with IFN, the cells were metabolically labelled with [^{35}S]-methionine for 20 minutes, the monolayers were washed with maintenance medium three times and incubated in the absence of radioactivity (chase) for up to 24 hours, in the presence or absence of IFN as appropriate. At various times, the cells were lysed and the relative levels of virus proteins were estimated by immunoprecipitation. The labelled polypeptides were visualised by phosphorimage analysis (Figure 3.3) and the results were consistent with the observations presented in the previous section; namely, IFN selectively altered the protein synthesis profile, so that in the presence of IFN nearly no L and HN were detected, the M levels were strongly reduced, whereas NP and P proteins expression levels were not significantly changed. Quantitation of the radioactivity in the precipitated virus protein bands is presented in Figure 3.4 (note that the relative levels of the various viral proteins in the absence or presence of IFN for each time point are compared with respect to their levels in the absence or presence of IFN in time 0 h, which is given a value of 100%). This showed that the half-life of each viral protein was not notably changed by IFN. Furthermore, this analysis revealed that the NP protein was relatively stable and only 30 to 40 % of NP had been degraded after a 24 hour chase. In contrast, the intracellular turnover of the M protein was more rapid and around 95 % had been degraded in both untreated and IFN treated infected cells after a 24 hour chase.

Figure 3.3 IFN is not inducing a quicker turnover of specific viral proteins in Vero cells infected with CPI-.

Vero cells were infected with CPI- at high m.o.i. (50 pfu/cell) and 12 hours later, exogenous IFN-α was (Panel B), or was not (Panel A), supplemented to the culture medium for 6 hours, after which time cells were metabolically labelled with [^{35}S]-methionine for 20 minutes. The monolayers were then washed with maintenance medium, incubated in the presence or absence of IFN, and lysed at various times after radiolabelling. Immunoprecipitation of the labelled viral polypeptides was performed using a mixture of appropriate monoclonal antibodies to SV5 proteins, and the precipitated polypeptides were then separated on a 4-12% PA gradient gel (Invitrogen) and visualized by phosphorimage analysis.

Figure 3.3


A) - IFN
CPI-
Chase (h)
0 0.5 1 2 4 6 8 24
Mock
L
HN
NP
P
M
V
B) + IFN
CPI-
Chase (h)
0 0.5 1 2 4 6 8 24
Mock
L
HN
NP
P
M
V

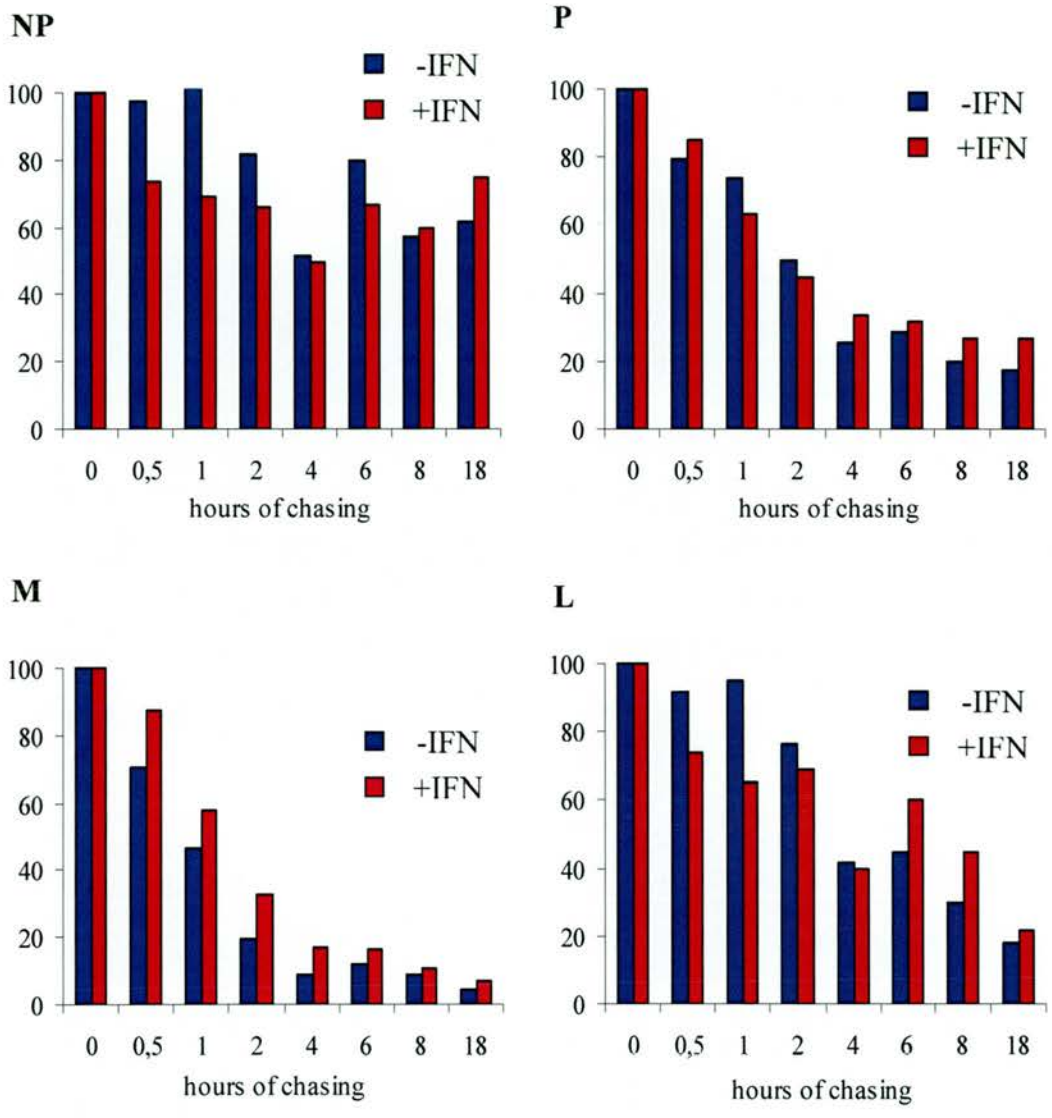


Figure 3.4 Quantification of the amounts of radioactivity in the "chased" newly synthesised viral proteins precipitated from Vero cells infected with CPI- in the presence or absence of IFN. For each protein analysed, the protein band at each time point in untreated (-IFN) and treated (+IFN) samples was normalized so that the respective protein band in untreated (-IFN) and treated (+IFN) samples, respectively, for time 0 h equalled 100 units.

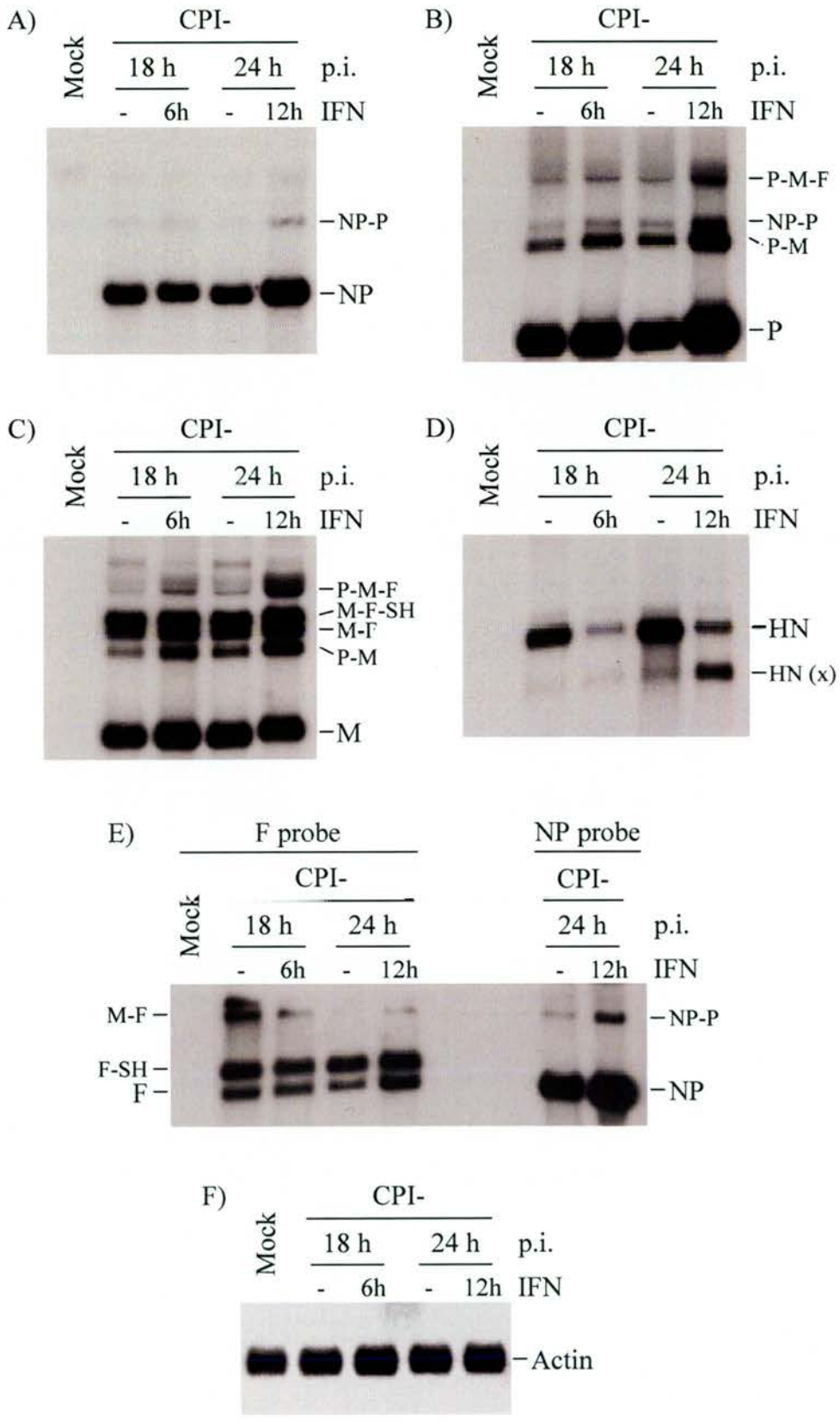
3.1.3 Interferon-α induces alteration in CPI- virus transcription

Given the striking change in the pattern of CPI- protein synthesis in response to IFN, the effect of IFN on the pattern of CPI- transcription was examined next. Vero cells were mock-infected or infected with CPI- and IFN was, or was not, added to the culture medium at 12 h p.i.At 6 and 12 hours post-treatment, total intracellular RNA was isolated from the cells and analysed by Northern blotting with [^{32}P] -labelled ssDNA probes specific for viral mRNAs from the NP, P, M, F and HN genes (Figure 3.5, Panels A to E). A replicate blot was probed for actin mRNA to confirm that similar amounts of RNA were loaded in each lane (Figure 3.5, Panel F). Monocistronic viral RNA corresponding to NP, P, M, F and HN genes, as well as larger RNAs corresponding to polycistronic read-through transcription products were detected. The monocistronic mRNAs were quantitated by phosphorimage analysis (Figure 3.6; note that the relative levels of the various viral mRNAs are compared with respect to their levels in the absence of IFN at either 6 or 12 h post treatment, which were each given a value of 100%). The results from this analysis revealed an alteration in both the levels and pattern of virus transcription in the presence of IFN. Surprisingly, there was an increase in the levels of NP and P mRNA in cells treated with IFN compared to untreated cells (Figure 3.5, Panels A and B, respectively). However, similar levels of M mRNA were detected in untreated and IFN treated infected cells (Figure 3.5, Panel C), but there was a significant decrease in HN mRNA in the presence of IFN (Figure 3.5, Panel D). These data show that IFN has different effects on virus gene expression, depending on the position of the gene in the genome, causing an increase in transcription of genes at the 3' end of the genome and a decrease in transcription of genes the further they are from the 3' terminus. Comparison of the read-through polycistronic RNAs on the blot probed for M mRNA support this observation; thus, the polycistronic mRNAs starting at the P gene (P/M and P/M/F) had a pattern similar to monocistronic P mRNA, but the polycistronic mRNAs starting at the M gene (M/F and M/F/SH) had a pattern similar to monocistronic M mRNA (Figure 3.5, Panel C). Analysis of the HN blot revealed a surprising effect of IFN expression. In the absence of IFN, the HN probe

Figure 3.5 Northern Blot analysis of positive-sense RNAs synthesised in Vero cells infected with CPI- in the presence of IFN.

Vero cells were infected with CPI- at a high m.o.i. and treated, or not treated (as a - IFN control), with IFN at 12 h p.i. Total intracellular RNA was isolated from these cells at 6 and 12 hours after addition of IFN and subjected to Northern Blot analysis. Hybridization was performed with [^{32}P]-labelled negative-sense DNA probes specific for viral mRNAs NP (A), P (B), M (C), HN (D), F mRNA (E) or the cellular actin mRNA (F).

Figure 3.5


A)
CPI-
Mock
18 h
24 h
p.i.
-
6h
-
12h
IFN
NP-P
NP
B)
CPI-
Mock
18 h
24 h
p.i.
-
6h
-
12h
IFN
P-M-F
NP-P
P-M
P
C)
CPI-
Mock
18 h
24 h
p.i.
-
6h
-
12h
IFN
P-M-F
M-F-SH
M-F
P-M
M
D)
CPI-
Mock
18 h
24 h
p.i.
-
6h
-
12h
IFN
HN
HN (x)
E)
F probe
NP probe
CPI-
CPI-
Mock
18 h
24 h
24 h
p.i.
-
6h
-
12h
-
12h
IFN
M-F
F-SH
F
NP-P
NP
F)
CPI-
Mock
18 h
24 h
p.i.
-
6h
-
12h
IFN
Actin

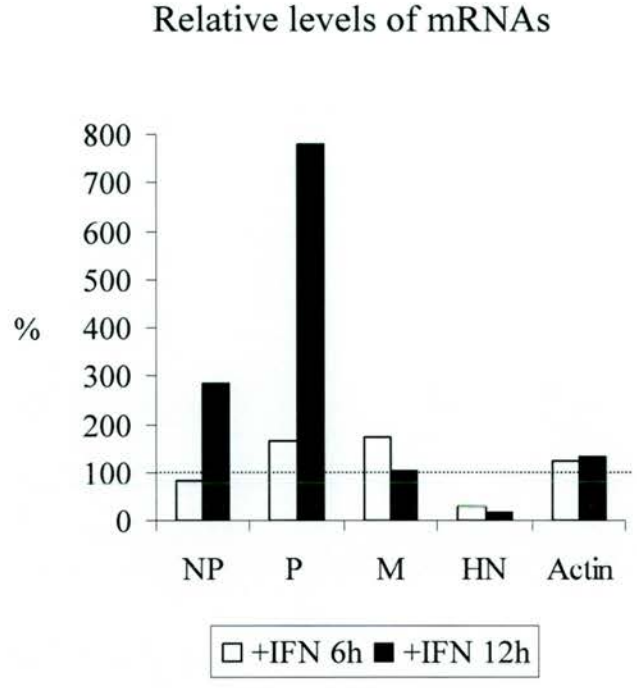


Figure 3.6 Quantitation was performed on the monocistronic mRNAs and for each indicated gene the amount of radioactivity in the RNA band in IFN treated samples was normalized to the amount of RNA, at the same time point, for the same gene in untreated samples (which was given a value of 100%, indicated by a dotted line in the figure).

detected the major monocistronic HN band (Figure 3.5, Panel D). A smaller faint band, labelled HN (x) could also be detected. Neither of these bands could be detected in the mock infected control lane, indicating that both were virus specific. Following 12 h treatment with IFN, the HN (x) band became prominent and was present at a higher level than the HN band. These data suggest that IFN treatment affects the size of the RNA produced from the HN gene.

To facilitate a direct comparison of the IFN-induced effects on virus transcription and protein synthesis, in this experiment virus protein synthesis was monitored in cells that were infected and treated with IFN in parallel with those used for the RNA analysis (Figure 3.7, Panel A). Quantitation of these results is presented in Figure 3.7, Panel B. From these results it was clear that although IFN alters the levels of transcription and protein expression of each gene, there was not complete concordance between these two processes. Thus, although there was a considerable increase in the relative levels of NP and P mRNAs (3-fold and 8-fold, respectively) in the presence of IFN, the expression levels of NP and P proteins were slightly decreased (approximately 2-fold and 1.5-fold, respectively). Moreover, while there were similar levels of mRNA from M gene, the expression levels of M protein were drastically decreased in the presence of IFN (approximately 30-fold in infected cells treated with IFN for 12 h). Significantly, whilst IFN considerably reduced the levels of HN mRNA (5-fold), a much stronger effect was observed in HN protein synthesis (20-fold decrease).

Overall, these data suggest that IFN affected SV5 gene expression in at least two ways, (i) by altering the virus transcription gradient, resulting in an increase in transcription of genes at the 3' end of the genome and a decrease in transcription of genes at the 5' end of the genome, and (ii) by inhibiting protein synthesis from all virus mRNAs.

3.1.4 Analysis of the polycistronic RNA products

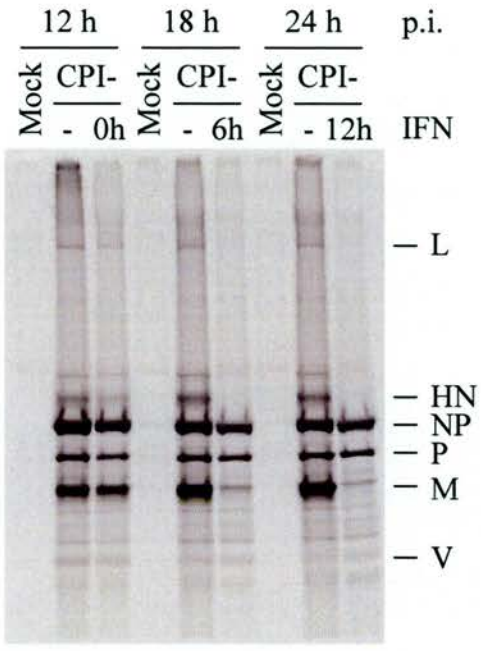
From the RNA analysis, new observations were found in respect to the polycistronic products that have not been reported before. It has been shown for a

Figure 3.7 Analysis of the relative levels of protein synthesis performed in parallel to Northern blotting analysis.

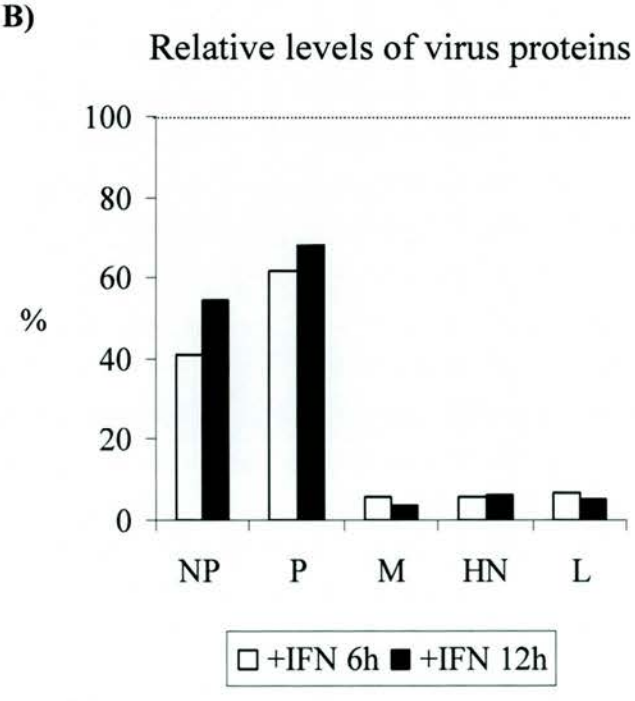
Vero cells were mock-infected or infected with CPI- at a high m.o.i. for 12 h, incubated in the presence or absence (-) of IFN-αA/D for 0, 6 or 12 h and metabolically labelled with [^{35}S]-methionine for 1 hour. Virus proteins were immunoprecipitated with a pool of mAbs to the NP, P, V, M and HN proteins, which were then separated on a 4-12% PAG gradient (Invitrogen) and visualized by phosphorimage analysis (Panel A). Quantitation of the labelled precipitated polypeptides in (A) was carried out and the amount of radioactivity of the protein bands in each lane of IFN treated samples was normalized so that the respective bands in the lane of untreated samples equalled 100% (indicated by a dotted line in Panel B).

Figure 3.7

A)


12 h
18 h
24 h
p.i.
Mock
CPI-
- 0h
Mock
CPI-
- 6h
Mock
CPI-
- 12h
IFN
L
HN
NP
P
M
V

B)


Relative levels of virus proteins
100
80
60
40
20
0
%
NP
P
M
HN
L
+IFN 6h
+IFN 12h

number of paramyxoviruses, including SV5, that read-through transcription is highest across the junction between the genes encoding the M protein and F protein. This naturally occurring M-F read-through has been proposed to be important for virus growth (Rassa & Parks, 1998). Indeed, in the Northern blot analysis presented above there was a considerable amount of the di-cistronic M-F read-through transcript product (Figure 3.5, Panel C). Additionally, it has been reported that with the exception of the M-F junction, each of the diverse SV5 junctions directs efficient gene end termination and downstream gene reinitiation (Paterson *et al.*, 1984; Rassa & Parks, 1998). Surprisingly, a disproportionate amount of the F-SH di-cistronic RNA relative to the monocistronic F mRNA was detected in all samples analysed (Figure 3.5, Panel E). The lower RNA band in the blot was confirmed to correspond to F mRNA, since duplicate samples were run in the same blot, and hybridised with a probe for F mRNA and a probe for NP mRNA which are very similar in size (NP mRNA is 1732 bp and F mRNA is 1718). On the other hand, the M-F and NP-P read-through products are also similar in size (3088 bp and 3037 bp, respectively) and thus the band above F mRNA was identified as F-SH di-cistronic RNA (2010 bp). The percentage of read-through was calculated as the fraction of total mRNA that was detected as a di-cistronic transcript with the downstream proximal gene (F-SH). In the experiment presented approximately 60% to 70% of the total F-specific RNA was detected as the di-cistronic F-SH species. It appears to be a significant amount and it is possible that, as observed for the M-F read-through, there is an importance of F-SH read-through transcription in the SV5 virus growth.

3.1.5 Interferon induced increase in the length of the poly (A) tail of CPI- mRNA

Careful examination of the Northern blots shown in Figure 3.5 suggested that the monocistronic mRNAs isolated from cells treated with IFN migrated slightly more slowly than mRNA from untreated cells, and that was particularly evident at 12 h post IFN treatment. As described in Chapter 1, at gene end sequences there is a stretch of several uridyl residues where the virus polymerase stutters resulting

in the addition of long poly (A) tails to the mRNAs. Thus, one possible explanation for an increase in the apparent size of the mRNAs was that there was an increase in the length of their poly (A) tails.

To examine this possibility, the length of the poly (A) tail on M mRNA from IFN treated or untreated cells was compared, using an assay described by Rassa and coworkers (Rassa *et al.*, 2000). The RT-PCR assay to measure the length of the poly (A) tails on an mRNA is schematically presented in Figure 3.8, and involved, first, ligation of an oligonucleotide (A) to the poly $(A)^+$ RNA. This oligonucleotide contains a 3'-amino blocking group so that no ligation occurs at this end, and the reaction takes place between the 3' end of the mRNA and the 5' end of the primer A. The ligated products are then reverse transcribed with a primer (A' in Figure 3.8) complementary to the anchor primer A. The resulting cDNAs are amplified in a PCR reaction using primer A' and a primer specific for the M gene that anneals within 65 nucleotides of the site of polyadenylation (B in Figure 3.8), in the presence of $[^{32}P]$-dATP so that the products can be visualized by autoradiography, or phosphorimage analysis, following polyacrylamide gel electrophoresis analysis. In this assay the DNA band that migrated slightly faster than the 0.1 kb marker represents M mRNA that was not polyadenylated, and the smear of DNA that migrated more slowly represents polyadenylated M mRNA (Figure 3.9, Panel A), as described previously (Rassa *et al.*, 2000). To allow quantitative analysis of the different sized products, the gel was analysed by performing a lane intensity profile analysis in which the phosphorimager software generates a plot of the signal intensity along the length of each lane of the gel (Figure 3.9, Panel B). Based on the sizes of the PCR products, it was clear that there was a significant increase in the average length of the poly (A) tails on M mRNA in the presence of IFN (by approximately 50 – 100 nucleotides as estimated by PAGE). Also, although the amount of radioactivity incorporated into the product representing non-polyadenylated RNA was slightly higher for the untreated sample compared to the IFN treated sample, the amount of radioactivity contained in the products representing polyadenylated M mRNA was higher in the IFN treated sample than in the untreated sample, indicating that a larger amount of

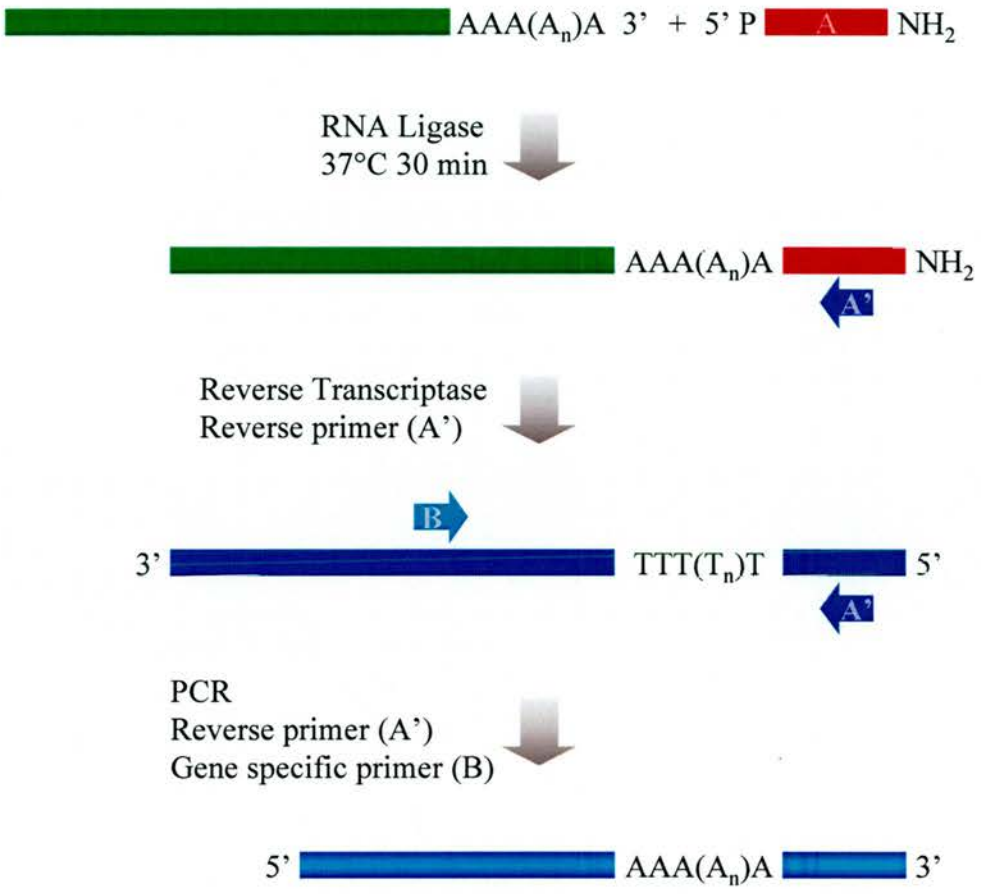


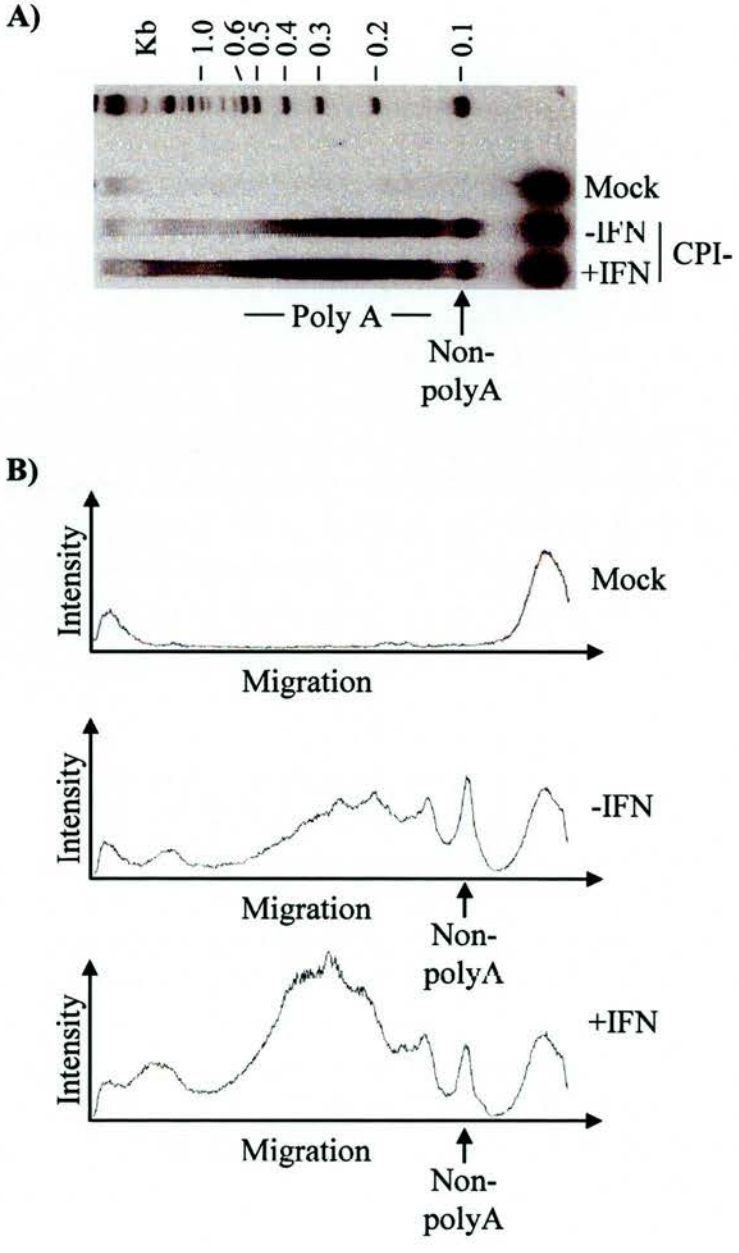
Figure 3.8 Schematic presentation of the assay to determine the poly A tail length on CPI- M mRNAs.

Poly $(A)^+$ RNA are ligated to a synthetic primer that contains a 3' amino modification to block ligation at this end (A) using RNA ligase. Reverse transcription reaction is performed using the ligated samples with a primer A' that is complementary to the primer A. M-gene-specific cDNAs are amplified by PCR using a primer specific for M gene (B) and primer A' along with [α-^{32}P]dATP. A portion of the PCR reaction product is analysed on a 5% PAGE, dried and exposed to film.

Figure 3.9 IFN affects the length of the poly (A) tail on CPI- M mRNA.

Poly (A) mRNA isolated from CPI- infected Vero cells that were treated with rHuIFN-αA/D at 12 h p.i. for 6 hours, or left untreated as a -IFN control, was used in the poly A tail length assay as described in the text, and the labelled products were analysed by electrophoresis on a 5% polyacrylamide gel (Panel A). The lanes containing samples derived from mock-infected and from infected -IFN or +IFN treated cells were analysed with a phosphorimage to generate a lane intensity profile, in which for each lane the signal intensity (y-axis) is plotted against the distance migrated (x-axis) (Panel B). The lane intensity profiles in Panel B are aligned with the polyacrylamide gel shown in Panel A.

Figure 3.9


A)
Kb
1.0
0.6
0.5
0.4
0.3
0.2
0.1
Mock
-IFN
+IFN
CPI-
Poly A
Non-polyA
B)
Intensity
Migration
Mock
-IFN
+IFN
Non-polyA

[^{32}P]-dATP was incorporated, which is consistent with longer A tracts in M mRNA isolated from IFN treated cells.

3.1.6 Interferon-α/β induces virus inclusion body formation in SV5 (CPI-) infected cells

Given that IFN significantly altered the pattern of CPI- virus transcription and protein synthesis, the effect of IFN on the distribution of virus proteins was next examined. Vero cells were infected with either CPI+ or CPI- for 12 hours before addition of IFN to the culture medium and the distribution of virus proteins observed at 1, 3 and 6 days p.i. by indirect immunofluorescence using a combination of anti-NP and anti-P mAbs. In the absence of IFN, NP and P proteins were primarily diffusely distributed throughout the cytoplasm in both CPI+ and CPI- infected cells, but could also be detected in small cytoplasmic inclusion bodies at all times in the infection time course (Figure 3.10). As expected, the addition of exogenous IFN to CPI+ infected cells had no effect on the distribution of virus proteins, which had a similar pattern to that observed in cells infected with CPI- in the absence of IFN (Panel A). In striking contrast, the distribution of CPI- virus proteins was dramatically changed following the addition of exogenous IFN (Panel B); the NP and P were primarily located in cytoplasmic inclusion bodies within infected cells, which increased in size with time post IFN treatment, whilst the M and HN proteins became undetectable (Figure 3.11, Panels A and B, respectively).

At the same time, Vero cells infected with either CPI- or CPI+ were also stained with an anti-peptide serum against peptides in the N and C terminus of the L protein. Whilst this also had a primarily diffuse cytoplasmic distribution in the absence of IFN in both CPI- and CPI+ infected cells (Figure 3.12), following IFN treatment L protein redistributed, together with NP and P, into inclusion bodies in CPI- infected cells (Panel A). As expected, the addition of exogenous of IFN to CPI+ infected cells did not affect the distribution of L, NP or P proteins (Panel B).

Figure 3.10 IFN induces accumulation of the NP and P proteins into large cytoplasmic inclusion bodies in cells infected with CPI- but not with CPI+.

Vero cells were either infected with CPI+ (Panel A) or CPI- (Panel B) at an m.o.i. of 50 pfu/cell and at 12 h p.i. rHuIFN-αA/D was added to the culture medium (+IFN), or cells were left untreated (as a -IFN control). Monolayers were fixed at 1, 3 and 6 days p.i. and the distribution of NP and P proteins was analysed by immunofluorescence using a monoclonal anti-NP (SV5-NP-a) and a monoclonal anti-P (SV5-P-e) antibodies, followed by a secondary anti-mouse IG Texas Red-conjugated antibody.

Figure 3.10

A) CPI+

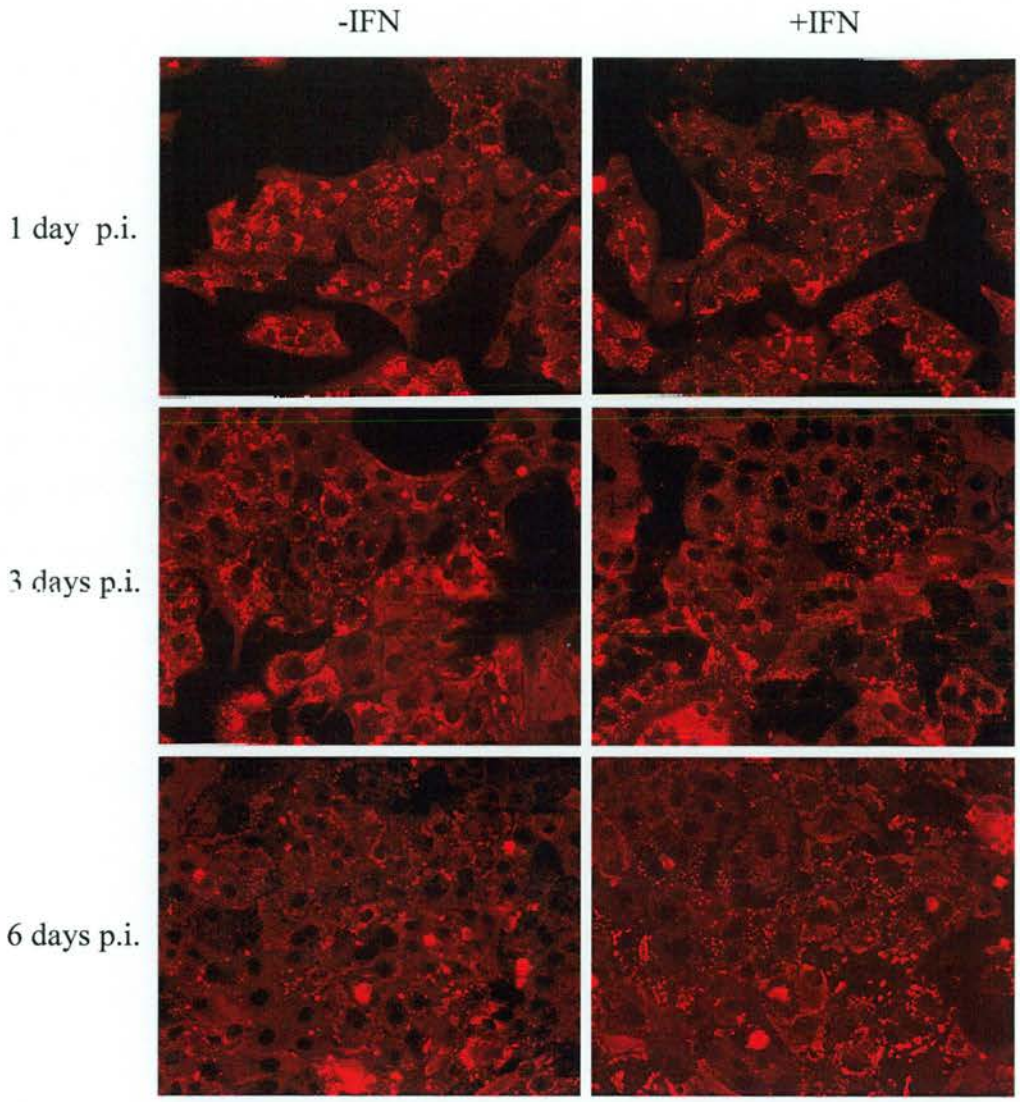


Figure 3.10

B) CPI-

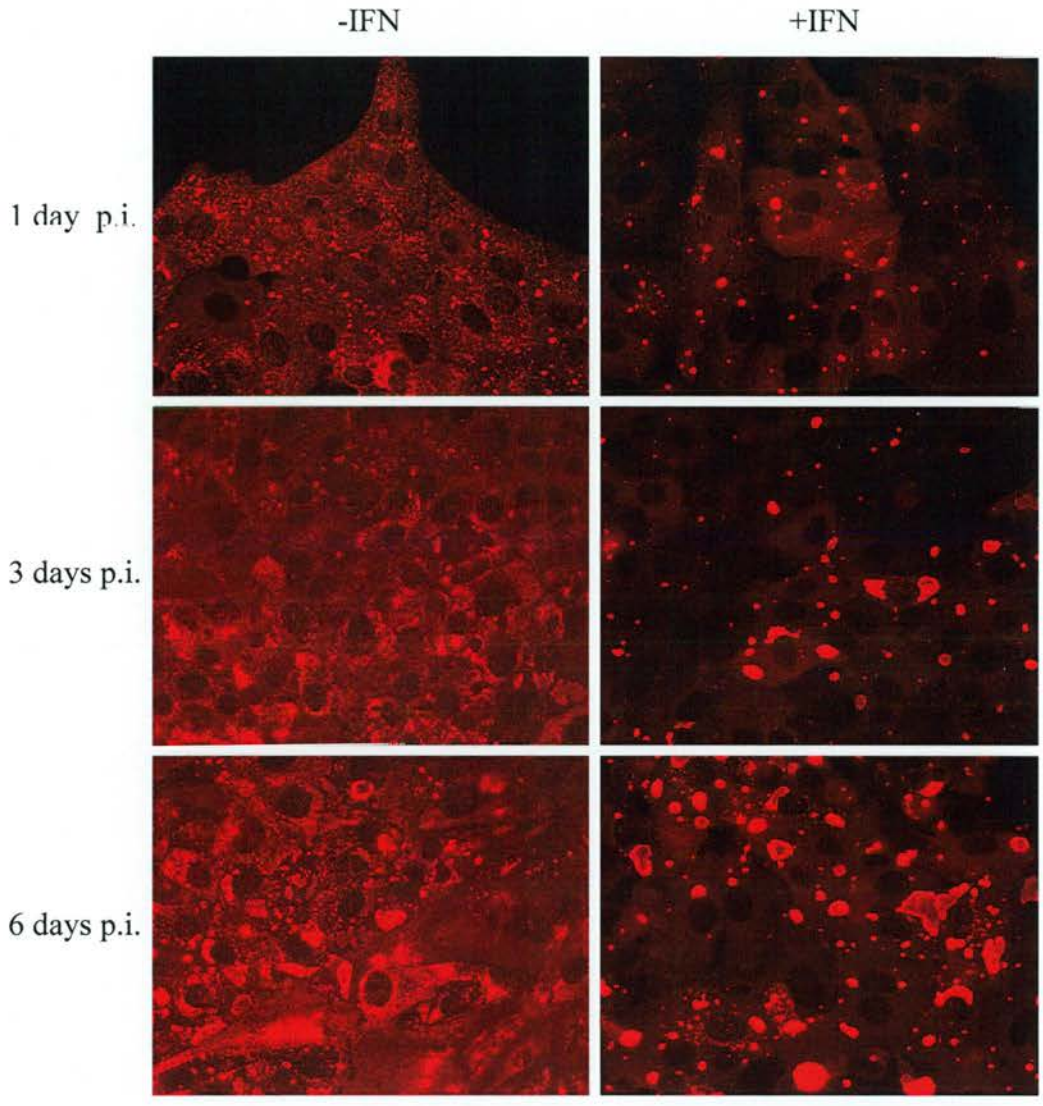


Figure 3.11 M and HN protein do not colocalize with the viral inclusion bodies.

Vero cells were infected with CPI- at an m.o.i. of 50 pfu/cell and at 12 h p.i. IFN was (+IFN), or was not (-IFN), added to the culture medium. Monolayers were fixed at 3 days p.i. and the distribution of M and HN proteins was analysed by double immunostaining using first a monoclonal antibody to detect M (SV5-M-h) or HN (SV5-HN-4a) proteins, followed by a secondary anti-mouse IG FITC-conjugated antibody or Texas Red-conjugated antibody, respectively (Panels A and B, respectively). Subsequently, a polyclonal antibody was used to detect P (V5 tag; Abcam), followed by a secondary anti-rabbit IG FITC-conjugated antibody or Texas Red-conjugated antibody.

Figure 3.11

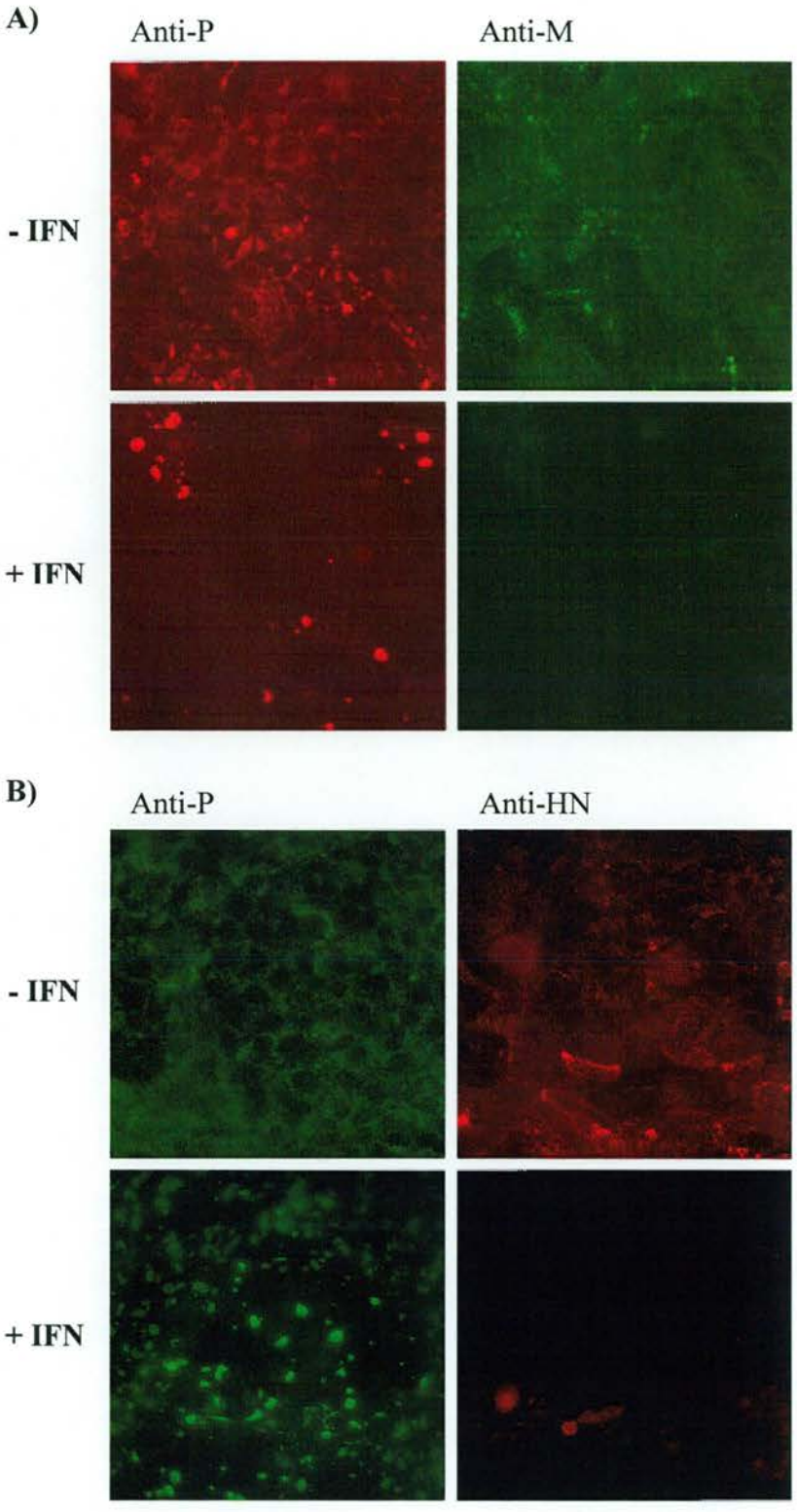
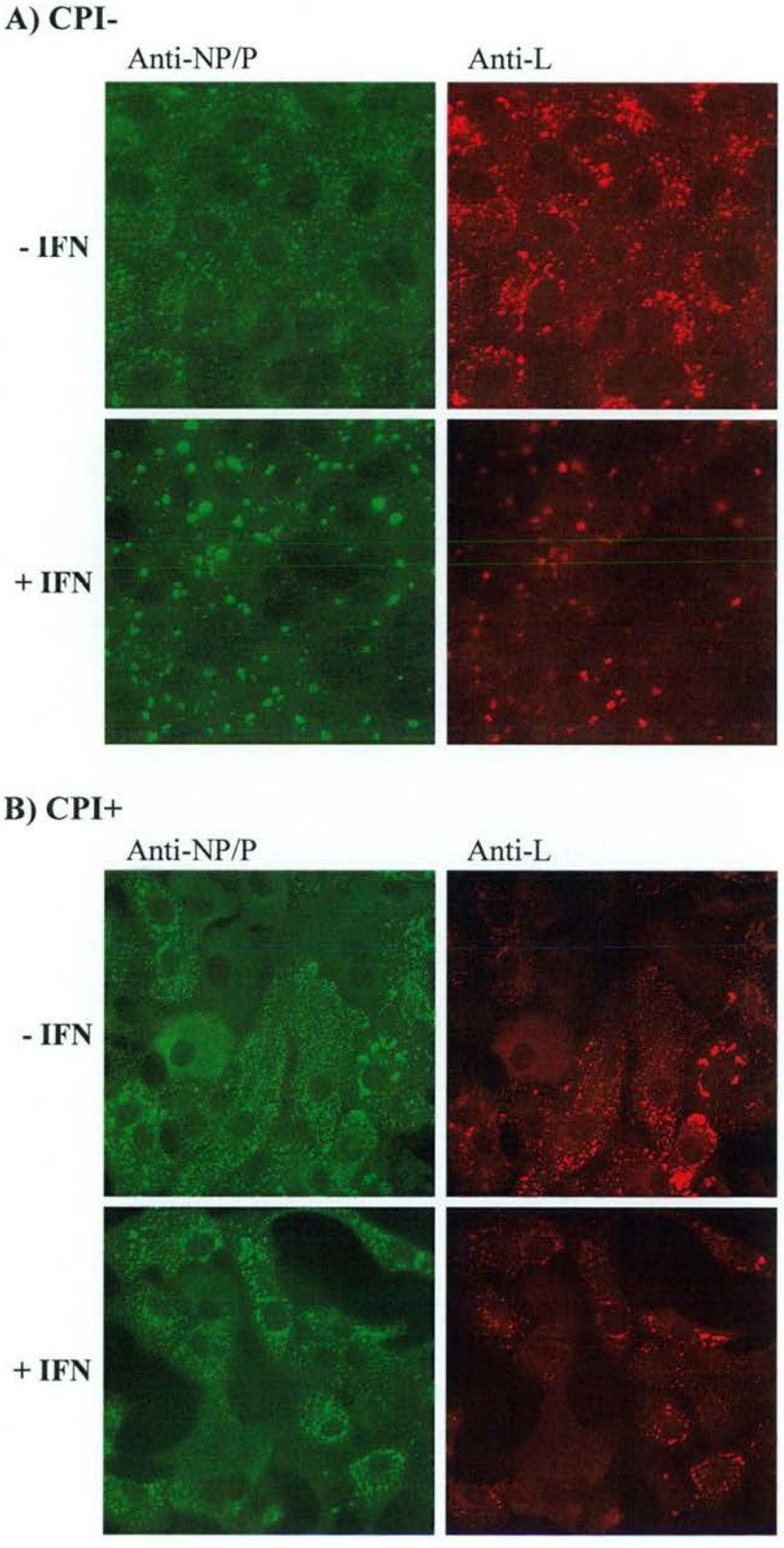


Figure 3.12 The large polymerase protein colocalizes with the NP and P proteins in the viral inclusion bodies.

Monolayers of Vero cells infected with either CPI- (Panel A) or CPI+ (Panel B) at a high m.o.i. (50pfu/cell), treated with IFN (+IFN) at 12 h p.i. or left untreated (-IFN), were fixed at 1 day p.i. and double staining fluorescence was carried out. First, cells were stained with a mixture of monoclonal anti-NP (SV5-NP-a) and anti-P (SV5-P-e) antibodies, followed by a secondary anti-mouse IG Texas Red-conjugated antibody. Subsequently, the L protein was detected with anti-peptide serum to the N and C terminus of L protein, followed by a secondary anti-rabbit IG FITC-conjugated antibody.

Figure 3.12



3.1.7 Interferon-γ does not have a significant effect on viral protein synthesis

Both, type I and type II of IFN activate antiviral defence mechanisms by inducing the expression of genes that encode proteins with antiviral effects. Although the signalling pathways and gene activation triggered by IFN-α/β and IFN-γ partially overlap, there are some genes selectively regulated by distinct IFNs. In light of differential induction of some of the antiviral proteins by IFN-α/β and IFN-γ, it was of interest to compare the consequences of addition of each of the two types of IFN on CPI- protein synthesis to examine whether IFN-γ induces the same change on the relative expression levels of CPI- proteins as IFN-α/β.

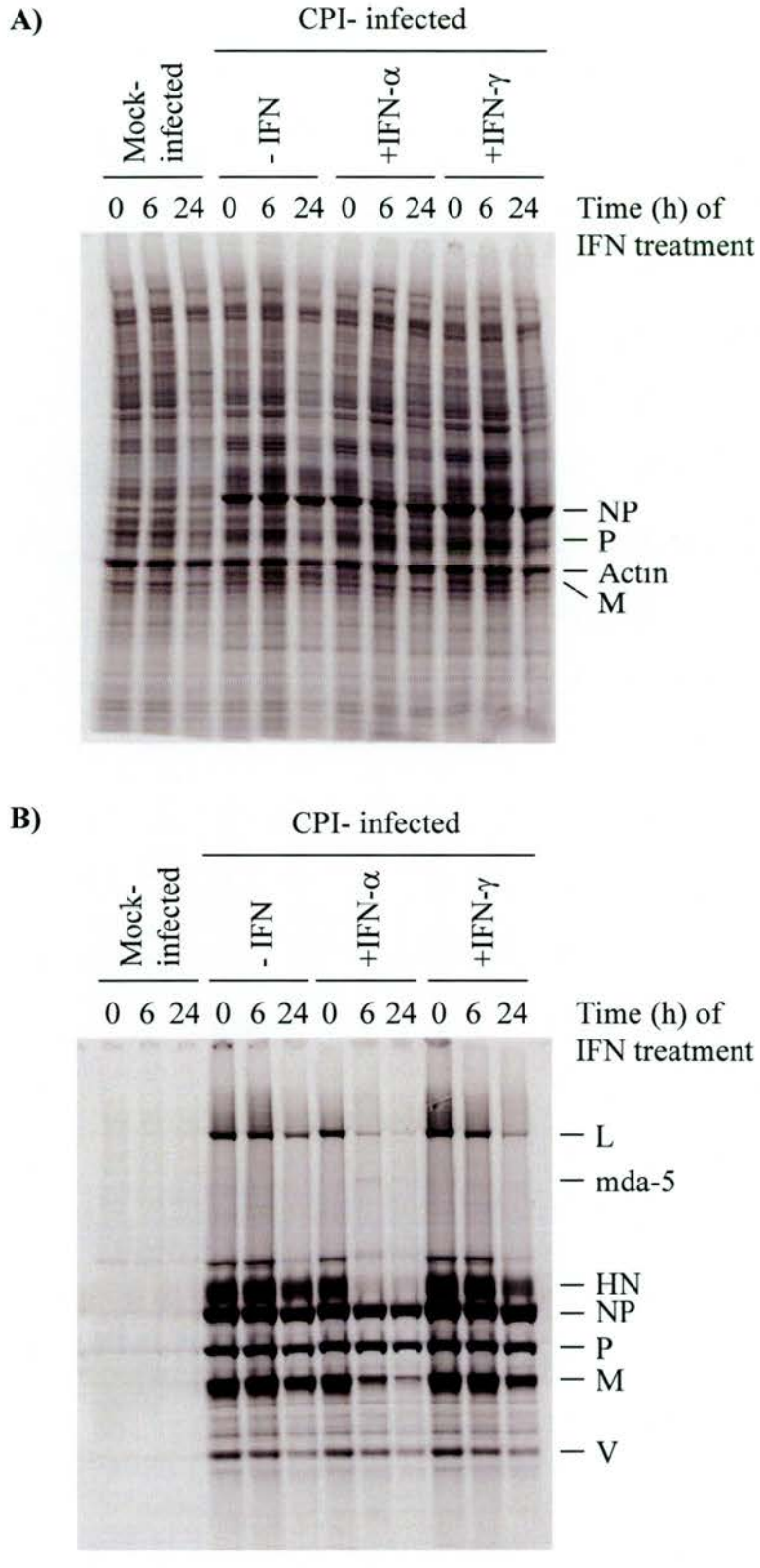
Vero cells were infected with CPI- at high m.o.i., and 12 hours later IFN-α or IFN-γ was, or was not, added to the culture medium. At different times after IFN treatment, the cells were metabolically labelled with [^{35}S]-methionine and the relative levels of virus proteins were estimated by immunoprecipitation using a pool of antibodies to NP, P, V, M and HN proteins, which were then separated in a gradient PAG and visualized by phosphorimager analysis (Figure 3.13). As shown in Panel B, IFN-γ, in contrast to IFN-α, did not significantly affect CPI- protein expression levels. In fact, the protein synthesis profile of CPI- in the presence of IFN-γ was very similar to that observed in untreated cells. Consistent with the results obtained previously, IFN-α had a striking effect on CPI- viral protein synthesis so that by 6 hours after the addition of IFN, low levels of M and L proteins and no HN protein could be detected. These results are consistent with previous observations, in which pre-treatment of 2fTGH (human diploid) cells with IFN-α significantly inhibited SV5 protein synthesis, whilst IFN-γ had only minor effects on the levels of SV5 proteins (Andrejeva *et al.*, 2002b).

Other consistent observation was that at 36 h p.i., HN, M and L proteins, but not NP and P proteins, were reduced in cells that were either treated with IFN-γ or left untreated. Levels of total cellular protein synthesis were also compared and

Figure 3.13 IFN-γ does not have an obvious affect on the expression levels of CPI- proteins in contrast to the dramatic affect of IFN-α.

Vero cells were infected with CPI- at a high m.o.i. (50 pfu/cell), and incubated with exogenous IFN-α or IFN-γ at 12 h p.i. or left untreated (-IFN). Cells were metabolically labelled with [^{35}S]-methionine for 1 hour at 0, 6 and 24 hours after addition, or no addition, of IFN. At the end of the radioisotope incubation period, cells were lysed and the protein content of the total cell extract was analysed by SDS-PAGE (Panel B). Virus proteins were precipitated from soluble antigen extracts of these cells with a pool of monoclonal antibodies to the NP, P, V, M, and HN proteins. The precipitated proteins from CPI- infected cells were subsequently analysed by SDS-PAGE (Panel A). Protein profiles were visualized by phosphorimage analysis.

Figure 3.13


A)
CPI- infected
Mock-infected
- IFN
+IFN-α
+IFN-γ
0 6 24 0 6 24 0 6 24 0 6 24
Time (h) of IFN treatment
NP
P
Actin
M
B)
CPI- infected
Mock-infected
- IFN
+IFN-α
+IFN-γ
0 6 24 0 6 24 0 6 24 0 6 24
Time (h) of IFN treatment
L
mda-5
HN
NP
P
M
V

showed that the extent of label incorporation was the same at each time point (Panel A).

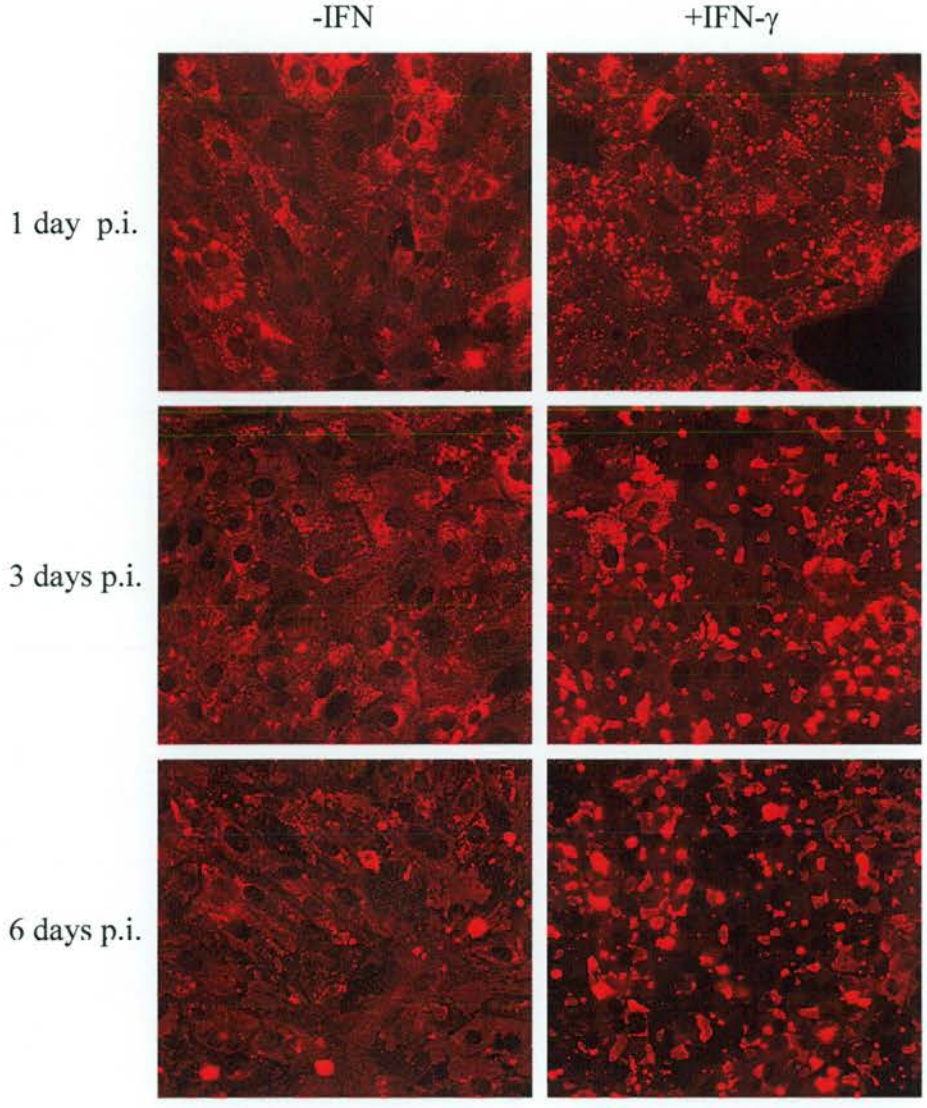
3.1.8 Interferon-γ appears to have a late effect on the distribution of NP and P proteins of CPI-

To determine whether the distribution patterns of NP and P proteins of CPI- were affected by IFN-γ in a similar way to that observed with IFN-α (Figure 3.10, Panel B) a similar analysis was performed as described above. As shown in Figure 3.14, in contrast to the early effect of IFN-α on the pattern of NP and P proteins of CPI- in infected cells, in which NP and P were in large inclusion bodies at 1 day p.i., the effect of IFN-γ on the distribution pattern of NP and P proteins only become as obvious as the effect of IFN-α by 3 and 6 days p.i. At 1 day p.i. although there was NP and P proteins in small cytoplasmic inclusions bodies in IFN-γ treated cells, these proteins were predominantly diffuse throughout the cytoplasm, whilst treatment with IFN-α had a more rapid effect such that at 1 day p.i. large inclusion bodies could be observed and very little amounts of NP and P were diffusely distributed (Figure 3.10, Panel B). However, IFN-γ did affect the distribution pattern of NP and P proteins, which were primarily located in cytoplasmic inclusion bodies by 3 and 6 days p.i., in contrast to the predominately diffuse distribution of these proteins in untreated cells (-IFN). Similarly to what was seen with IFN-α, the size of inclusion bodies increased with the time of IFN treatment.

Figure 3.14 Immunofluorescence analysis of NP and P protein distribution pattern in Vero cells infected with CPI-, in the presence or absence of IFN-γ.

Vero cells were infected with CPI- at an m.o.i. of 50 pfu/cell and at 12 h p.i. the culture medium was, or was not, supplemented with exogenous IFN-γ (+IFN-γ) or left untreated (-IFN). The cells were fixed at 1, 3 and 6 days p.i. and stained with a monoclonal anti-NP (SV5-NP-a) and a monoclonal anti-P (SV5-P-e) antibodies, followed by a secondary anti-mouse IG Texas Red-conjugated antibody.

Figure 3.14



3.2 Antiviral effects of interferon pre-treatment of cells prior to infection with paramyxoviruses

3.2.1 Interferon limits the replication of strains of SV5 that block interferon signalling and enhances inclusion body formation if cells are pre-treated with interferon prior to infection

Although most wild type (wt) strains of SV5, including CPI+, block IFN signalling and limit the production of IFN by infected cells, their ability to circumvent the IFN response is not absolute. This has been demonstrated by the fact that such strains form larger plaques in monolayers of MRC-5 and Hep-2 human cells, which have been engineered to be non-responsive to IFN (Young *et al.*, 2003). To observe how IFN may limit the spread of SV5 strains that block IFN signalling, Vero cells were treated with IFN for 14 hours or left untreated as a control, and subsequently infected with either CPI- or CPI+ in the continued presence or absence of IFN as appropriate. At 12 h, 18 h and 24 h p.i., the cells were metabolically labelled with [^{35}S]-methionine for 1 hour and the virus proteins were isolated by immunoprecipitation to estimate the relative levels of virus protein synthesis (Figure 3.15). As expected, in the absence of IFN both CPI- and CPI+ produced high levels of virus proteins at each time point. However, in IFN pre-treated cells the pattern of both CPI- and CPI+ protein synthesis was dramatically altered. At 12 h p.i. there was an overall reduction in NP and P protein synthesis, and very little M, and no HN or L could be detected. This pattern remained the same throughout the time course in CPI- infected cells. However, in CPI+ infected cells the levels of virus protein synthesis increased as the infection progressed with the pattern becoming closer to that observed in untreated cells by 24 h p.i. It has been previously shown that wt SV5 can degrade STAT1 in cells which have entered an antiviral state (Didcock *et al.*, 1999b). Therefore, presumably STAT1 was degraded in CPI+ (but not CPI-) infected cells and as a consequence these cells could not maintain an antiviral state indefinitely in the absence of IFN signalling. Nevertheless, pre-treatment of cells with IFN

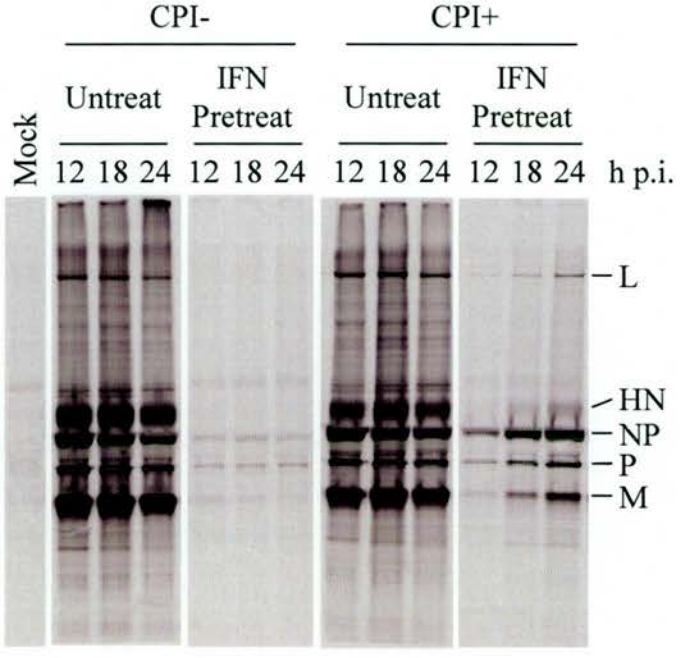


Figure 3.15 Effect of IFN pre-treatment on CPI- and CPI+ protein synthesis in Vero cells.

Cells were pre-treated with rHuIFN-αA/D (or left untreated) for 14 hours prior to infection with CPI- or CPI+ at an m.o.i. of 10 pfu/cell (in continuous presence of IFN). At 12, 18 and 24 hours p.i. cells were metabolically labelled with [^{35}S]-methionine for 1 hour. Virus proteins were immunoprecipitated using a mixture of mAbs to the NP, P, M and HN proteins, which were separated on a 4-12% PA gradient gel (Invitrogen) and visualized by phosphorimage analysis.

initially altered the pattern of CPI+ protein synthesis in a manner to that described earlier for CPI- infected cells that were post-treated with IFN (Figure 3.1).

Consequently, the effect of IFN pre-treatment on the distribution of virus proteins in infected cells was next examined. Vero cells were, or were not, pre-treated with IFN for 14 h and then infected at a high m.o.i. with either CPI- or CPI+ virus. Cell monolayers were fixed at 1, 3 and 6 days p.i. and examined by immunofluorescence using a mixture of monoclonal antibodies to NP and P proteins. From this analysis it was clear that pre-treatment of Vero cells with IFN significantly inhibited the replication of both CPI+ and CPI- (Figure 3.16), such that even though the monolayers were infected at a high m.o.i., at 1 day p.i. a significant proportion did not become positive for virus antigen, and in those cells which were positive the NP and P proteins were located in small cytoplasmic inclusion bodies (Panel A). However, by 3 and 6 days p.i. the size of the inclusion bodies got slightly larger with time, consistent with the finding that although CPI- protein synthesis was inhibited in IFN pre-treated cells, low levels of NP and P synthesis occurred (Panels B and C). In IFN pre-treated cells infected with CPI+, at 1 day p.i., although the majority of NP and P were located in small cytoplasmic inclusion bodies, NP and P were also distributed throughout the cytoplasm in a few cells. By 3 and 6 days p.i. the proportion of cells strongly positive for virus antigen was much higher in monolayers that had been infected with CPI+ as compared to CPI-, although in a significant proportion of cells NP and P were still primarily located in cytoplasmic inclusion bodies. Also an increasing proportion of CPI+, but not CPI-, infected cells became positive for HN as the time course progressed (data not shown). Since wt SV5 can eventually degrade STAT1 in cells which have entered an antiviral state (Didcock *et al.*, 1999b), presumably in the case of cells infected with CPI+ these cells cannot remain in an antiviral state in the absence of continued signalling (due to a loss of STAT1), and suppress virus replication indefinitely. Consequently, although in the presence of IFN at 6 days p.i. a significant proportion of cells in monolayers infected with CPI- remained uninfected, all cells were positive for virus antigen in monolayers of cells infected with CPI+ (Panel C).

Figure 3.16 Pre-treatment with IFN affects the distribution of NP and P proteins and the replication of both strains of SV5, CPI- and CPI+.

Vero cells were left untreated or pre-treated with IFN-α for 14 hours before infection with CPI- or CPI+ an m.o.i. of 10 pfu/cell (in continuous presence of IFN). At 1, 3 and 6 days p.i. the distribution of viral proteins was analysed by immunofluorescence using a monoclonal anti-NP (SV5-NP-a) and a monoclonal anti-P (SV5-P-e) antibodies, followed by a secondary anti-mouse IG Texas Red-conjugated antibody. In addition, cells were stained with DAPI for nuclear staining.

Figure 3.16

A) 1 day p.i.

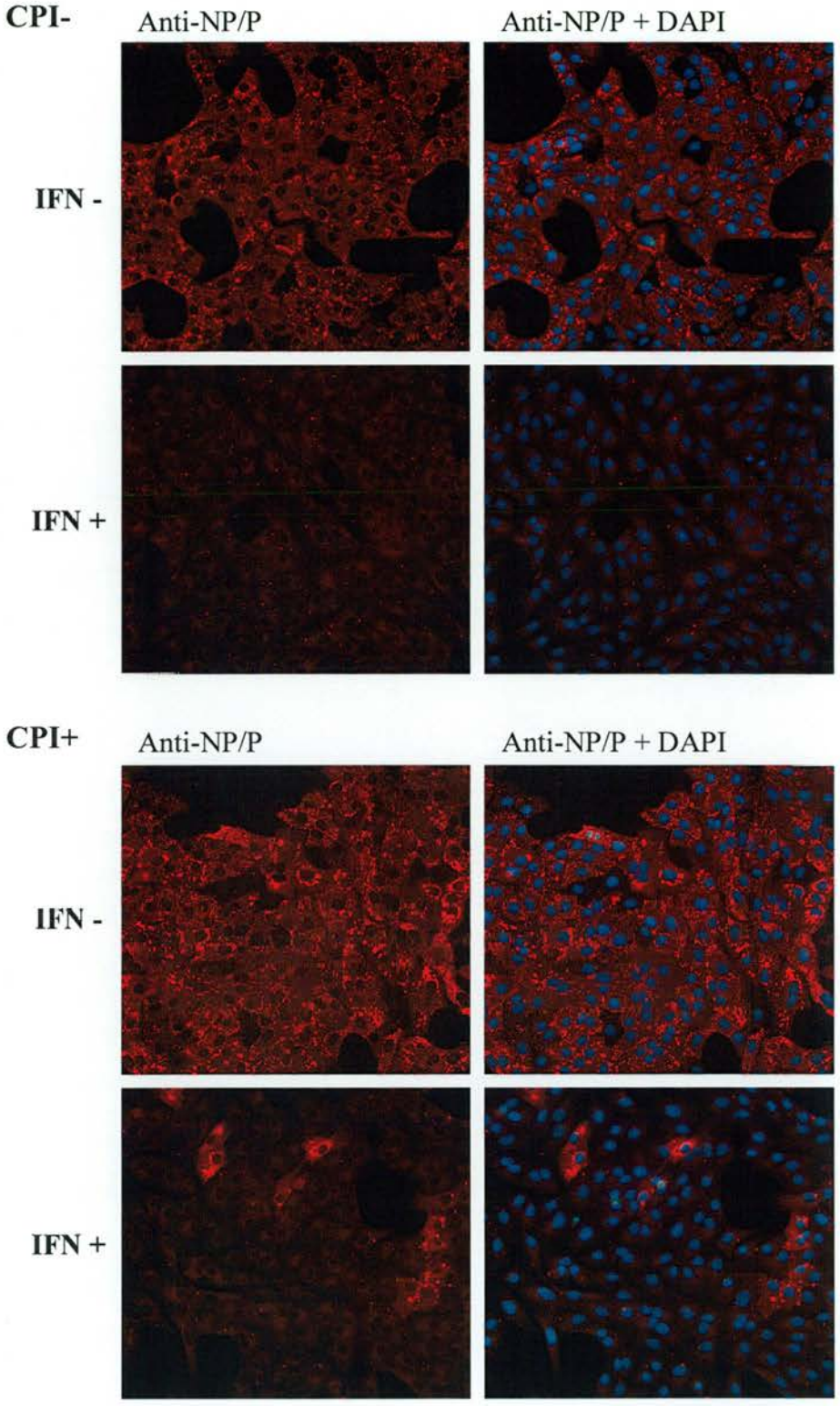


Figure 3.16

B) 3 days p.i.

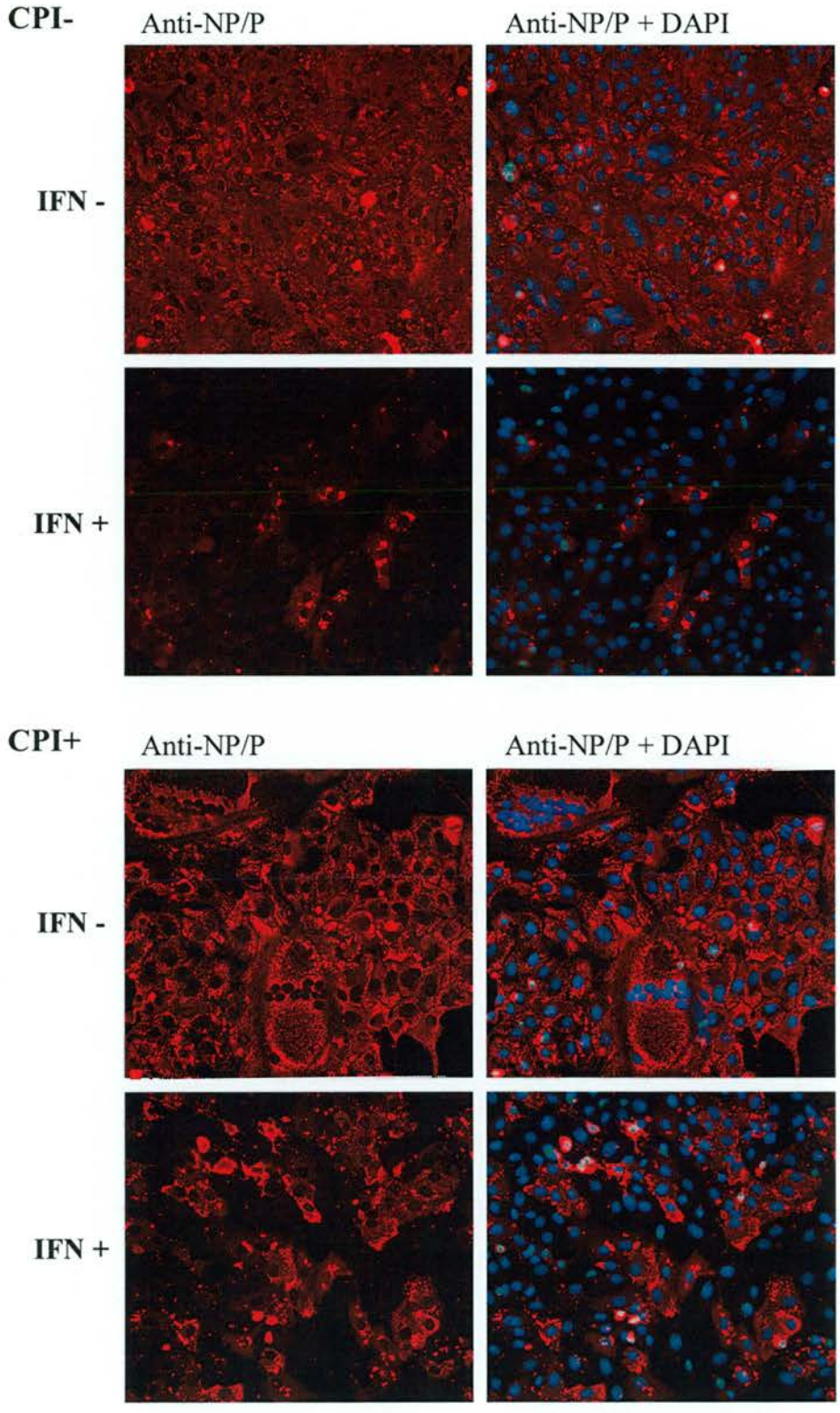
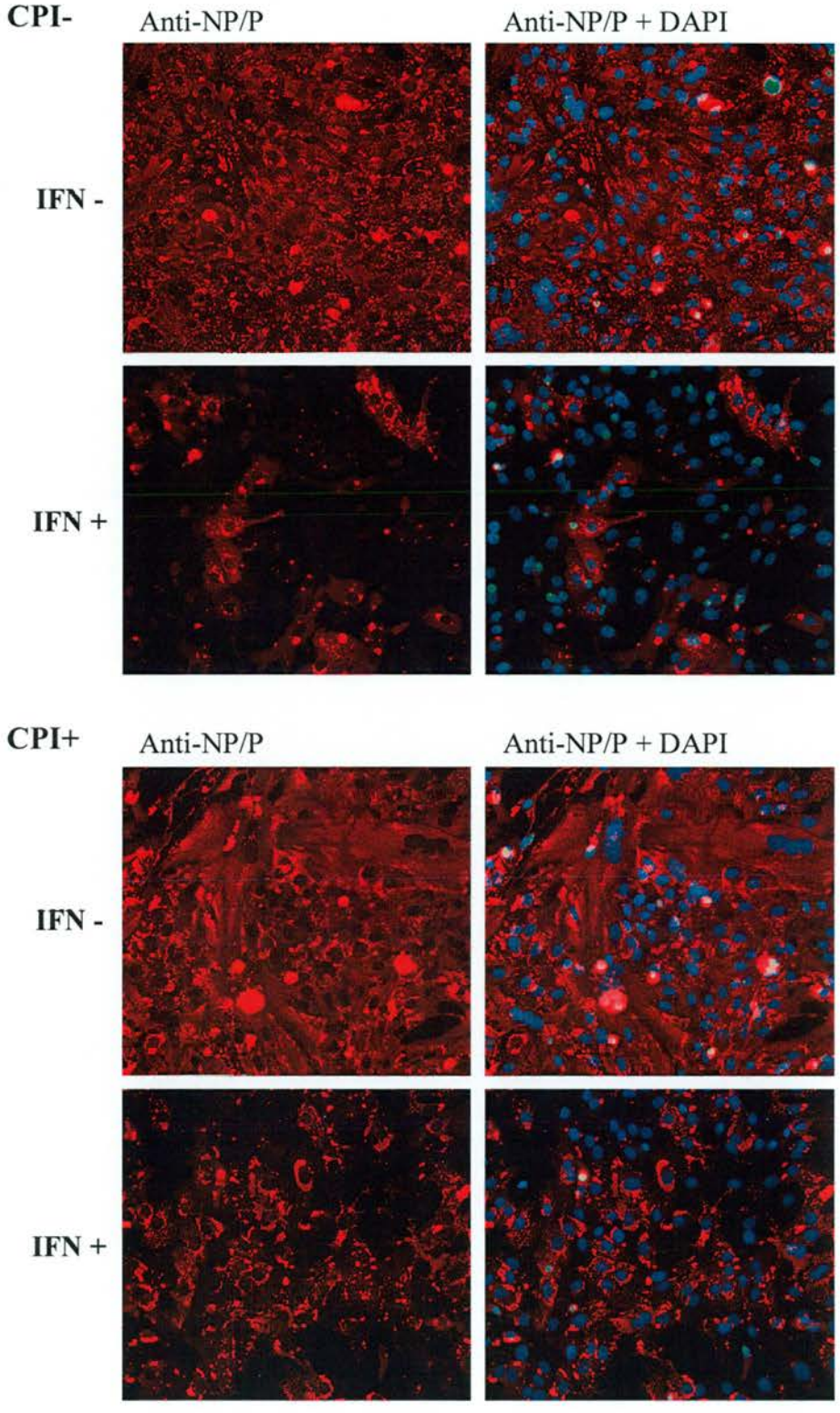


Figure 3.16

C) 6 days p.i.



3.2.2 Effect of interferon on the spread of CPI- and CPI+ viruses within a cell monolayer

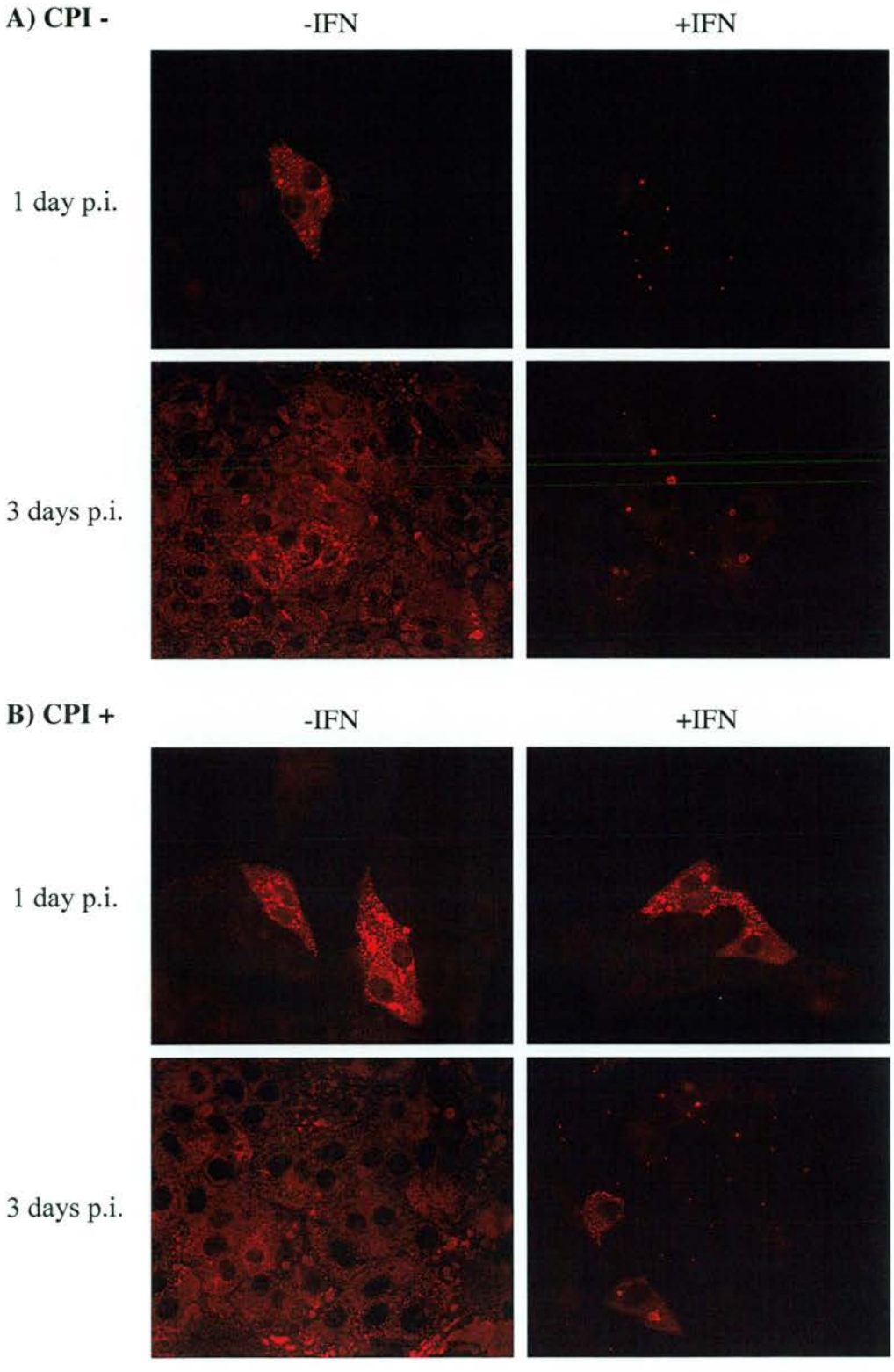
Given that pre-treatment of cells with IFN clearly induces an antiviral state within cells, which initially inhibits replication of both IFN-sensitive and IFN-resistant strains of SV5, it was of interest to examine the consequences of addition of IFN on the ability of these viruses to spread within a cell monolayer. Vero cells were infected with either CPI- or CPI+ at 0.01 pfu per cell and at 12 h p.i. IFN was added so that replication could be fully established in the absence of an IFN-induced response in the initially infected cells. The infected monolayers were examined at 1, 3 and 6 days p.i. by immunofluorescence, using monoclonal antibodies to the NP and P proteins. In the absence of IFN, both CPI- and CPI+ spread rapidly through the cell monolayer, and all the cells were infected by 3 days p.i. (Figure 3.17). However, the addition of IFN at 12 h p.i. induced an antiviral state in uninfected cells, which restricted the spread of both CPI- and CPI+ from cell-to-cell. Consequently, at 3 days p.i only a few cells were positive for virus antigen, and in those positive cells NP and P proteins were found in cytoplasmic inclusion bodies. In cell monolayers that had been infected with CPI-, although those inclusion bodies became larger with time p.i., there was no obvious spread of the virus from the initial foci of infection. Nevertheless, most cells that had been initially infected remained alive at 6 days p.i., and IFN did not clear cells from virus infection. Although IFN delayed the spread of the virus in monolayers infected with CPI+, nevertheless the cells could not maintain the IFN-induced antiviral state (once STAT1 had been degraded), thereby allowing the virus to replicate and to spread from the initial foci of infection, and support a productive infection (Figure 3.18).

In parallel experiments, the consequences of the addition of IFN on the pattern of virus protein synthesis were examined, following low m.o.i. of Vero cells with CPI+. The results showed that the addition of IFN had a dramatic effect on the rate of synthesis of CPI+ proteins (Figure 3.19). Thus, by 36 h p.i. significantly less protein synthesis had occurred in IFN treated cells compared to untreated cells. However, by 3 days p.i. in cells treated with IFN there were significant

Figure 3.17 Cell-to-cell spread of CPI- and CPI+ and analysis of NP and P proteins distribution in Vero cells infected with low m.o.i., in the presence or absence of IFN.

Vero cells were infected at 0.01 pfu/cell with CPI- (Panel A) or CPI+ (Panel B) and 12 h later, IFN-α was, or was not, added to the culture medium. Monolayers were fixed at 1 and 3 days p.i. and immunofluorescence analysis was performed with a mixture of monoclonal antibodies to NP (SV5-NP-a) and P (SV5-P-e) proteins, followed by a secondary anti-mouse IG Texas Red-conjugated antibody.

Figure 3.17



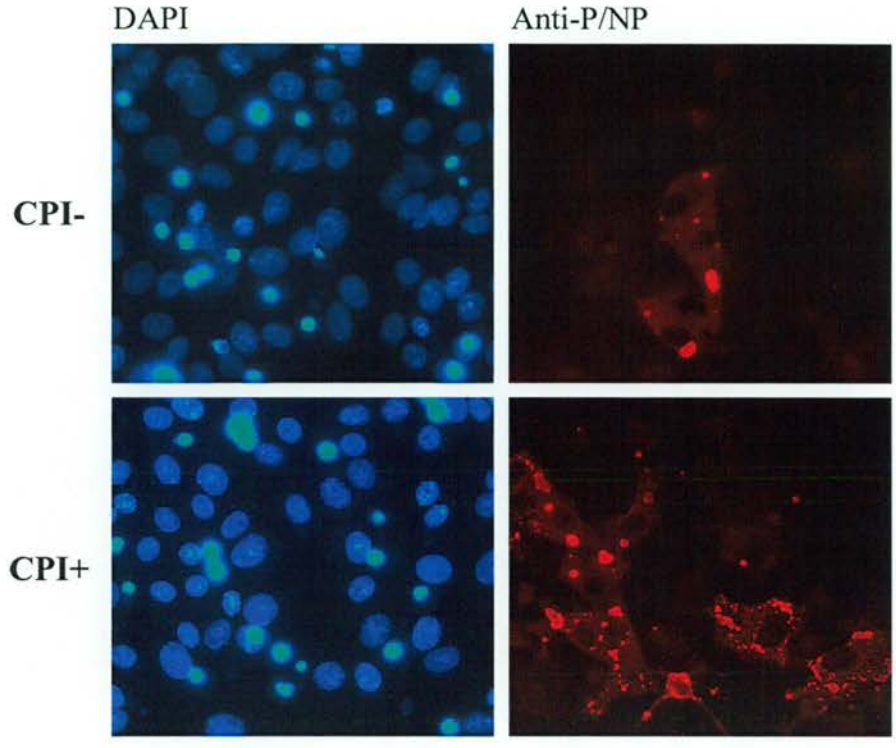


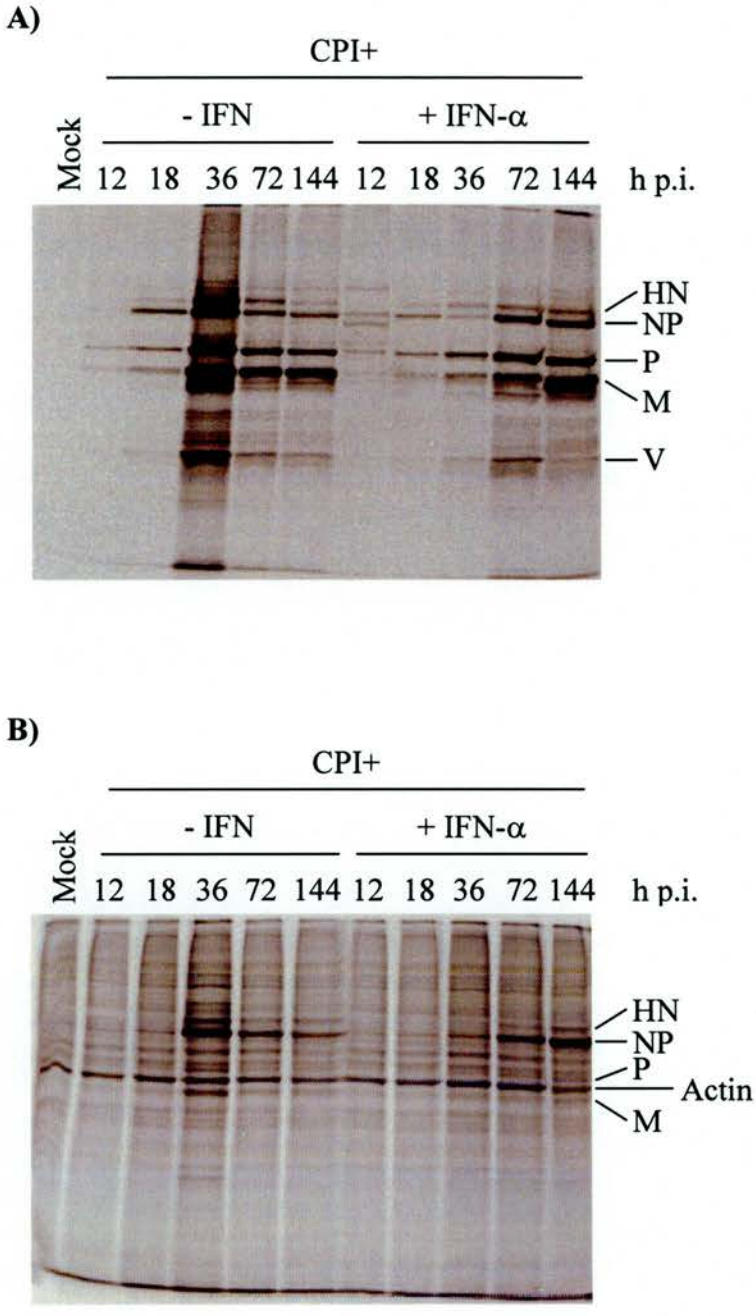
Figure 3.18 In the presence of IFN, CPI+ spreads within a monolayer of Vero cells more efficiently than CPI-.

Vero cells were infected with CPI- or CPI+ at a low m.o.i (approximately 0.01 pfu/cell) and incubated with IFN-α at 12 h p.i. The cells were fixed at 6 days 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.

Figure 3.19 Treatment with IFN following low m.o.i. of Vero cells delays CPI+ viral protein synthesis.

Vero cells were infected with CPI+ at an m.o.i. of 0.01 pfu/cell and treated, or not treated, with exogenous IFN-α at 12 h p.i. Cells were metabolically labelled with [^{35}S]-methionine for 1 hour at various times p.i. and NP, P, V, M and HN proteins immune precipitated using a mixture of appropriate mAbs. The precipitated proteins (Panel A) and the proteins in the total cell extracts (Panel B) were subsequently analysed by SDS-PAGE and visualized by phosphorimage analysis.

Figure 3.19


A)
CPI+
- IFN
+ IFN-α
Mock
12 18 36 72 144 12 18 36 72 144 h p.i.
HN
NP
P
M
V
B)
CPI+
- IFN
+ IFN-α
Mock
12 18 36 72 144 12 18 36 72 144 h p.i.
HN
NP
P
Actin
M

amounts of virus protein being made, although a change in the relative levels of the various virus proteins synthesised was observed. Thus, the ratio of M compared to NP and P being synthesised at 3 days p.i. in IFN-treated cells, was less than that observed at 36 h p.i. in untreated cells. Presumably, the reason for the observed increase with time in CPI+ protein synthesis in IFN treated cells was because in some of the cells STAT1 had been degraded and the cells had subsequently gone out of an IFN-induced antiviral state, thereby removing the restriction on virus protein synthesis. It is also of interest to note that at late times p.i. there was a significant reduction in the overall levels of CPI+ virus proteins synthesis in untreated cells, even though the majority of cells survived the infection (Panel B). It is possible that when virus proteins accumulate at late times p.i., the viral polymerase switches into a replicative mode, and further copies of genomic RNA are produced instead of synthesis of mRNAs. However, evidence to show this remains to be established.

3.2.3 Growth of CPI- and CPI+ in Vero cells in the absence and in the presence of interferon

Clear differences were observed in the distribution and expression levels of CPI- and CPI+ viral proteins in the presence and absence of IFN. Consequently, it was of relevance to compare the yield of CPI- and CPI+ at various times following infection of Vero cells that were pre-treated with IFN (for 14 hours prior to infection) or IFN post-treated (at 8 h p.i.) or left untreated. The amounts of infectious virus released into the medium were determined by plaque assay in Vero cells, the results of which are presented in Figure 3.20.

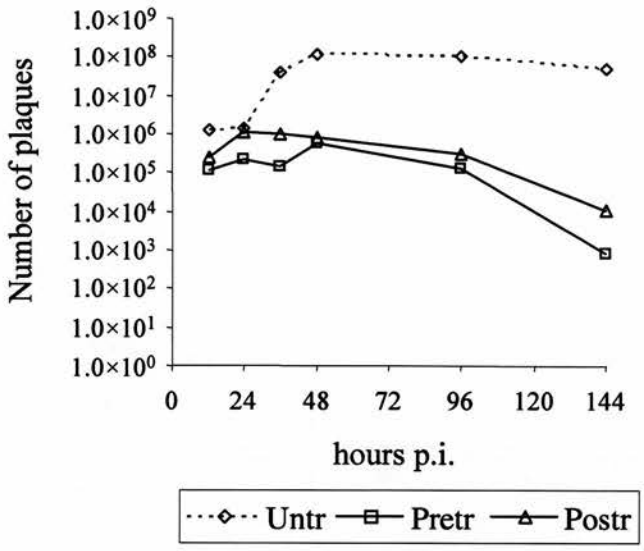
There was significant difference between CPI- and CPI+ yields. In the absence of IFN, CPI- grew to much higher titres than CPI+ until 48 h p.i., which is in agreement with the higher (than the wt SV5) growth kinetics of a recombinant SV5 mutant (rSV5-V/P-CPI-; engineered to encode the same V/P amino acid substitutions found in CPI- strain that contribute to the inability to block IFN signalling) at high m.o.i. (Wansley & Parks, 2002). After the highest release of

Figure 3.20 CPI- and CPI+ virus yields from Vero cells that were left untreated, IFN pre-treated or IFN post-treated.

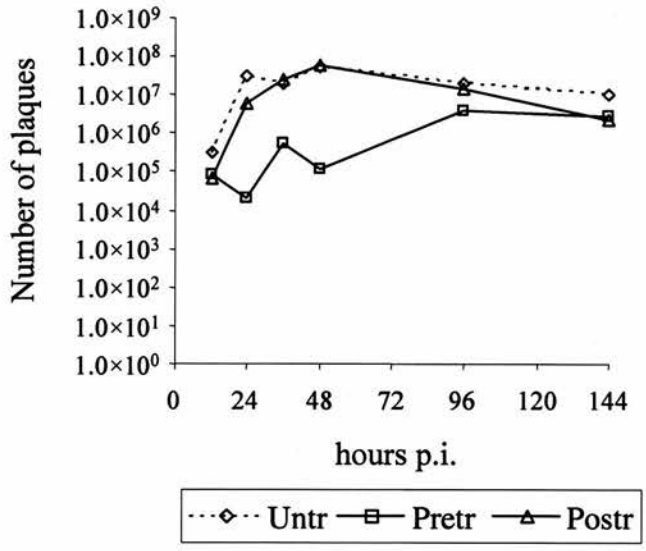
Vero cells that were or were not treated with IFN for 14 hours prior to infection (pre-treated) were infected with CPI- or CPI+ at high m.o.i. (50 pfu/cell). After 1 hour of incubation with virus inoculum, cells were incubated with fresh maintenance medium at 37 °C. A set of CPI- or CPI+ infected cells was treated with IFN at 12 hours p.i. (post-treated). The amount of infectious CPI- (A) and CPI+ (B) viruses released into the medium was determined at different times p.i. by plaque assays on Vero cells.

Figure 3.20

A) CPI-



B) CPI+



both CPI- and CPI+ viruses that was reached at 48 h p.i., the production of both viruses decreased. The decline on the amounts of infectious virus released at later times p.i. is possibly correlated with the decrease in the levels of protein synthesis observed at later times p.i. The presence of IFN drastically reduced CPI- virus yield, and the amounts of virus released from pre-treated cells was lower than from post-treated cells. Although IFN post-treatment of CPI+ infected cells did not significantly affect the virus yield, IFN pre-treatment considerably decreased the production of this IFN-resistant virus. These results, coupled to the protein synthesis levels of CPI- and CPI+ in the correspondent situations of infection and IFN treatment, suggest a link between the viral proteins expression levels and the amounts of virus released.

3.2.4 The spread of CPI- is enhanced in cells that permanently express the V protein of wild type SV5 (W3)

Given that the addition of exogenous IFN to Vero cells, which do not produce IFN but respond to IFN, affected the spread of both CPI- and CPI+ viruses, it was of relevance to examine what happens in cells which can produce and respond to IFN and in cells which have been engineered so that they cannot respond to IFN. Thus, a comparison of the spread of CPI- and CPI+ viruses was performed in naïve MRC-5 cells and MRC-5 cells that constitutively express the V protein of SV5 (MRC-5/SV5-V) and as a consequence no longer respond to IFN. MRC-5 and MRC-5/SV5-V cells were infected with CPI- or CPI+ at a low m.o.i. and 12 hours later the cells were treated with IFN or left untreated. Monolayers were fixed at 1, 3 and 6 days p.i. and examined following staining with the appropriate anti-NP and anti-P monoclonal antibodies.

The results from this analysis revealed that CPI- was rescued from the IFN-induced antiviral state action in cells that constitutively express the V protein of wt SV5 (Figure 3.21). In naïve MRC-5 cells, CPI- grew poorly in the absence of exogenous IFN, which is likely to be due to small amounts of IFN released by infected cells that induce an antiviral state in surrounding uninfected cells, and

Figure 3.21 Spread of CPI- and CPI+ in the presence of IFN is favoured in human cells that constitutively express the V protein of wild type SV5.

MRC5 human cells that constitutively express SV5 V (MRC5/SV5-V; Panels C and D) or not (MRC5; Panels A and B) were infected with CPI- or CPI+ at a low m.o.i. and 12 h later the culture medium was supplemented with IFN-α (+IFN) or left untreated (-IFN). The cells were fixed at 1, 3 and 6 days p.i. and immunostained using the monoclonal anti-NP (SV5-NP-a) and anti-P (SV5-P-e) antibodies, followed by a secondary anti-mouse IG Texas Red-conjugated antibody.

Figure 3.21

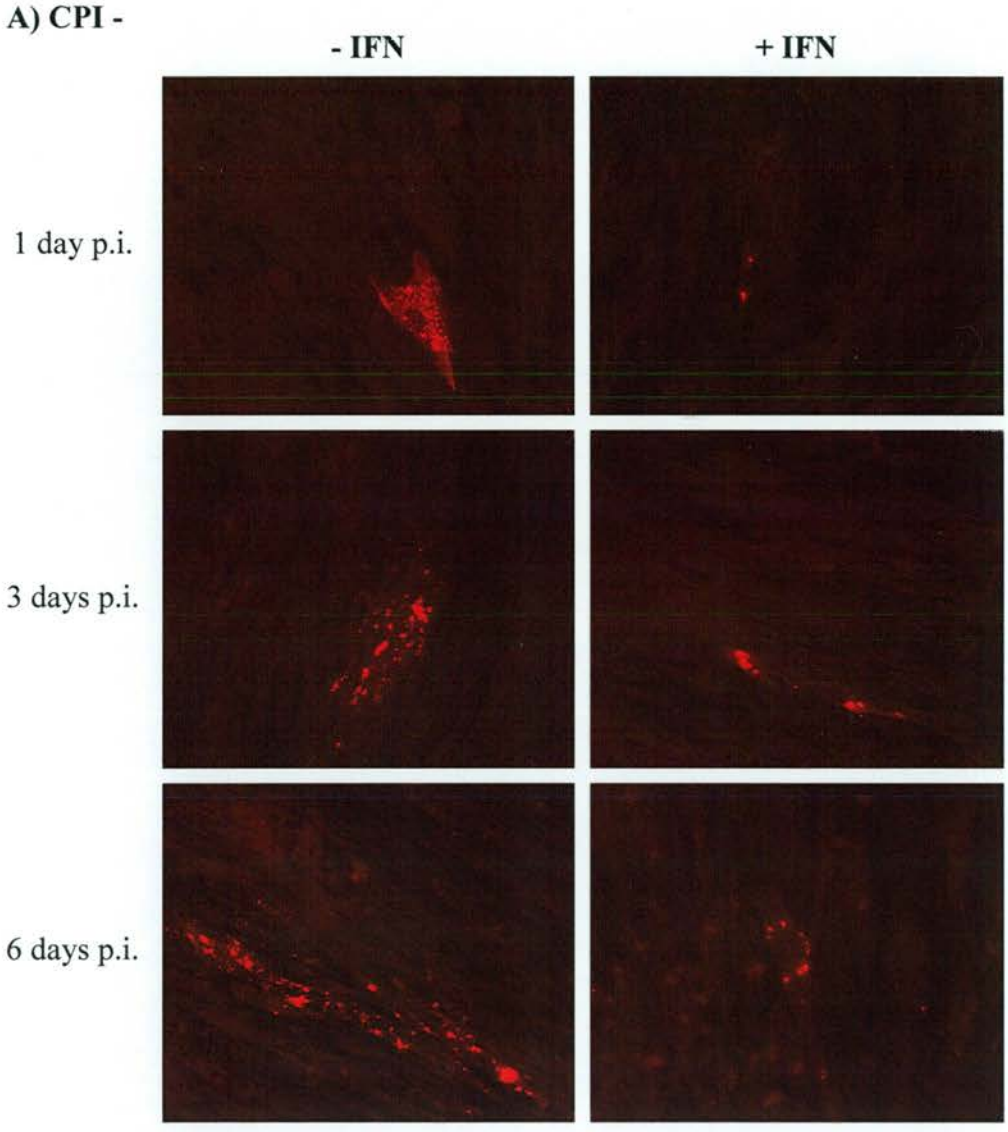


Figure 3.21

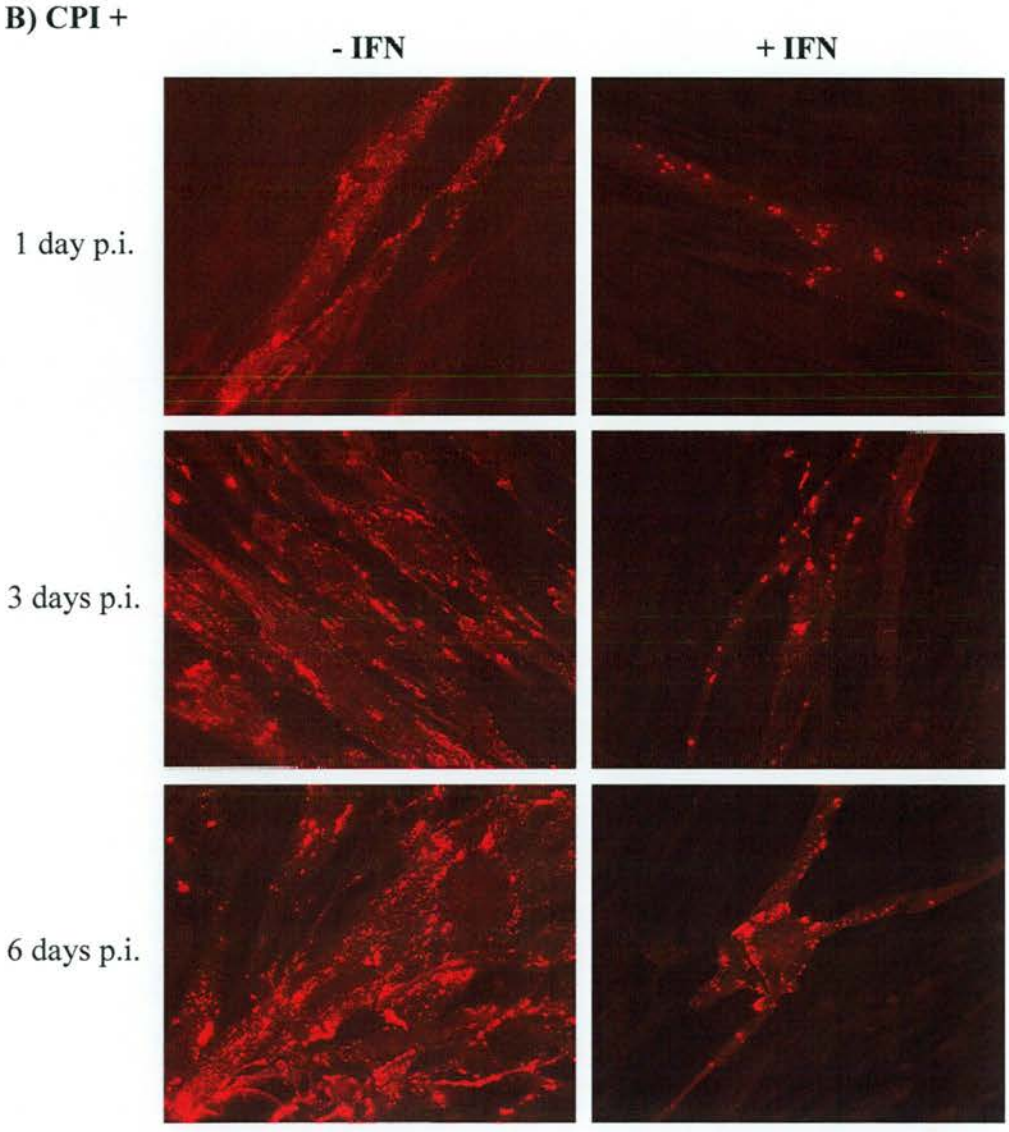


Figure 3.21

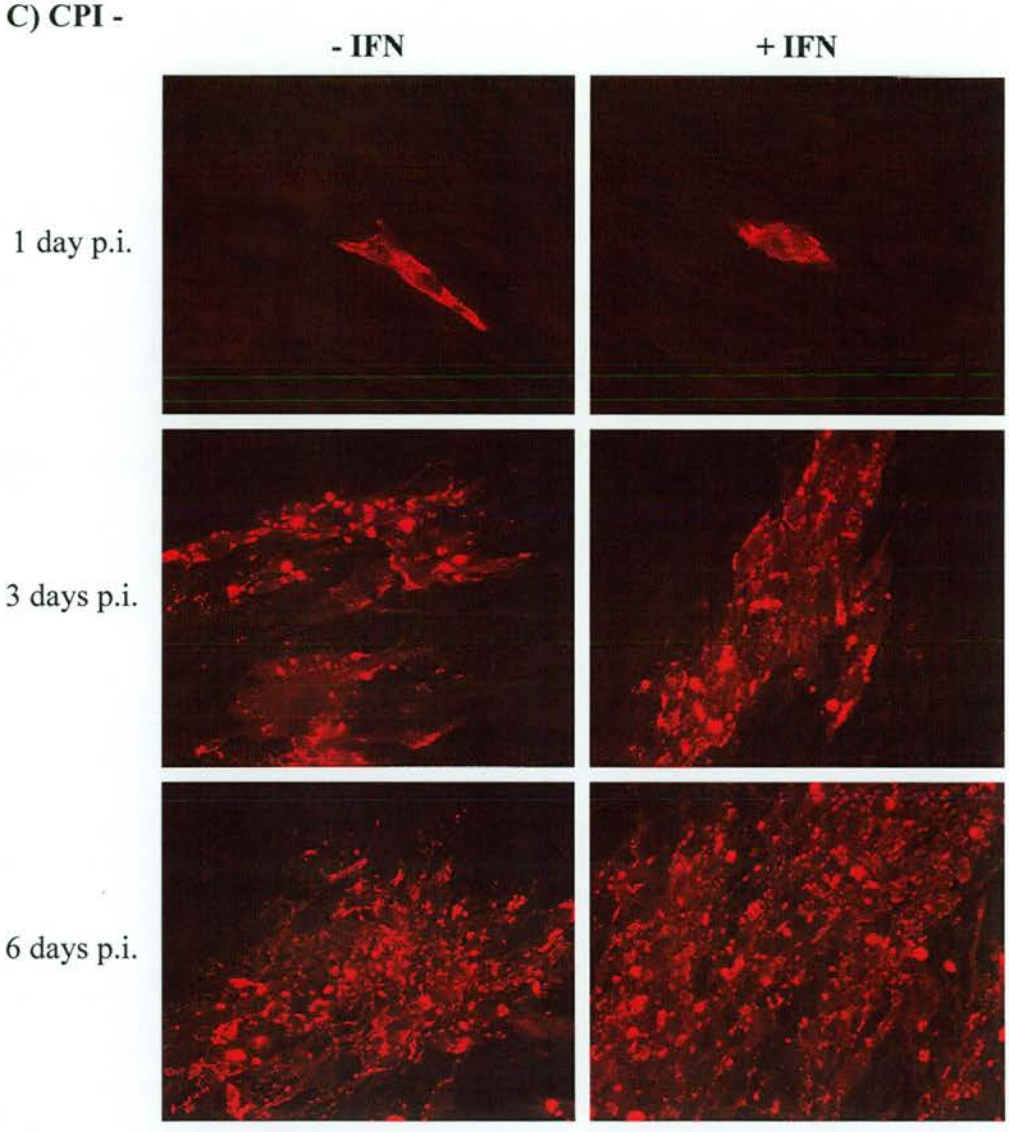
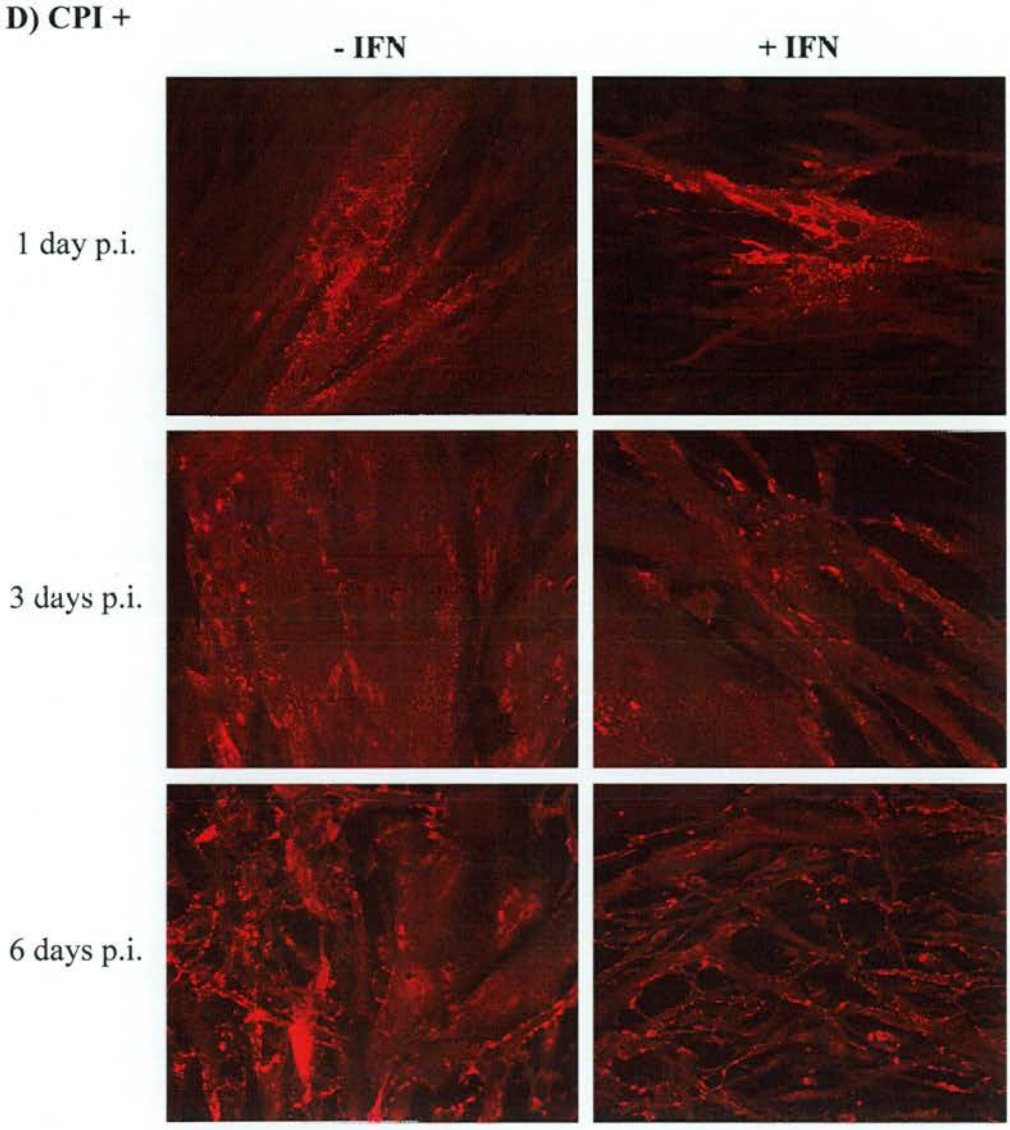


Figure 3.21



thus delays the spread of CPI- (Panel A). As expected, the addition of exogenous IFN to the naïve MRC-5 cells restricted the spread of both CPI- and CPI+ (Panels A and B, respectively). However, this effect was more evident in CPI- infected monolayers, in which most of the cells remained uninfected. Although, unlike CPI-, CPI+ spread through monolayers of MRC-5 cells in the absence of exogenous IFN, nevertheless CPI+ spread even more rapidly through MRC-5/SV5-V cells, presumably because MRC-5 cells also produce small amounts of IFN in response to CPI+. This analysis indicated that the expression of V protein in these cells prevented the establishment of an IFN antiviral state and thus rescued CPI- and CPI+ from the restrictions usually established by IFN, favouring the spread of these viruses within a monolayer of cells. Furthermore, this experiment also revealed that while treatment with IFN of naïve cells led to the accumulation of NP and P proteins in inclusion bodies, the distribution of these proteins was more diffuse in cells expressing the V protein.

3.2.5 Effect of interferon on MuV, hPIV2, hPIV3 and RSV

In light of the observed IFN induction of an antiviral state within uninfected cells, which significantly delay the spread of SV5, it was of interest to investigate next whether IFN similarly affected the replication of other paramyxoviruses. Vero cells were infected with MuV, hPIV2, RSV or hPIV3 at 0.01 pfu per cell, IFN was, or was not, added at 12 h p.i. and the spread of these viruses was monitored by immunofluorescence.

As shown in Figure 3.22, from these experiments it was clear that IFN delayed the spread of MuV and PIV2 (Panels A and B, respectively) in a similar manner to CPI+, in a way that the speed of virus spread was slowed down and the formation of inclusion bodies was induced. In striking contrast, IFN did not have such an obvious effect on the spread of RSV (Panel C) or PIV3 (Panel D). These latter results suggested that PIV3 and RSV replicate relatively efficiently in cells that are already in an IFN-induced antiviral state prior to infection.

This could either be because these viruses produce factors which inhibit the

Figure 3.22 Treatment with IFN has an effect on the spread of MuV and hPIV2, but not of RSV or hPIV3.

Vero cells were infected at an m.o.i. of 0.01 pfu/cell with MuV (A), hPIV2 (B), RSV (C) or hPIV3 (D), and 12 hours later IFN-α was, or was not, added to the culture medium. Monolayers were fixed at 1, 3 and 6 days p.i. and stained with appropriate monoclonal antibodies to detect the respective viruses, followed by a secondary anti-mouse IG Texas Red-conjugated antibody.

Figure 3.22

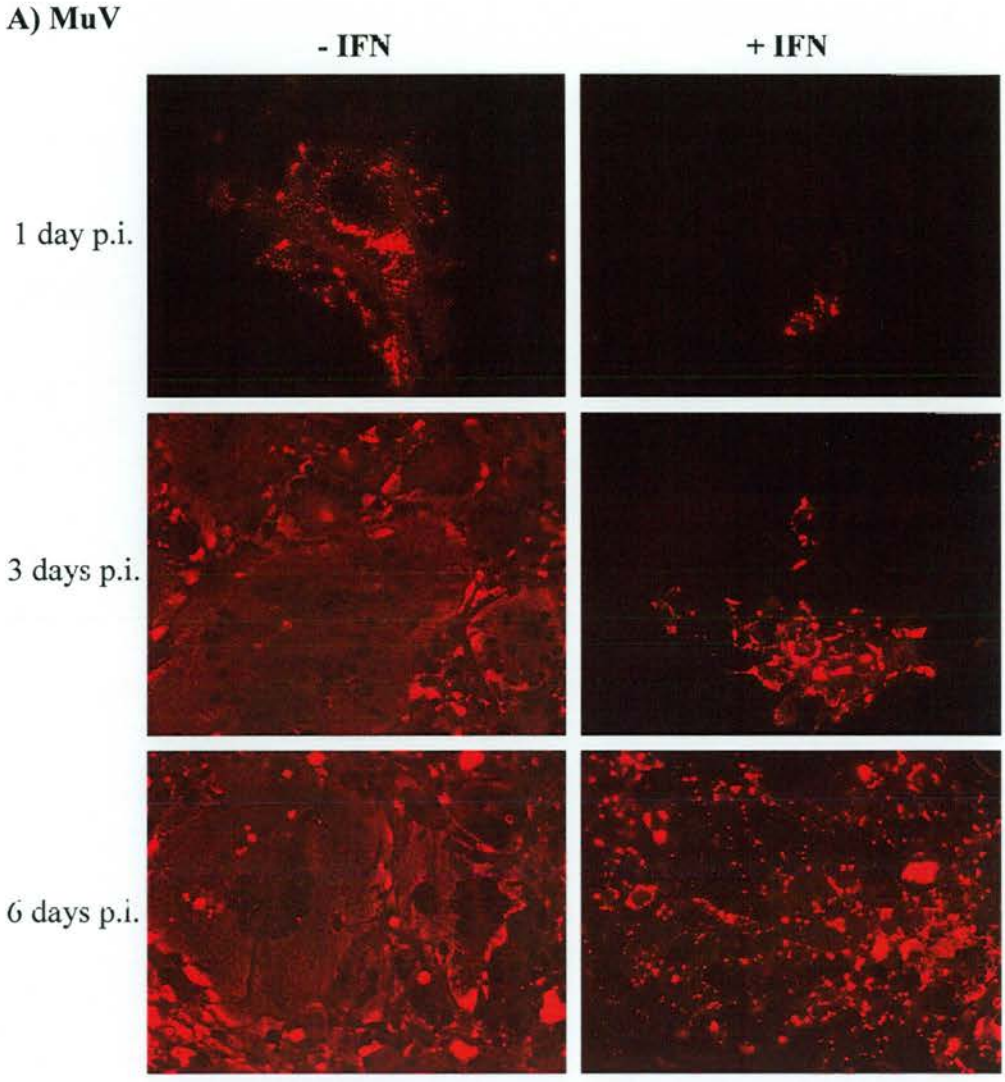


Figure 3.22

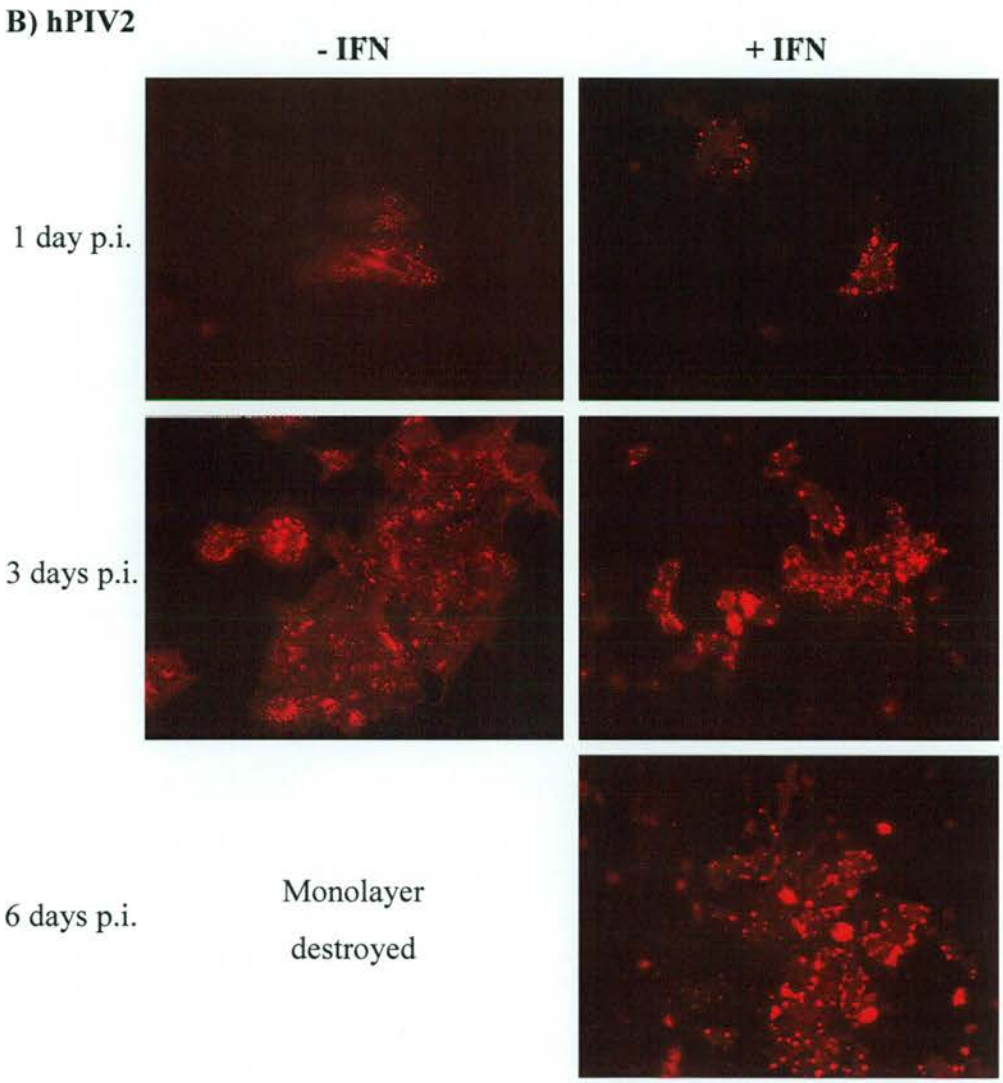

B) hPIV2
- IFN
+ IFN
1 day p.i.
3 days p.i.
6 days p.i.
Monolayer
destroyed

Figure 3.22

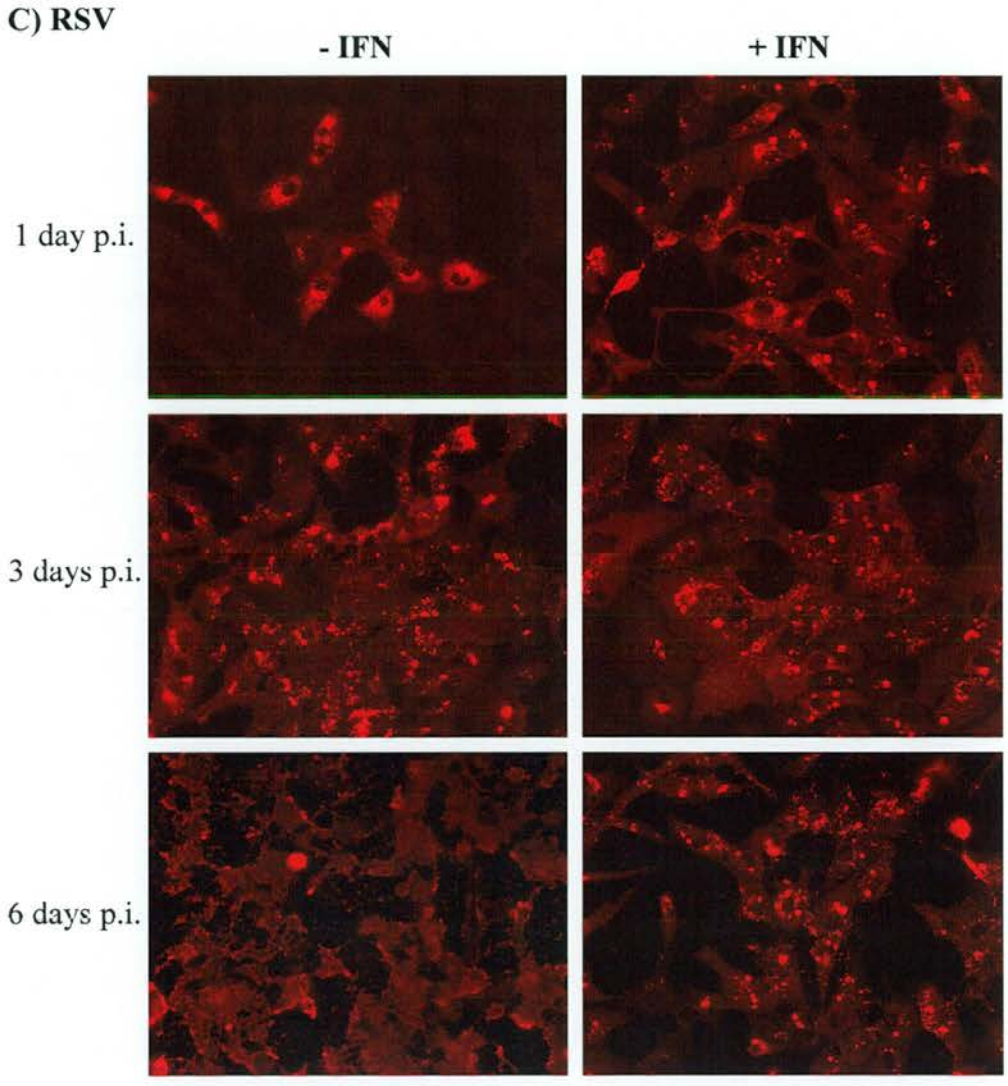
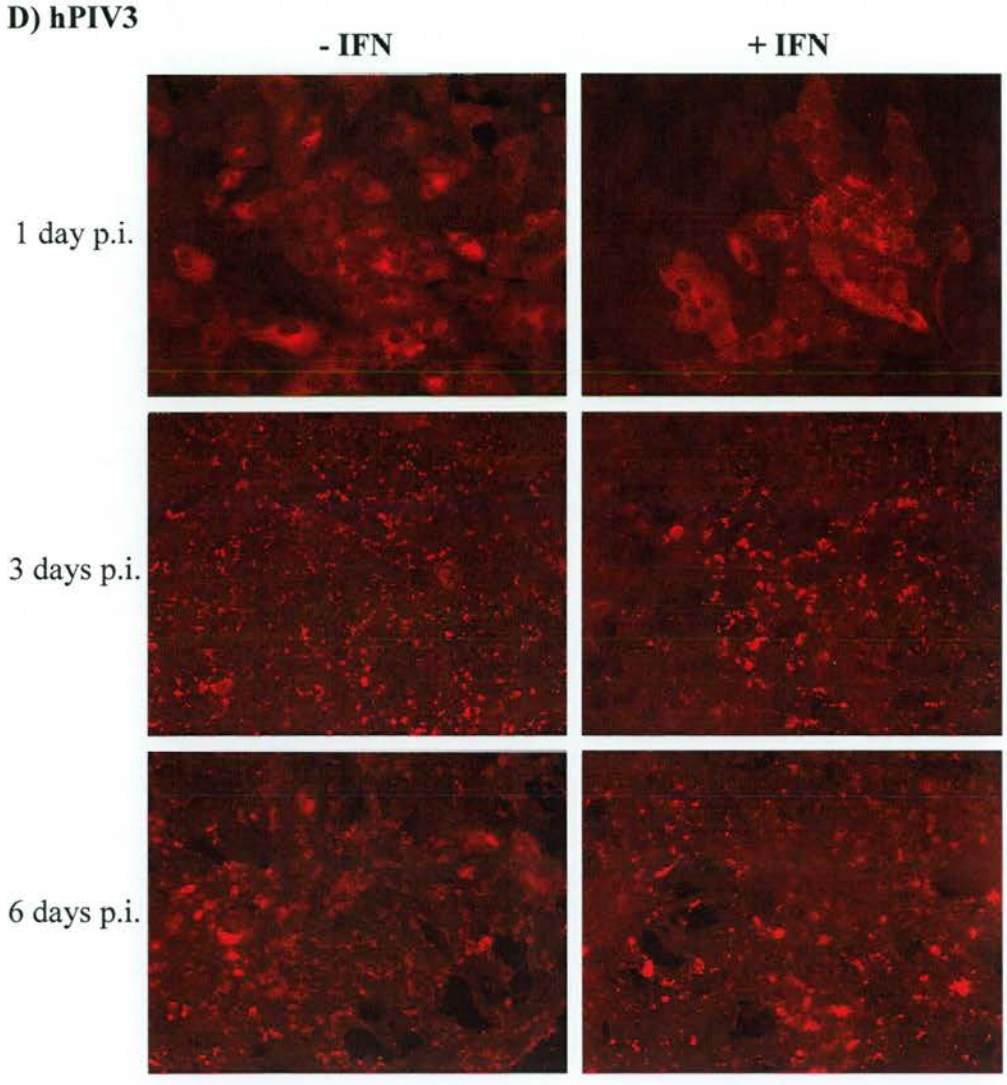


Figure 3.22



antiviral products induced by IFN that inhibit the replication of SV5, PIV2 and MuV, or because the replication of PIV3 and RSV are insensitive to these products.

If RSV does specifically inhibit IFN-induced enzymes, which interfered with the replication of SV5, then RSV may rescue CPI- from the antiviral effect of IFN. It was clear from the results of examination of a dual infection of Vero cells with CPI- and RSV, that SV5 inhibited the replication of RSV. The RSV protein synthesis occurring in cells infected with CPI- and RSV in simultaneous was strongly inhibited (Figure 3.23). In addition, immunofluorescence analysis of those monolayers showed that only a few cells of these monolayers were positive for RSV virus antigen, whilst CPI- productively infected the cells such that nearly all cells of the monolayer were positive for CPI- virus antigen, as seen by immunofluorescence analysis (Figure 3.24, Panel B). Thus, it was not possible to ascertain whether RSV could rescue CPI- from an IFN-induced antiviral state given that RSV replication was inhibited by CPI-. In cells infected with CPI- and RSV at the same time, the pattern of CPI- protein synthesis was altered in the presence of IFN likewise to the alteration observed in Vero cells infected with CPI- in the presence of IFN (Figure 3.23).

These observations suggest that SV5 inhibited the replication of RSV, which could either be because SV5 induces a cellular enzyme which can inhibit RSV but that is not normally activated by RSV alone or because the replication of SV5 directly inhibits RSV.

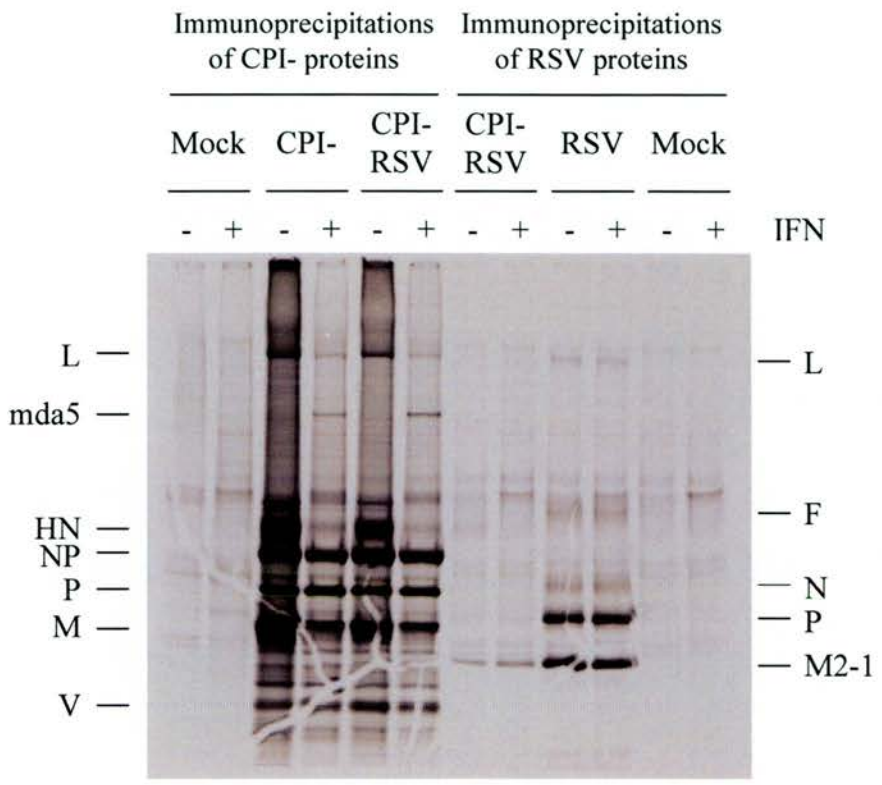


Figure 3.23 Effect of IFN on CPI- and RSV proteins synthesis in Vero cells infected with CPI- or RSV individually or infected with CPI- and RSV simultaneously.

Vero cells were infected with CPI- or RSV separately or with CPI- and RSV together at a high m.o.i. and 12 hours later IFN was, or was not, added to the culture media. The cells were metabolically labelled with [^{35}S]-methionine at 6 hours after IFN treatment. The RSV proteins were immunoprecipitated using a commercial mix of anti-RSV antibodies from either cells infected with RSV only and cells infected with both CPI- and RSV. In parallel, NP, P, V, M and HN proteins of CPI- were immunoprecipitated using a mixture of appropriate antibodies from each cells infected with CPI- alone and cells infected with RSV and CPI- together. The precipitated proteins were subsequently separated on a 4-12% PA gradient gel (Invitrogen) and visualized by phosphorimage analysis.

Figure 3.24 Efficiency of infection of Vero cells with CPI- or RSV or both CPI- and RSV in the absence and in the presence of IFN by immunofluorescence analysis.

The cell monolayers infected at high m.o.i. with CPI- or RSV separately (Panel A) or CPI- and RSV together (Panel B) and treated, or not treated, with IFN at 12 h p.i. were fixed at 6 hours after IFN treatment to control virus infection of each virus. Staining of RSV and CPI- was carried out with appropriate monoclonal or polyclonal antibodies to detect the respective viruses, followed by a secondary anti-mouse IG Texas Red-conjugated antibody or secondary anti-rabbit IG FITC-conjugated antibody.

Figure 3.24

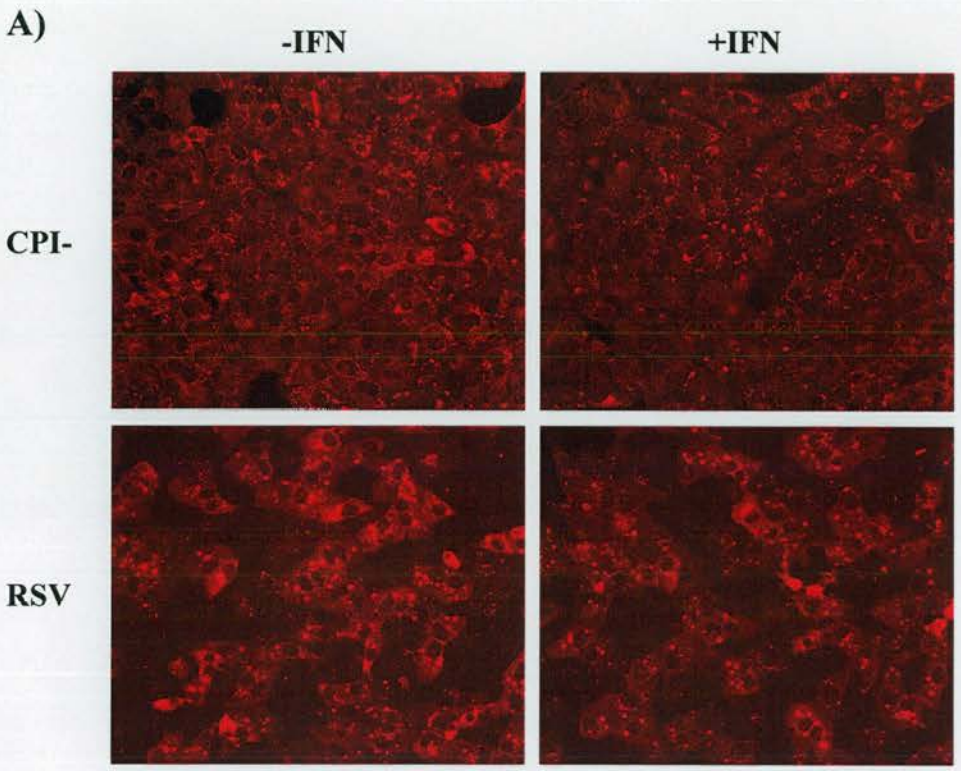
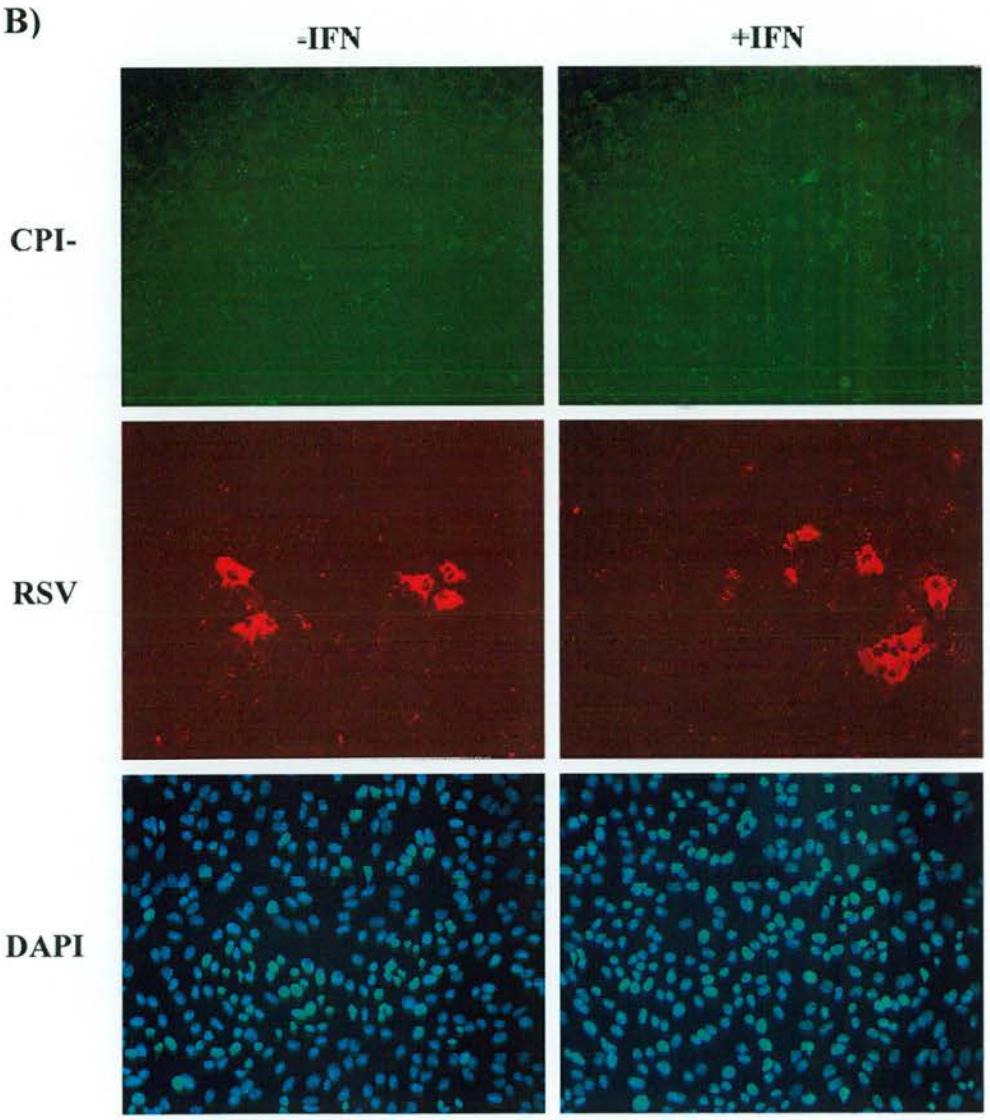


Figure 3.24



3.3 Interferon modulation of SV5 persistence

It has been previously suggested that SV5 may establish persistent infections by remaining inactive in cytoplasmic inclusion bodies, from which it may subsequently be reactivated (Chatziandreou *et al.*, 2002; Fearns *et al.*, 1994). Experiments in murine cells infected with SV5 revealed that, although no signs of virus replication were shown by immunofluorescence, nondefective virus could be recovered from those cells, which contained distinct cytoplasmic inclusion bodies (Fearns *et al.*, 1994). It was suggested that SV5 genomes may remain inactive in those cytoplasmic viral aggregates and once conditions become permissive for virus replication, SV5 can be reactivated. More recently, cytoplasmic inclusion bodies were also observed in canine cells infected with the IFN-sensitive SV5 strain (CPI-), and it was suggested that the occurrence of inclusion bodies might denote that the virus has entered a quiescent state (Chatziandreou, 2002).

To examine how IFN may modulate the establishment and maintenance of SV5 persistence, the Vero cell model was further developed. Vero cells were infected with CPI- in the presence of IFN (or absence as a control) and the cells were passaged for a long period. CPI- virus infection was monitored by immunofluorescence at various times, and the effect of removing IFN at various times from the cells was also examined.

Three different cultures of CPI- infected Vero cells were set up; those in which the cells were treated with IFN 14 hours prior to infection, those in which IFN was added at 8 h p.i. and untreated cultures. The infected monolayers were examined at 1, 3 and 6 days p.i. by immunostaining, using a mixture of monoclonal antibodies specific for the NP and P proteins (Figure 3.25). As shown in previous sections, in the absence of IFN, NP and P proteins were primarily distributed diffusely throughout the cytoplasm, although small inclusion bodies could also be detected (Panel A). In IFN-treated cells NP and P proteins were primarily in cytoplasmic inclusion bodies, which increased in size with time post IFN treatment (Panels B and C). However, although cells were infected with a high m.o.i., pre-treatment of cells with IFN appeared to completely prevent

Figure 3.25 Treatment with IFN affects the replication of CPI- but it does not clear cells from virus infection.

Vero cells were pre-treated with IFN (or left untreated) for 14 hours prior to infection with CPI- at a high m.o.i. (10 pfu/cell). Cultures of untreated infected cells were also treated with IFN at 8 h p.i. (or left untreated). The cells monolayers were fixed at 1, 3 and 6 days p.i. and immunofluorescence analysis was performed 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.

Figure 3.25

A) Untreated

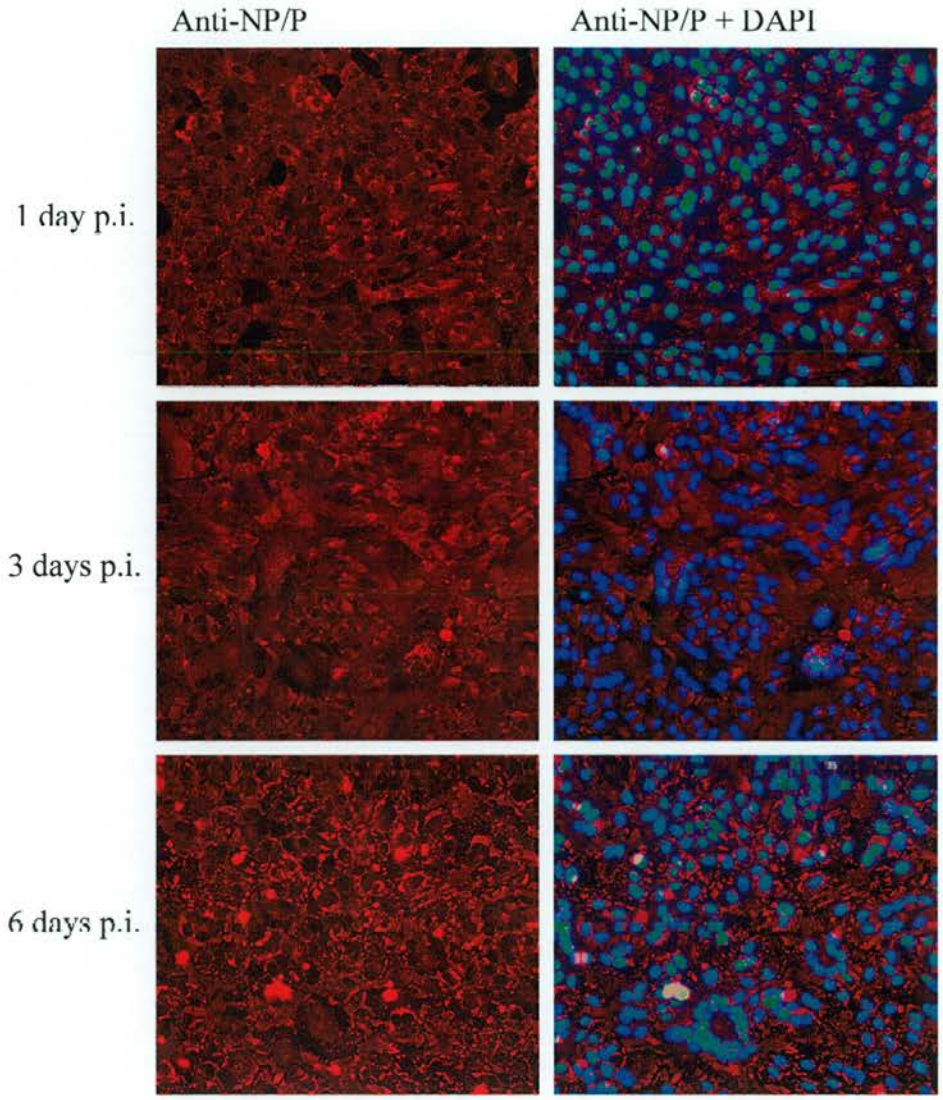


Figure 3.25

B) Pre-treated

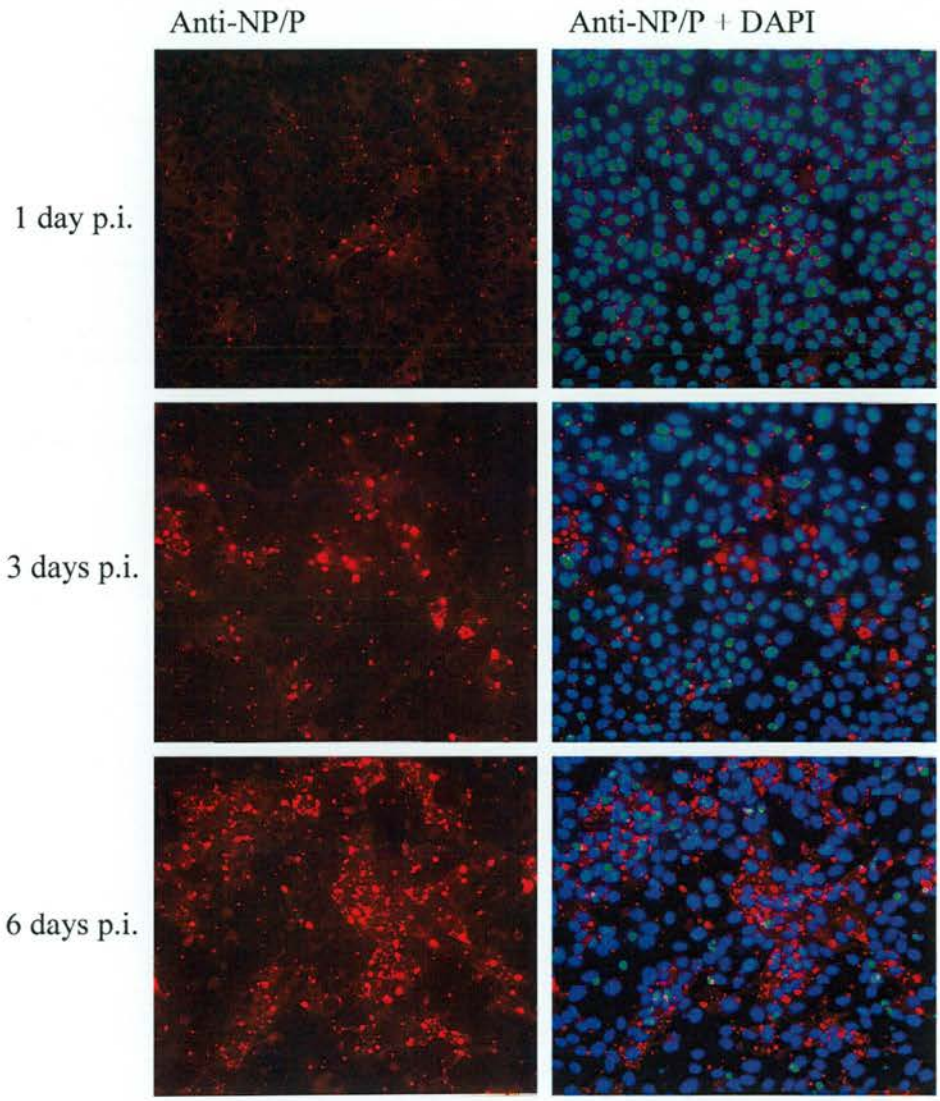
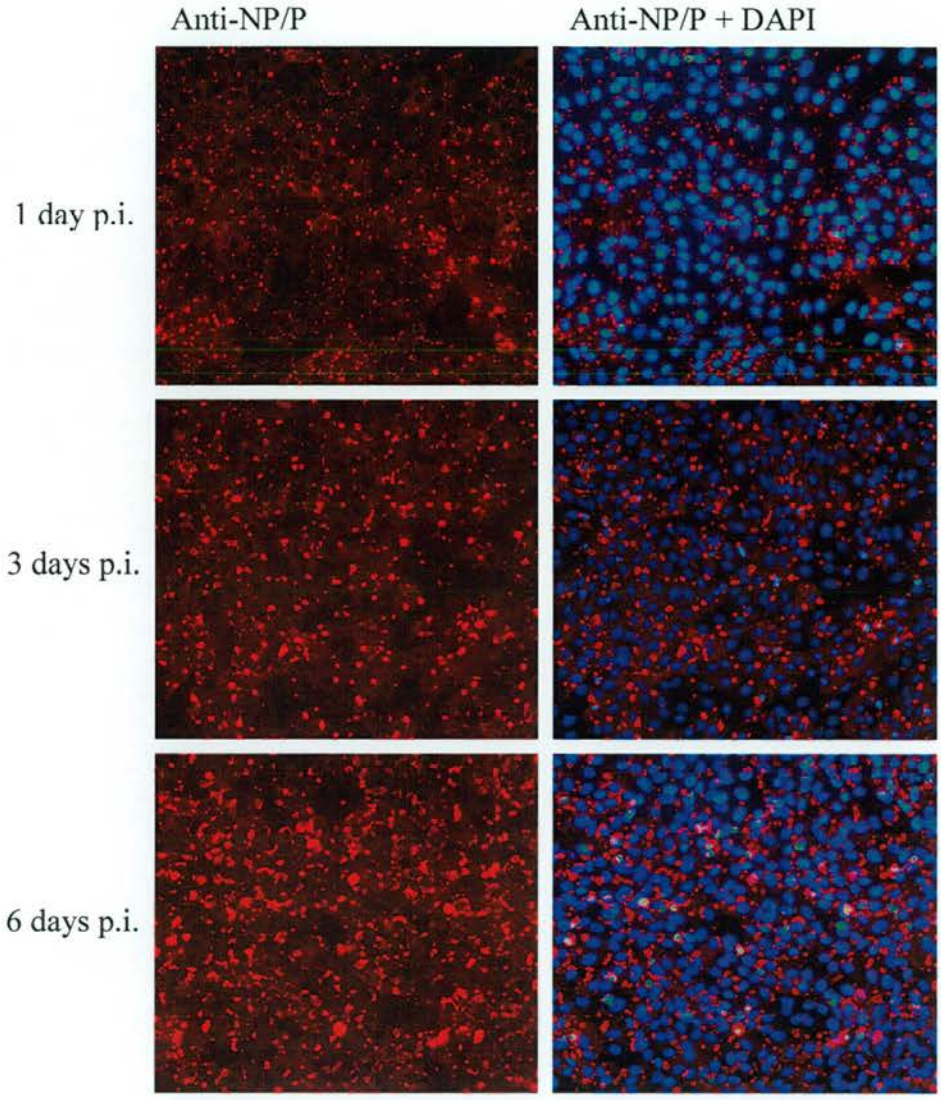


Figure 3.25

C) Post-treated



some cells from becoming infected as seen in monolayers of pre-treated cells, in which even at 6 days p.i. a small proportion of cells remained uninfected.

These cells were passaged twice in the presence of IFN (pre-treated and post-treated cells) or in the absence of IFN (untreated cells) and at 14 days p.i. the IFN was, or was not, withdrawn from the IFN-treated cells. At the same time IFN was, or was not, added to cultures of infected cells that had not previously been treated with IFN. As shown in Figure 3.26, the continued culture of cells in the presence of IFN for 20 days failed to clear the infection, and the cytoplasm of the infected cells contained viral inclusion bodies (Panels B and C). However, when IFN was withdrawn from the IFN-treated cells at 14 days p.i., the NP and P proteins redistributed such that by 17 days p.i. (3 days after removal of IFN from the cells) they were primarily diffuse, a pattern reminiscent of infected cells in the absence of IFN (Panels B and C). With regard to the cells that were initially infected with CPI- in the absence of IFN, it is of note that the majority of cells did not die from the infection and even at 20 days p.i. the NP and P proteins had a "normal" diffuse pattern. However, the addition of IFN at 14 days p.i. to those cell cultures results in the formation of cytoplasmic inclusion bodies.

CPI- infected Vero cells were passaged in the continuous presence of IFN for a period of up to six months. During this time the cells remained persistently infected, and immunofluorescence analysis showed the presence of large inclusion bodies in infected cells (Figure 3.27, Panel A). However, upon removal of IFN, the cells began to synthesise normal levels of virus proteins within a couple of days. The distribution of NP and P proteins in these cells was similar to that in Vero cells acutely infected with CPI- in the absence of IFN, and HN could be readily detected on the cell surface (Panel B).

Figure 3.26 IFN withdrawn from IFN treated-cells redistributes NP and P from cytoplasmic inclusion bodies into a cytoplasmic diffuse distribution, while the addition of IFN to untreated infected cells induces the formation of viral inclusion bodies.

IFN pre-treated and post-treated Vero cells infected with CPI- had been passaged in continuous presence of IFN, while untreated infected cells were passaged in the absence of IFN. At 14 days p.i. IFN was, or was not, removed from pre-treated and post-treated cells (Panels B and C, respectively) and was, or was not, added to untreated cells (Panel A). In the following 1, 3 and 6 days (i.e. 15, 17 and 20 days p.i.), cells were fixed and immunostained 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.

Figure 3.26

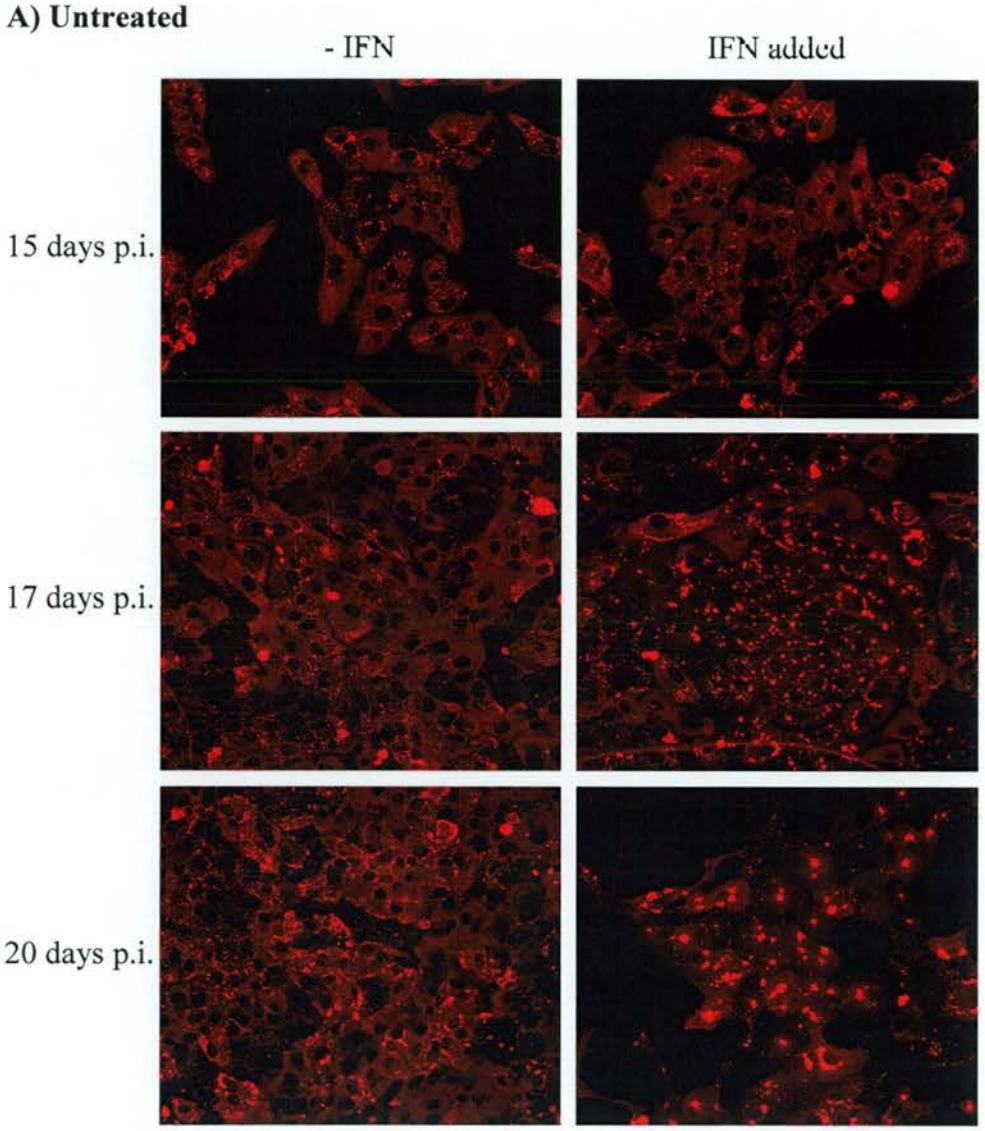


Figure 3.26

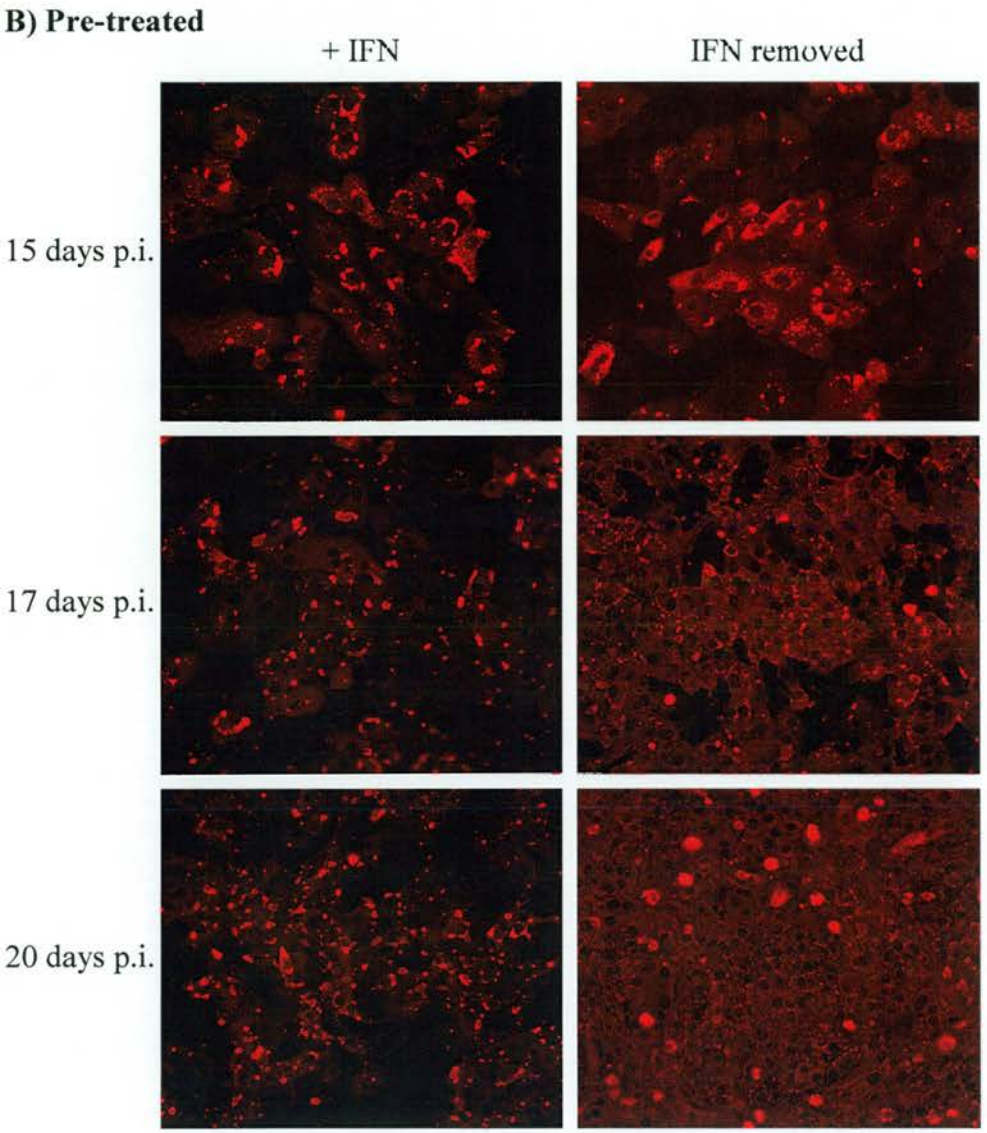
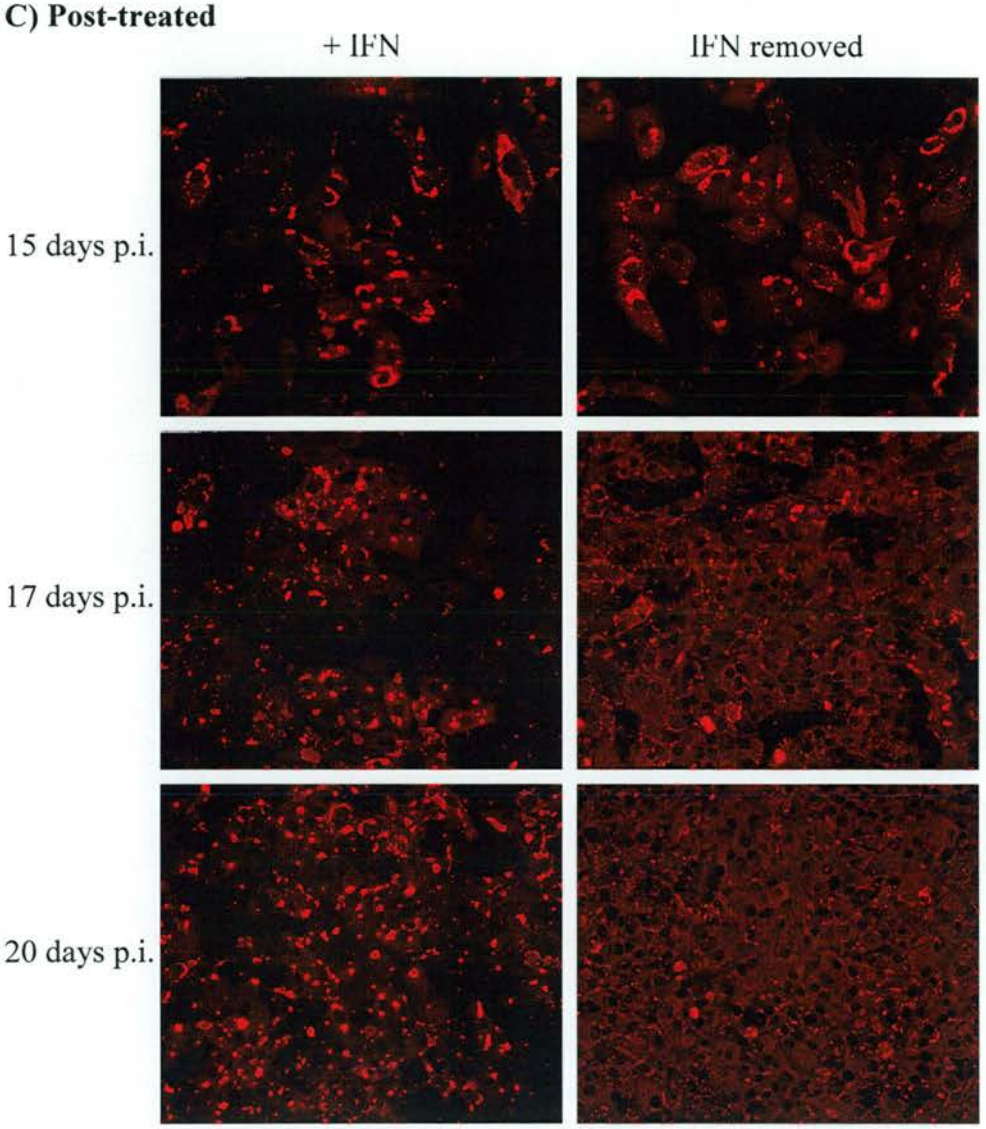


Figure 3.26

C) Post-treated



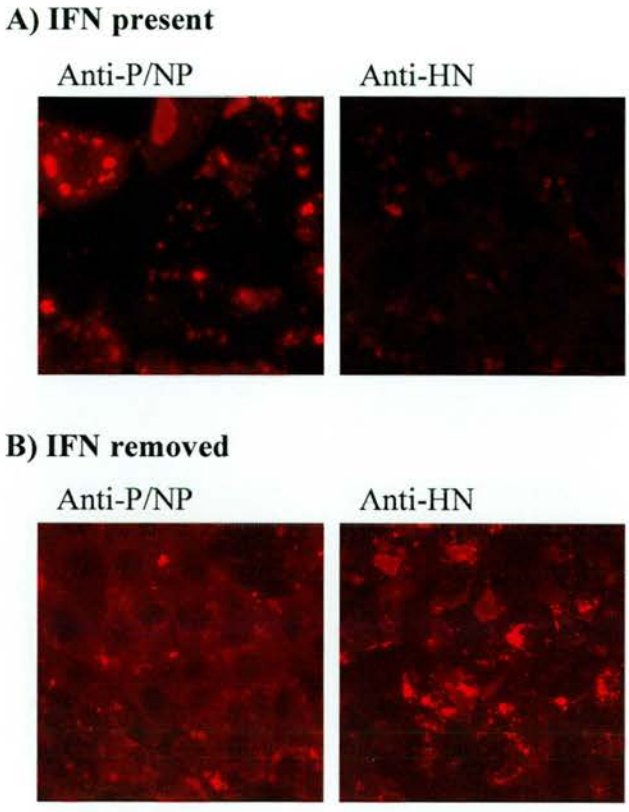


Figure 3.27 Immunofluorescence analysis showing that the NP and P proteins are located in cytoplasmic inclusion bodies in persistently infected Vero cells.

Distribution of NP and P proteins and expression levels of HN protein in cells persistently infected with CPI- that had been passaged in the continuous presence of IFN for up to 6 months (A). When IFN was removed at time of passage (B), NP and P proteins redistributed into a more diffuse pattern and the expression levels of HN increased.

3.4 Mechanism of interferon-induced inhibition of SV5 replication and induction of inclusion bodies

In the previous sections many of the effects of IFN on the replication cycle of SV5 were documented. In the long term our lab wishes to define at the molecular level how IFN mediates these effects. Clearly IFN must be inducing cellular proteins, which alter the pattern of SV5 transcription and protein synthesis, as well as inducing the formation of inclusion bodies.

3.4.1 The role of MxA

Previous work has documented that MxA, which is rapidly induced by IFN-α/β in response to acute viral infections, plays an important role in the antiviral response and can inhibit the replication of a number of RNA viruses. Furthermore, in a study with La Crosse virus it was shown that MxA sequesters the viral nucleocapsid protein to form an assembly of elongated tubular structures resulting in cytoplasmic inclusions, which is likely to account for the block in viral genome replication (Haller & Kochs, 2002). Therefore, the effects of MxA on the replication cycle of SV5, as well as whether MxA induced the formation of inclusion bodies, were initially examined.

3.4.1.1 Effect of MxA on CPI- virus yield and plaque formation

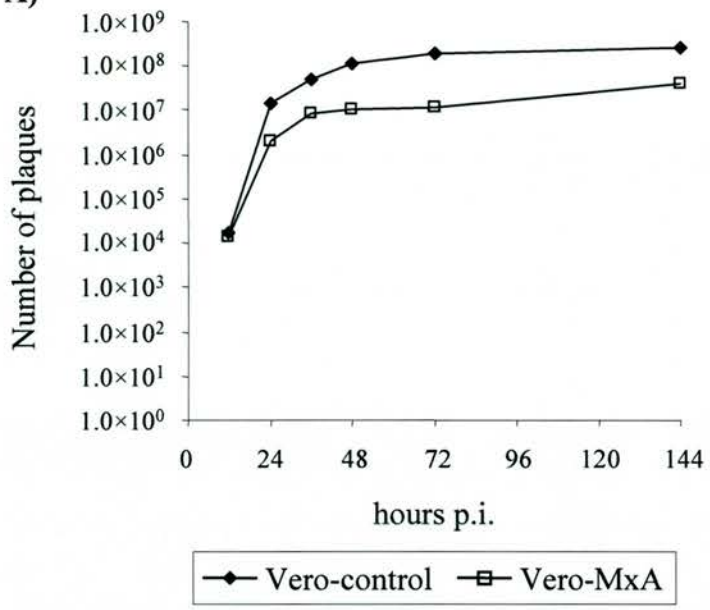
In the first series of experiments a growth cycle of SV5 was performed in Vero cells that do, or do not, constitutively express the MxA protein. Naïve Vero and MxA-expressing Vero cells were infected with CPI- at a high m.o.i. and the amounts of infectious virus released into the medium following infection were determined at various times p.i. by plaque assay in Vero cells. The results obtained showed that CPI- virus titres were reduced in MxA-expressing cells, indicating an effect of MxA on the replication of CPI- (Figure 3.28, Panel A). The cytopathic effect of CPI- was also examined in MxA-expressing and non-

Figure 3.28 MxA affects CPI- virus growth and plaque formation.

Naïve Vero and MxA-expressing Vero cells in 25 cm^2 flasks were infected with CPI- at high m.o.i. (50 pfu/cell) for 1 hour, at which time the inoculum was replaced with fresh maintenance medium and cells incubated at 37 °C. At various times p.i. an aliquot of medium was taken and the amount of infectious virus released into the medium was estimated by plaque assay on Vero cells (A). A comparison of size and number of the plaques formed by CPI- was carried out in naïve Vero cells and MxA-expressing cells (B).

Figure 3.28

A)



B)

Vero-control

(154×10^8 plaques)

Vero-MxA

(49×10^8 plaques)

MxA-expressing Vero cells. The number of plaques formed and the size of these plaques were decreased in MxA-expressing cells (Panel B).

3.4.1.2 Effect of MxA on CPI- virus protein synthesis

Given the effect of MxA on the growth cycle of CPI-, it was next of interest to ascertain whether MxA was responsible for the IFN-induced alteration in the pattern of SV5 protein synthesis. Naïve Vero cells and MxA-expressing Vero cells were infected with CPI- at a high m.o.i. to ensure that all cells were infected, and 12 hours later IFN was, or was not, added to the culture medium (as a control for the effect of IFN on the pattern of protein synthesis). At different times after IFN treatment, the cells were metabolically labelled with [^{35}S]-methionine and the relative levels of virus proteins were estimated both by examining the relative levels of proteins in total cell extracts and by immunoprecipitation (Figure 3.29; Panels A and B, respectively). As expected, in naïve Vero cells the relative expression levels of NP, P and V proteins were not significantly different in IFN treated compared to non-treated cells, while there was an obvious reduction in the relative levels of the M, HN and L proteins. If MxA were the IFN induced antiviral protein whole responsible for this alteration on protein synthesis pattern, it would be expected to see the same decline in M protein expression in MxA-expressing infected cells even in the absence of IFN. However, this was not the case, and the levels of M expressed in Vero-MxA cells in the absence of IFN were very similar to those observed in naïve Vero cells in the absence of IFN. Yet, Vero-MxA cells were responding to IFN as addition of IFN affected the synthesis of M protein in the same way as in naïve Vero cells treated with IFN. Therefore, the alteration in the relative levels of viral proteins seems to be caused by some other IFN-induced factor, but not MxA protein.

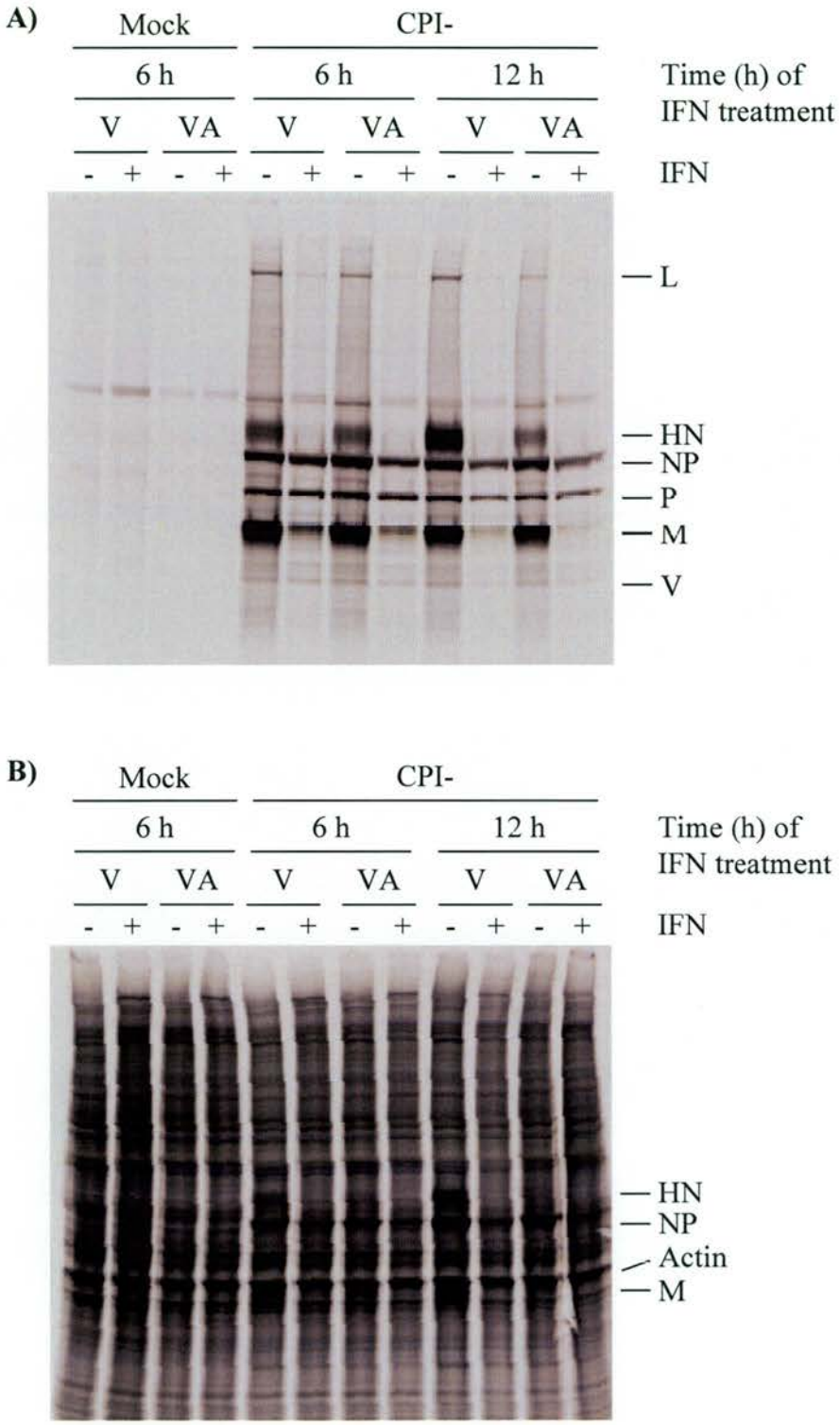
3.4.1.3 MxA does not co-localize with SV5 viral inclusion bodies but has an effect on their formation

As described in Chapter 1, various studies involving the role of MxA on RNA virus replication showed that the viral target recognition by MxA is virus and cell

Figure 3.29 MxA is not responsible for the observed decrease on the relative expression levels of CPI- protein synthesis in the presence of IFN.

Naïve Vero cells (V) and MxA-expressing Vero cells (VA) were infected with CPI- at an m.o.i. of 50 pfu/cell, and at 12 h p.i. the cells were, or were not, treated with exogenous IFN. At 6 and 12 hours later cells were metabolically labelled with [^{35}S]-methionine for 1 hour, and lysed at the end of the radioisotope incubation period. The protein content of the total cell extract was analysed by SDS-PAGE (Panel B). Virus proteins were precipitated from soluble antigen extracts of these cells with a pool of monoclonal antibodies to the NP, P, V, M, and HN proteins. The precipitated proteins from CPI- infected cells were subsequently separated on SDS-PAGE and visualized by phosphorimage analysis (Panel A).

Figure 3.29


A)
Mock
CPI-
6 h
6 h
12 h
Time (h) of IFN treatment
V
VA
V
VA
V
VA
- + - + - + - + - + - +
IFN
L
HN
NP
P
M
V
B)
Mock
CPI-
6 h
6 h
12 h
Time (h) of IFN treatment
V
VA
V
VA
V
VA
- + - + - + - + - + - +
IFN
HN
NP
Actin
M

type specific, and thus MxA inhibit a diverse array of viruses at different phases of their life cycle depending on the virus and the type of the host cell. It is thus possible that MxA does not have an effect on SV5 by inhibiting transcription or mRNA translation, but it does have an effect in other stage of SV5 replication cycle, such as transportation of nucleocapsids, which would be reflected in an altered distribution of NP and P proteins. Thus, whether the MxA protein was colocalized with the SV5 inclusion bodies or involved in their formation was next examined.

Naïve Vero cells and MxA-expressing Vero cells were infected with CPI- and were, or were not, treated with IFN at 12 h p.i. At 1 and 3 days p.i. the cells were stained for the cellular MxA protein and the viral NP and P proteins. As shown in Figure 3.30, MxA does not directly induce inclusion body formation as at 1 day p.i. the diffuse distribution of NP and P proteins observed in MxA-expressing Vero cells was similar to that observed in naïve infected cells (Panel A). However, following the addition of IFN to either the naïve or the MxA-expressing cells, NP and P proteins redistributed into distinct cytoplasmic inclusion bodies (Panel B). Furthermore, MxA did not co-localize with the viral inclusion bodies and MxA was diffusely distributed throughout the cytoplasm both in cells that had or had not been treated with IFN. However, by 3 days p.i., there was a clear difference in the distribution of NP and P proteins in MxA-expressing cells in comparison with non-MxA-expressing cells; there was more NP and P in inclusion bodies when MxA was being expressed, yet there was no colocalization of P protein with MxA protein (Panel C). In the presence of IFN, both naïve and MxA-expressing cells infected with CPI- contained cytoplasmic inclusion bodies (Panel D), but MxA was excluded from the viral inclusion bodies.

To confirm that MxA was not directly involved in the induction of viral inclusion bodies, the effect of IFN treatment on the distribution of NP and P proteins was examined in human Hep-2 cells, which do not express endogenous MxA protein (Landis *et al.*, 1998), as confirmed by imunoblot analysis (Figure 3.31, Panel A). If MxA was the IFN induced factor responsible for the effect on the distribution of NP and P viral proteins in the presence of IFN, then in Hep-2 cells a normal

Figure 3.30 MxA does not colocalize with SV5 inclusion bodies, but it appears to induce their formation at later times p.i.

Vero naïve cells and MxA- expressing Vero cells were infected with CPI- at high m.o.i. (50 pfu/cell) and at 12 h p.i. the medium was (IFN+), or was not (IFN-), supplemented with IFN. The infected monolayers were fixed and examined at 1 day p.i. (Panels A and B are untreated and IFN treated, respectively) and 3 days p.i. (Panels C and D are untreated and IFN treated, respectively) by double-staining immunofluorescence. Cells were first stained with a polyclonal antibody specific for MxA, followed by a secondary anti-rabbit IG FITC-conjugated antibody. Subsequently, the NP and P proteins were detected with a mixture of monoclonal anti-NP (SV5-NP-a) and anti-P (SV5-P-e) antibodies, followed by a secondary anti-mouse IG Texas Red-conjugated antibody.

Figure 3.30

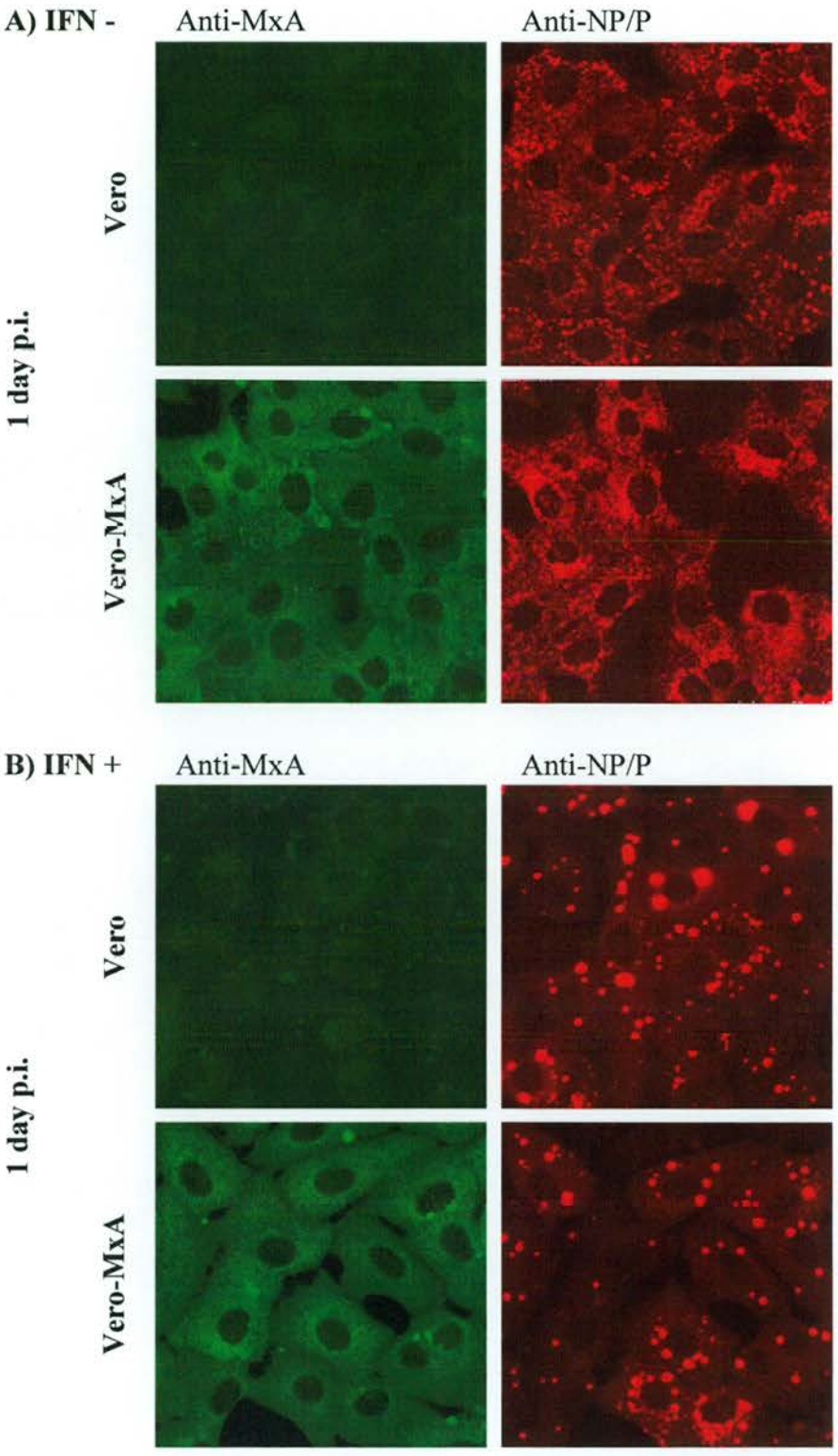

A) IFN -
Anti-MxA
Anti-NP/P
1 day p.i.
Vero
Vero-MxA
B) IFN +
Anti-MxA
Anti-NP/P
1 day p.i.
Vero
Vero-MxA

Figure 3.30

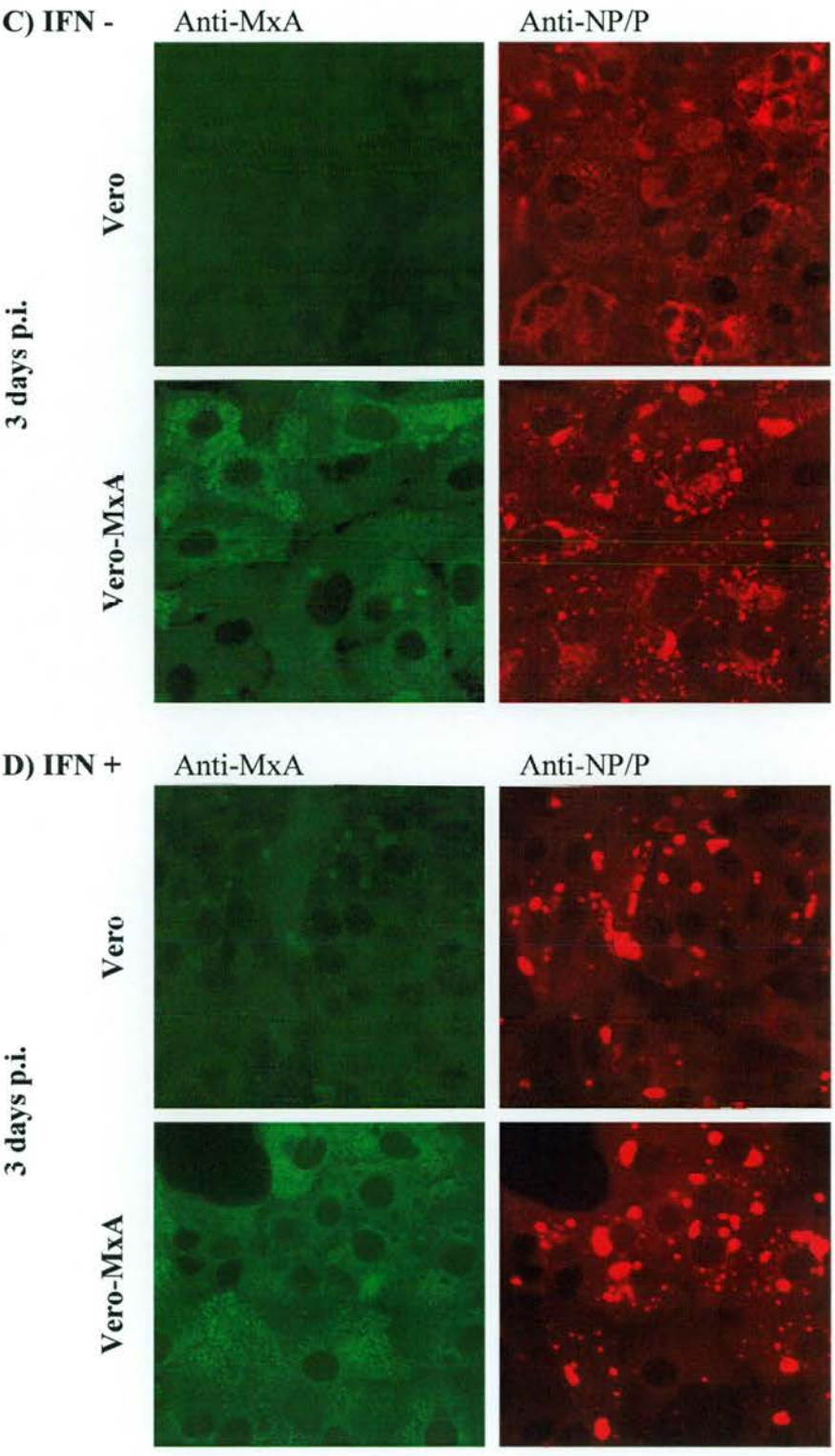
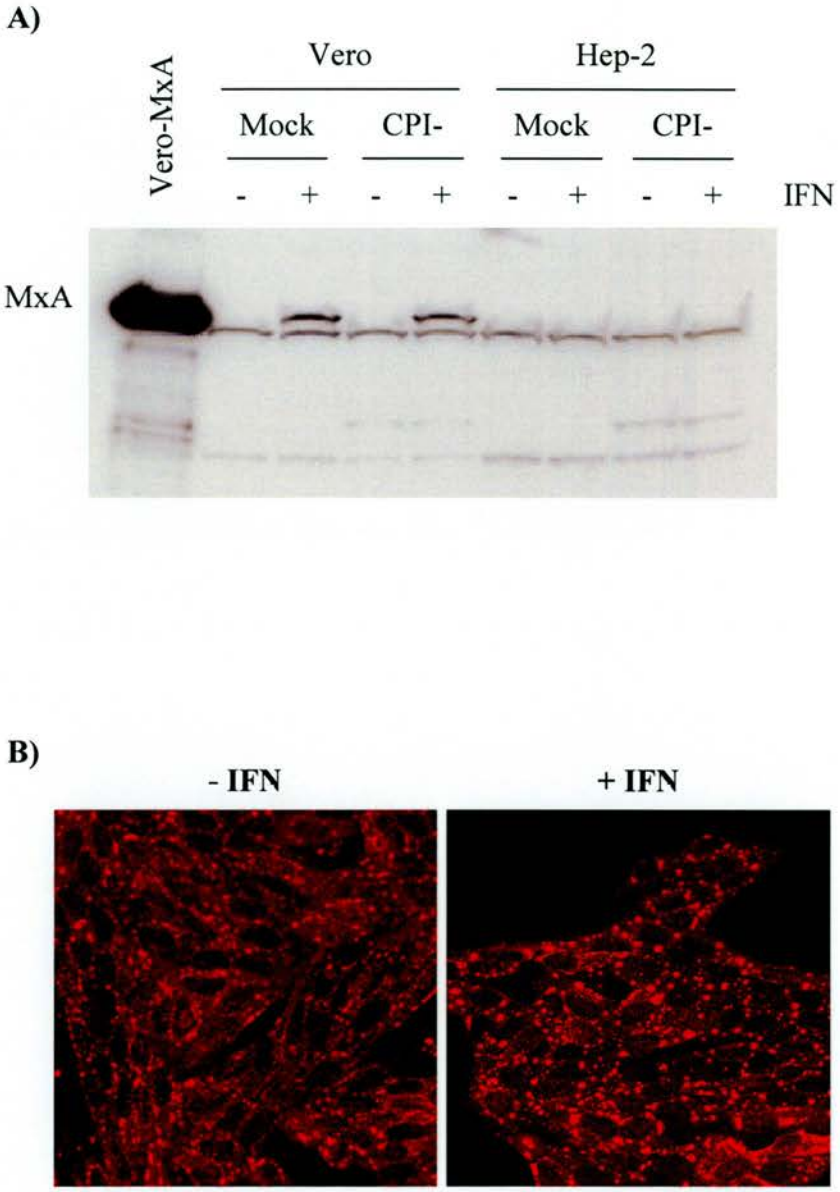

C) IFN -
Anti-MxA
Anti-NP/P
3 days p.i.
Vero
Vero-MxA
D) IFN +
Anti-MxA
Anti-NP/P
3 days p.i.
Vero
Vero-MxA

Figure 3.31 Distribution of NP and P proteins in CPI- infected Hep-2 cells.

Panel A- Hep-2 cells do not express endogenous MxA protein
Vero cells and Hep-2 cells were mock-infected or infected with CPI- at a high m.o.i. (50 pfu/cell) and induced or not with IFN at 8 h p.i. At 24 h p.i. the cells were lysed and the proteins contained in total cell extracts were separated by SDS-PAGE. Immunoblot analysis was subsequently performed using polyclonal anti-MxA antibody.

Panel B- Hep-2 cells were infected with CPI- at an m.o.i. of 10 pfu/cell, treated or not treated with exogenous IFN at 8 h p.i. and the cells were fixed at 1 day p.i. Immunofluorescence analysis was carried out using monoclonal antibodies to NP (SV5-NP-a) and P (SV5-P-e), followed by a secondary anti-mouse IG Texas Red-conjugated antibody.

Figure 3.31



diffuse pattern of those proteins should be observed. This was not the case, and NP and P proteins were distributed primarily in cytoplasmic inclusion bodies in Hep-2 cells infected with CPI- (Figure 3.31, Panel B). Hep-2 cells respond to viral infection by producing IFN, which explains the similar pattern of NP and P proteins in untreated and IFN treated infected cells.

Overall, from the observations presented above it was clear that MxA may contribute to but does not appear to be the absolute responsible for the observed effect of IFN on SV5 protein synthesis and inclusion body formation, although MxA did have an effect on CPI- replication at later times p.i.

3.4.2 The role of PKR

The possible involvement of other cellular proteins induced by IFN are currently in study, such as PKR that is known to inhibit the protein synthesis and which is an essential component of innate antiviral resistance (as described in Chapter 1). As part of the ongoing studies, within our group, Hep-2 cell lines that express various IFN antagonists have been isolated, including those which express the E3L (Hep-2/E3L) and NS1 (Hep-2/NS1) proteins of vaccinia virus and Influenza A virus, respectively. Both E3L and NS1 proteins bind to double-strand RNA to inhibit the activation of PKR. Therefore, it was of interest to examine the pattern of CPI- protein synthesis in these cells and compare it with those in Hep-2 naïve cells and Hep-2 cells that constitutively express the V protein of SV5. Hep-2 naïve cells produce IFN in response to viral infection, and therefore in these cells would be possible to control the effects of IFN on CPI- protein synthesis. In contrast, Hep-2 cells that constitutively express the V protein of SV5 (Hep-2/V), which limits IFN production and blocks IFN response, are used as a control for CPI- protein synthesis in the absence of an IFN-induced antiviral state.

The cells were infected with CPI- at a high m.o.i., treated with IFN at 12 h p.i. (or left untreated) and 6 hours later the cells were metabolically labelled with [^{35}S]-methionine for 1 hour and immunoprecipitation of virus proteins was carried out with a pool of antibodies specific for the NP, P, M, HN and L proteins. The

precipitated proteins and the proteins present in the total cell extracts were subsequently separated on 4-12% PA gradient gels and visualized by phosphorimage analysis (Figure 3.32). As expected, given that Hep-2 naïve cells produce IFN in response to viral infection, the protein synthesis of CPI- was altered in these cells in a similar way to that observed in Vero cells infected with CPI- in the presence of IFN, in which there was an obvious reduction in the relative levels of M, HN, and L proteins. In infected Hep-2/V cells, the relative expression levels of CPI- proteins were not altered, and all viral proteins were being expressed as observed in CPI- infected Vero cells in the absence of IFN. Furthermore, the results suggested a possible role of PKR on the alteration of CPI- protein synthesis, as in Hep-2/E3L and Hep-2/NS1 infected cells, in which PKR was inhibited, the expression levels of M, HN and L proteins was enhanced. Thus, the ratio of M and HN proteins compared to NP and P proteins being synthesised in Hep-2/E3L and Hep-2/NS1 were higher than in Hep-2/naïve. However, the same ratio of M and HN proteins compared to NP and P proteins was much higher in Hep-2/V than in Hep-2/E3L and Hep-2/NS1.

These preliminary results suggest that the IFN-induced PKR protein may play a role in the alteration of CPI- protein synthesis observed in the presence of IFN, although it does not appear to be the only responsible, as the viral protein levels when PKR was inhibited were not as great as those in the absence of an IFN-induced antiviral state (CPI- infection in Hep-2/V cells and in Vero cells in the absence of IFN).

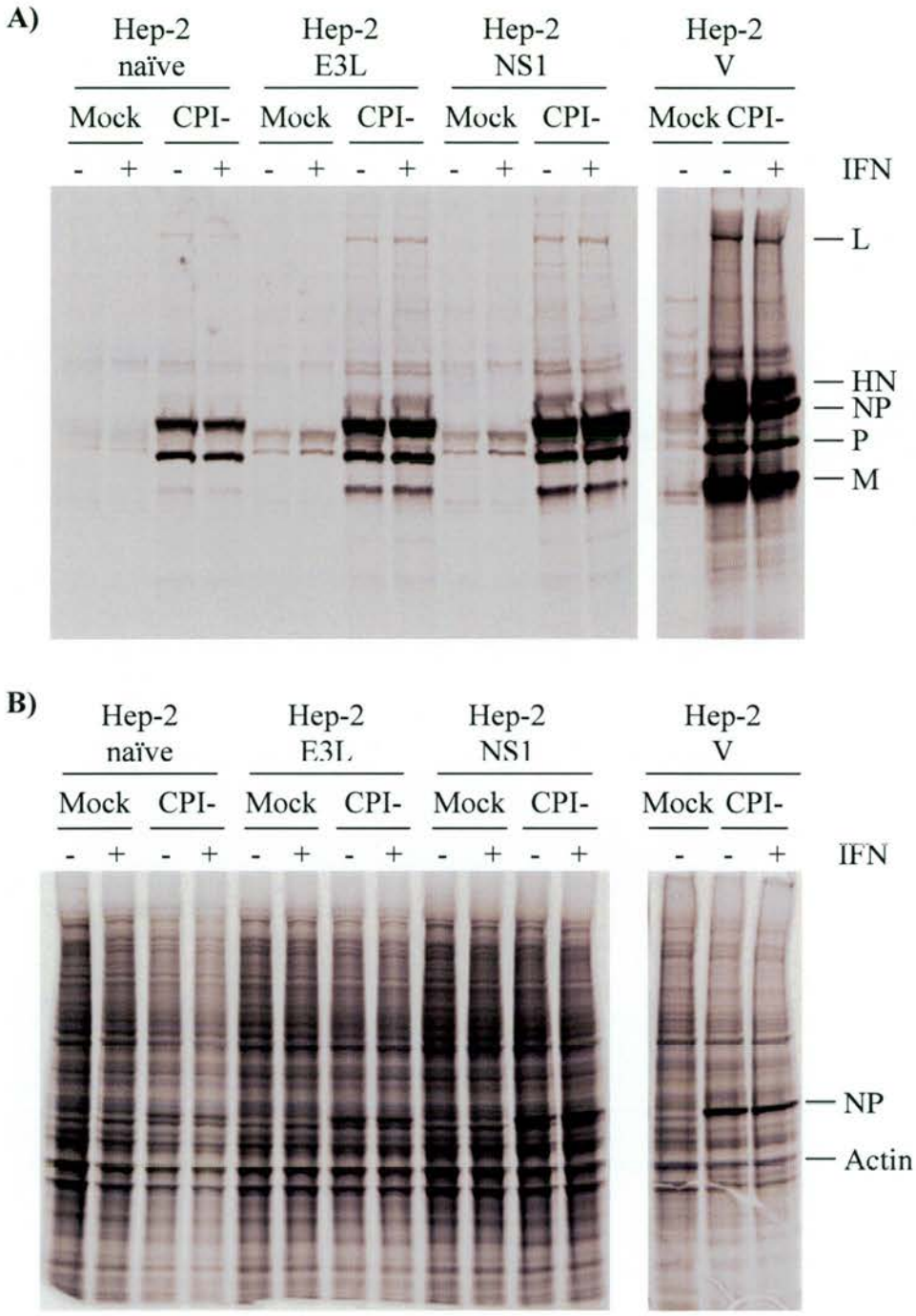
3.4.3 Colocalization studies of the inclusion bodies with cellular membrane proteins

It has been shown for a number of DNA viruses, such as poxviruses, iridoviruses, and African swine virus, that the cytoplasm is favoured as a site of assembly, which takes place in perinuclear viral factories that contain high levels of viral structural proteins, viral DNA, and amorphous membranous material used to produce viral envelopes. Further analysis of the viral factories, which by

Figure 3.32 Expression of E3L and NS1 viral proteins, which inhibit the activation of PKR protein, results on an increase of the expression levels of HN and M proteins.

Hep-2 naïve, Hep-2/E3L, Hep-2/NS1 and Hep-2/V cells were infected with CPI- at an m.o.i. of 50 pfu/cell and 12 hours later treated with IFN (or left untreated). The cells were metabolically labelled with [^{35}S]-methionine for 1 hour at 18 h p.i. and immunoprecipitation of virus was carried out from soluble antigen extracts of these cells using monoclonal antibodies specific for the SV5 NP, P, M, HN and L proteins. The labelled precipitated proteins (Panel A) and the labelled polypeptides present in the total cell extracts (Panel B) were analysed by SDS-PAGE and visualized by phosphorimage analysis.

Figure 3.32


A)
Hep-2 naïve
Hep-2 E3L
Hep-2 NS1
Hep-2 V
Mock
CPI-
IFN
L
HN
NP
P
M
B)
Hep-2 naïve
Hep-2 E3L
Hep-2 NS1
Hep-2 V
Mock
CPI-
IFN
NP
Actin

immunofluorescence appear as a large inclusion body, suggested that the compact perinuclear location of the viral factory was dependent on an intact microtubule network and, by implication, that microtubules are used to transport proteins to viral factories. In addition, the results presented in that analysis showed that those perinuclear structures caused rearrangement of intermediate filaments and the collapse of vimentin into characteristic cages, which recruited mitochondria and cellular chaperones (Heath *et al.*, 2001). To further analyse the nature of the SV5 inclusion bodies and their intracellular location, colocalization studies were therefore carried out using antibodies specific for cellular proteins that localized to various cellular compartments. The marker proteins examined are typical for the cellular compartments shown in Table 3.1.

Table 3.1- Marker proteins and respective cellular compartments studied to observe possible localization of SV5 inclusion bodies.

Marker proteins	Cellular compartment
Actin	Cytoskeleton
Vimentin	Rough endoplasmic reticulum
Tubulin (β)	Microtubules
Sec13	COP II vesicles
Syntaxin17	Smooth endoplasmic reticulum
Galactosyltransferase (Gal-T)	Trans-Golgi compartment
Mannose 6-Phosphate-Receptor (M6PR)	Endosomes
p63	Rough endoplasmic reticulum
p53	Endoplasmic reticulum-Golgi intermediate compartment
Lysosome associated membrane protein 1 (LAMP-70)	Lysosomes
Giantin	Cis-Golgi compartment
70-kDa peroxisomal membrane (PMP-70)	Peroxisomes
Caveolin	Caveolae

However, when double-immunostaining of Vero cell monolayers infected with SV5 was performed using antibodies against the viral P and/or NP protein and the cell proteins mentioned above, no colocalization of the inclusion bodies with any of these cell markers could be seen (Figure 3.33). Furthermore, there was no

rearrangement of intermediate filaments or collapse of vimentin, as seen in Figure 3.33 (Panel labelled vimentin; the viral proteins are stained in red and vimentin stained in green). It is also of note that the filamentous network formed by tubulin throughout the cell was not disrupted by SV5, but there was a partial exclusion of tubulin from the area occupied by the viral inclusion bodies (In the panel labelled tubulin, the viral proteins are stained in red and tubulin stained in green).

Overall, this analysis indicated no association between the viral inclusion bodies and the cellular compartments analysed.

Figure 3.33 Membrane proteins of representative cellular compartments are excluded from the viral inclusion bodies.

Vero cells were infected with SV5 at a low m.o.i. (0.01 pfu per cell) and treated with IFN at 8 h p.i., or left untreated as a control. The cell monolayers were examined at 3 days p.i. by double-staining immunofluorescence. For each cell marker examined, the cells were first stained for SV5 viral inclusion bodies and a cell marker protein by using specific antibodies. Antigen-bound primary antibodies were detected with fluorophore (Cy2, Cy3 or Cy5)- conjugated secondary antibodies. The panels show the merged images, where for the panels showing Vimentin, Tubulin (β), Sec13, Gal-T, p63, p53, LAMP-70, Giantin, PMP-70 and Caveolin, the green signal identifies these cell markers and the viral NP and P proteins are shown in red. In the actin, syntaxin17 and M6PR panels these cellular proteins are identified in red and the viral proteins in green. The cellular DNA was labeled with DAPI (blue).

Figure 3.33

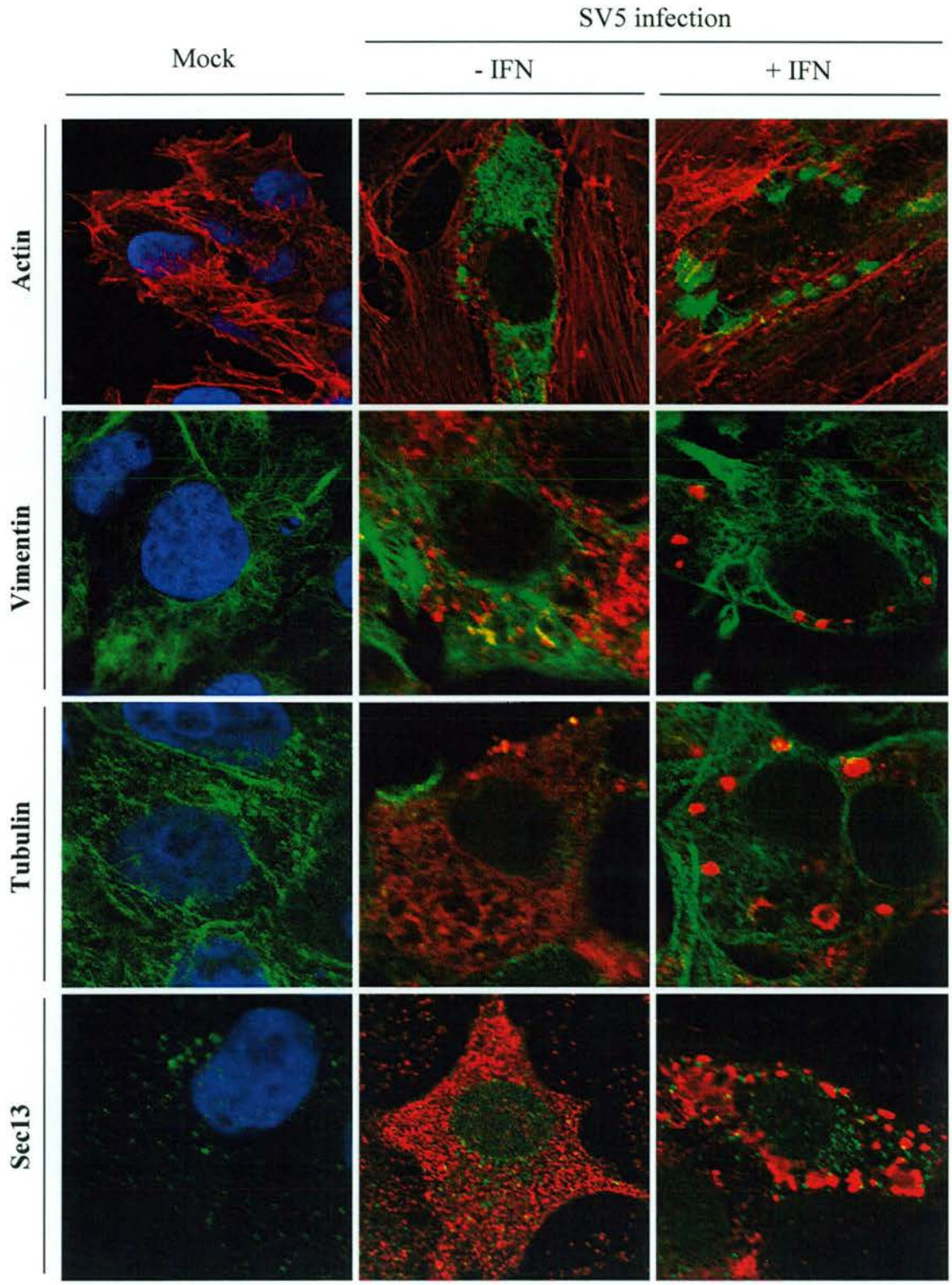


Figure 3.33

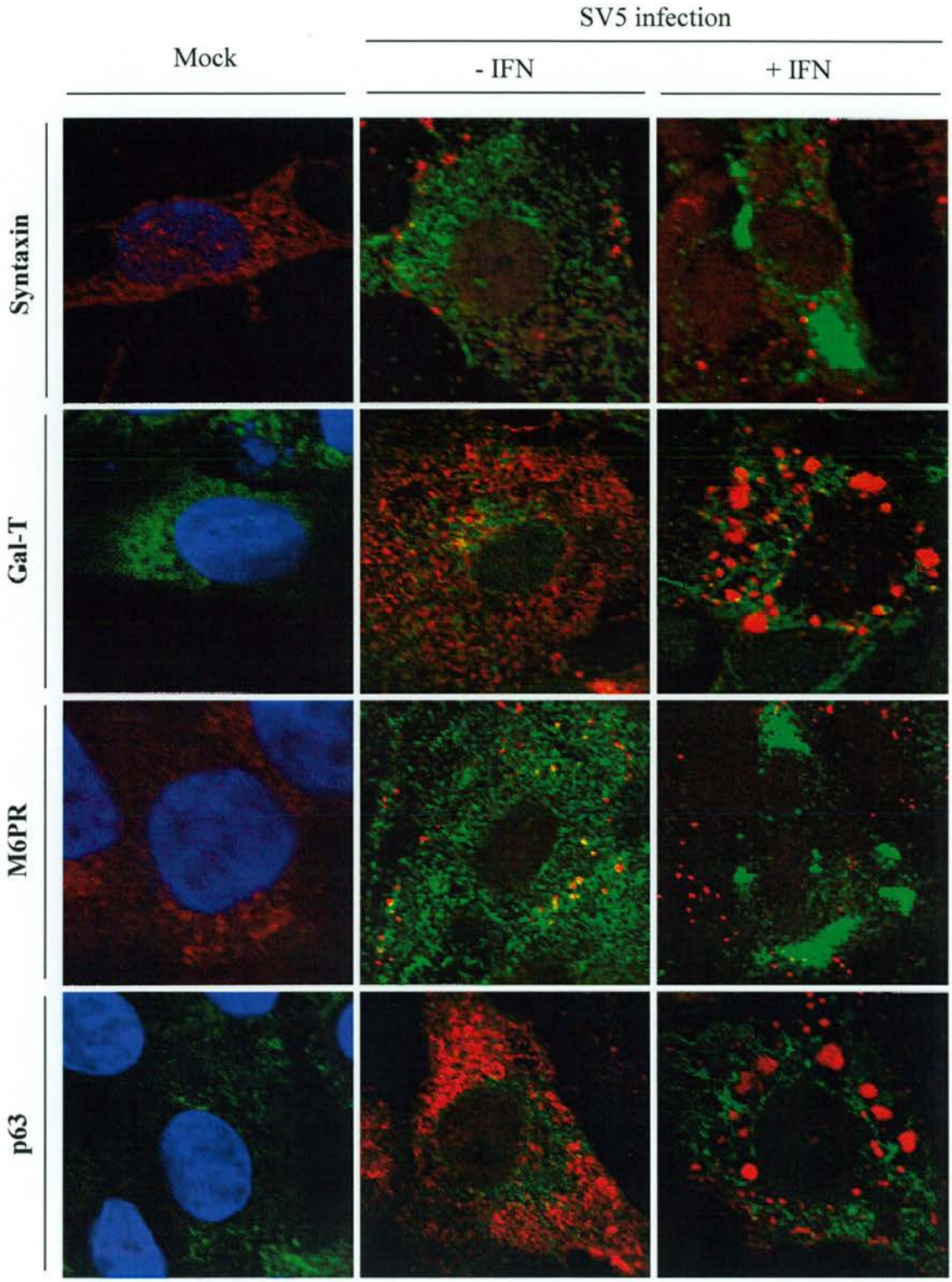
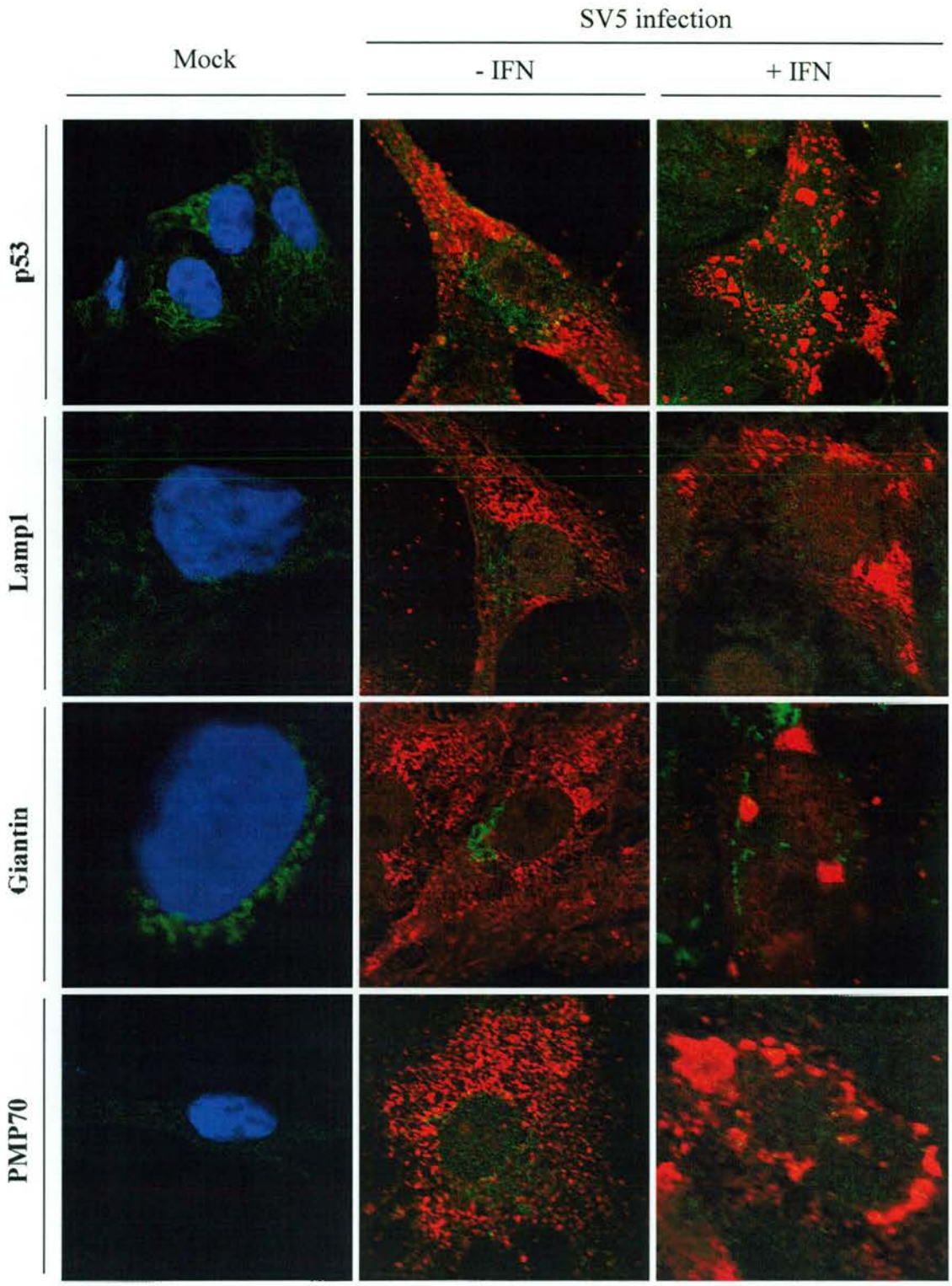


Figure 3.33



4. DISCUSSION

Previous reports have shown that SV5 interferes with IFN-mediated defence responses. SV5 targets STAT1 for degradation, thereby blocking both IFN-α/β and IFN-γ signalling within infected cells (Didcock *et al.*, 1999b). In addition, SV5 specifically limits the production of IFN by virus infected cells (Andrejeva *et al.*, 2004; He *et al.*, 2002; Poole *et al.*, 2002). However, it is known that SV5 does not completely prevent cells from secreting IFN. *In vivo*, immune cells, such as plasmocytoid dendritic cells and activated subsets of lymphocytes, will also secrete large amounts of IFN in response to virus infection, which could exert effects in virus-infected cells (Colonna *et al.*, 2004). In the work presented in this thesis a new model system was developed to further define how IFN inhibits the replication of SV5. In this model, the SV5 variant CPI- was used and, since this virus is unable to block IFN signalling, any effects of IFN would be expected to be more evident than with a wild-type virus. This virus was used to infect Vero cells, which although able to respond to IFN, do not produce IFN, and therefore IFN could be added under controlled conditions to observe the effects of IFN on virus macromolecular synthesis.

Experimental data presented in this report revealed important observations that have contributed to identifying a signalling intermediate involved in a presently uncharacterized intracellular dsRNA signalling pathway that results in activation of the IFN-β promoter. Moreover, these findings have contributed to revealing the mechanism by which SV5 and other paramyxoviruses limit IFN production, which was previously not completely clear. While examining the effect of IFN on the replication of CPI-, a substantial increase in the levels of a 150 kDa protein, co-immunoprecipitated with virus proteins from cells treated with exogenous IFN (Figure 3.1), was noted. Subsequent experiments by others within our laboratory confirmed that this protein specifically interacts with the V protein and is induced by IFN. The protein was then identified as the melanoma differentiation-associated gene 5 product, and found to play a central role in an intracellular signal transduction pathway that can lead to the activation of the IFN-β promoter. Furthermore, mda-5 was shown to be a binding target for the V proteins of

paramyxoviruses, which enables way these viruses to limit IFN production (Andrejeva *et al.*, 2004). mda-5 is a DexD/H-box-containing RNA helicase that contains a caspase recruitment domain (CARD) at the amino-terminus, and has been implicated in the regulation of the growth and differentiation of melanoma cells (Kang *et al.*, 2002). Interestingly, at the same time, another study reported that retinoic acid-inducible gene I (RIG-I), a close relative of mda-5, has similar properties to mda-5 in IFN-β production induced by dsRNA. Thus, RIG-I also has a CARD domain at the N-terminus, in addition to an RNA helicase domain, which recognises dsRNA and regulates signal transduction in an ATPase-dependent manner. The CARD domain was found to be responsible for transmitting "downstream" signals leading to the activation of transcription factors, IRF-3 and NF-κB, and subsequent activation of antiviral functions induced by those factors (Yoneyama *et al.*, 2004). Lately, a molecule has been identified, named IFN-β promoter stimulator 1 (IPS-1), that when over-expressed induced type I IFN and IFN-inducible genes through activation of IRF-3, IRF-7 and NF-κB. The observed induction of IFN mediated by IPS-1 required both TBK1 and IKKε protein kinases. Comparative analysis has shown that IPS-1 shared homology with the CARD domains of RIG-I and mda-5, and the N-terminal CARD-like structure of IPS-1 was also revealed to associate with the N-terminal CARD-containing region of RIG-I and mda-5. Therefore, IPS-1 is believed to be an adaptor molecule acting downstream of RIG-I and mda-5 in activation of IFN-β promoter (Kawai *et al.*, 2005).

4.1 Effect of interferon on the transcription and replication of SV5

Although the V protein of SV5 specifically reduces the production of IFN by infected cells through its interaction with mda-5, it does not completely prevent cells from secreting IFN. As mentioned above, immune cells also produce and release IFN upon viral infection, and therefore these IFNs could affect virus replication by inducing an antiviral state in cells prior to their infection with SV5. Results presented in this thesis, as well as elsewhere (Andrejeva *et al.*, 2002b;

Chatziandreou *et al.*, 2002; Didcock *et al.*, 1999a; Wansley *et al.*, 2005), have shown that cells in an IFN-induced antiviral state severely restrict SV5 replication. However, if cells in an IFN-induced antiviral state are infected with strains of SV5, such as CPI+, which block IFN signalling by targeting STAT1 degradation, they cannot maintain the antiviral state once STAT1 has been degraded. As a consequence, the virus can, after an initial delay, replicate normally and spread from an initial focus of infection. Therefore, the model system described above was used to examine the effect of IFN on the transcription and replication of SV5. Using this model, in which Vero cells were infected with CPI- and exogenous IFN was added to infected cells once virus replication was well established, it was possible to monitor the effects of IFN on virus molecular synthesis in the absence of virus countermeasures. The results from these experiments demonstrated that IFN induces an antiviral state in cells which results in an alteration of the patterns of both SV5 transcription and protein synthesis, as well as in an alteration of the intracellular distribution of virus proteins.

The experimental data resultant from the analysis of virus proteins isolated by immunoprecipitation from infected cells showed that the relative expression levels of NP, P, and V proteins were not significantly different between IFN-treated and untreated cells. However, IFN-treatment did cause an obvious reduction in the relative levels of M, HN, and L proteins (Figure 3.1, Panel A). Analysis of total cell extracts confirmed that IFN treatment significantly inhibited expression of the M protein but had little effect on synthesis of NP, P, and V proteins, as well as cellular proteins (Figure 3.1, Panel B). These results suggest that IFN causes a specific down-regulation in the expression of genes that are downstream of the V/P gene. As expected, in contrast to CPI-, the addition of IFN to cells infected with a strain of SV5 (CPI+) that blocks IFN signalling had no effect on the relative levels of virus protein synthesis (Figure 3.2). Additionally, the effect of IFN treatment on the relative levels of CPI- protein synthesis was demonstrated not to be an increased turnover of the proteins induced by IFN, as shown in Chapter 3 (Figure 3.3 and Figure 3.4).

The results presented in Chapter 3 demonstrated that there were at least three effects of IFN treatment on CPI- virus mRNA transcription. Firstly, IFN caused a change in the virus transcription gradient, which resulted in increased levels of mRNA from genes towards the 3' end of the genome, and a decrease in the levels of mRNA from genes towards the 5' end of the genome (Figure 3.5). Secondly, IFN caused an alteration in the length of the poly (A) tail of virus-specific mRNAs (Figure 3.9). Thirdly, IFN caused an alteration in the transcription of HN mRNA with a significant increase in the level of truncated mRNA (HN (x); Figure 3.5, Panel D). The nature of HN (x) is unknown, but it is likely to be either an HN gene transcription product that has been initiated or terminated inappropriately or an HN mRNA degradation product, perhaps through the action of an IFN-induced enzyme such as RNase L. A plausible explanation for each of these three effects on mRNA expression is that IFN treatment results in a change in the processivity of the virus polymerase. It may be that IFN induces a modification in the virus polymerase complex that alters the polymerase function (Figure 4.1). If polymerase processivity were reduced, the polymerase/transcription complex might be relatively unstable and prone to disengaging, even during mRNA synthesis within gene coding regions, thus accounting for the truncated HN (x) mRNA species. In addition, a reduction in polymerase processivity could result in excessive stuttering during polyadenylation, and thus providing a possible explanation for the increased length of the poly (A) tails of viral mRNA. Both these effects could increase the probability of the polymerase disengaging from the template, within genes and at the gene junctions, respectively, thereby explaining the observed general decrease in mRNA levels encoded by genes farther from the single 3'- proximal polymerase entry site. Furthermore, if the polymerase that prematurely dissociated from the template then reinitiates mRNA synthesis at the 3' end of the genome, this might explain why higher levels of NP and P mRNA were transcribed in cells in the presence of exogenous IFN. If this model is correct, it is unclear why there seems to be less chance of the polymerase disengaging the template between the NP and P genes than at the other gene junctions, but this could reflect effects of sequence variations at the different intergenic regions. In this respect, it is of note that it has been reported that sequence diversity at SV5

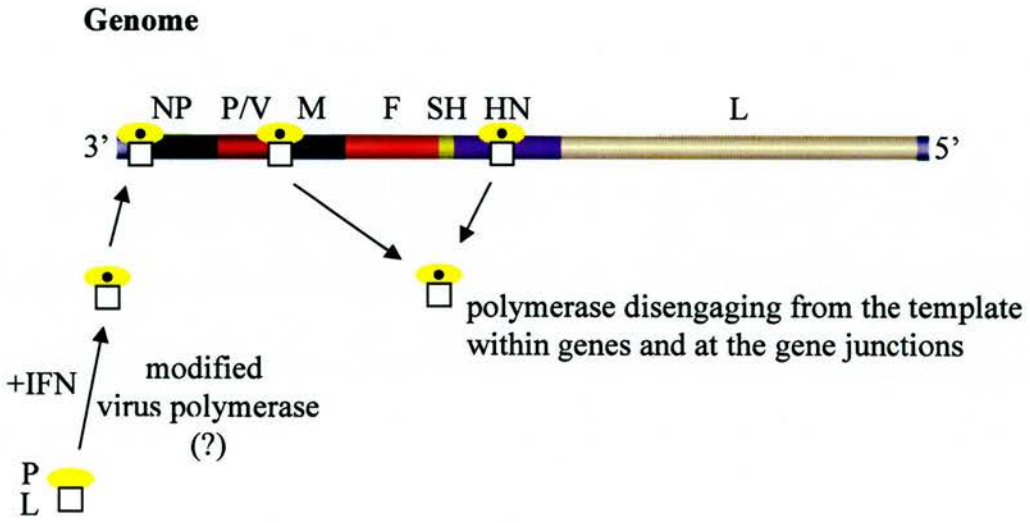


Figure 4.1 Schematic representation of the present hypothesis for the selective effect of IFN on SV5 transcription.

gene junctions may differentially affect SV5 gene expression and hence provide additional control of virus transcription (He & Lamb, 1999; Rassa & Parks, 1999; Rassa *et al.*, 2000). However, further studies will be necessary to fully ascertain how IFN induces the observed effects on CPI- virus transcription and whether the effects are primarily on termination-initiation or chain elongation. Furthermore, it is possible that IFN induces alterations in the stability of virus mRNAs, although it is unlikely that any gross changes in mRNA stability could explain the steeper gradient of mRNA transcription from the 3' to the 5' end of the genome observed upon IFN treatment.

A direct comparison between mRNA accumulation and protein synthesis showed that there was not a complete correlation between these processes (Figure 3.7). For example, in cells infected with CPI-, higher levels of NP and P mRNAs were synthesized in IFN-treated cells than in untreated cells, but the overall level of NP and P proteins synthesis was slightly decreased in IFN-treated cells compared to untreated cells. More strikingly, while the levels of M mRNA were similar in IFN-treated and untreated cells, the levels of M protein were dramatically reduced in IFN-treated cells. These results suggest that in addition to IFN-induced effects on virus transcription, virus protein synthesis was also independently repressed, perhaps through the induction of PKR and 2'-5' oligoadenylate synthetase. However, since there was little discernible effect of IFN on the overall level of cellular protein synthesis in IFN-treated cells infected with CPI- (Figure 3.1, Panel B), if IFN does induce specific inhibition of virus protein synthesis, the effector mechanisms must be acting locally within cells in areas where virus replication is occurring, for example through double-stranded RNA activation of PKR and 2'-5' oligoadenylate synthetase. As presented in Chapter 3, it is possible that PKR plays a role in the observed alteration on the relative levels of CPI- protein synthesis by IFN treatment. Experimental data showed that in Hep-2 cells that constitutively express the NS1 and the E3L proteins of Influenza A virus and vaccinia virus, respectively, which are IFN antagonists that block the activation of PKR (described in Introduction), the expression levels of M, HN, and L proteins were enhanced. However, these results are preliminary and close examination and experimental investigation of the effect of PKR inhibition on the

pattern of protein synthesis will be undertaken to ascertain whether PKR is the IFN-inducible protein responsible for the observed effects of IFN on SV5 protein synthesis.

As described above (Introduction) the major components of the virus polymerase are the large protein catalytic subunit and the phosphoprotein cofactor. Attempts were made to try to find possible modifications on the phosphoprotein from infected cells treated with exogenous IFN (such as a change in the phosphorylation pattern of the protein), by both two-dimensional polyacrylamide gel electrophoresis and MALDI-TOF (Matrix Assisted Laser Desorption /Ionization- Time Of Flight) mass spectrometry analysis. The results from these experiments did not reveal any obvious differences in the phosphoprotein isolated from CPI- infected cells that were, or were not, treated with exogenous IFN (data not shown). Antibodies against the large protein catalytic subunit of the virus polymerase have been recently prepared in our laboratory, and thus further experimental investigation of the virus polymerase complex should be carried out in order to elucidate how IFN is affecting viral transcription.

In addition to demonstrating that IFN can induce changes to the patterns of virus transcription and protein synthesis, results presented in this report also showed that IFN changes the intracellular distribution of the virus proteins synthesized. Thus, following IFN treatment of CPI- infected Vero cells, the NP and P proteins rapidly became localized in cytoplasmic inclusion bodies, whereas in the absence of IFN, the majority of NP and P proteins were more evenly distributed throughout the cytoplasm. Interestingly, inclusion bodies in IFN-treated cells increased in size as the infection progressed (Figure 3.10, Panel B), consistent with the observation that the synthesis of NP and P continued over this time period in the presence of IFN (data not shown). In contrast, the M and HN proteins became undetectable (Figure 3.11), which is also consistent with the decrease in the expression levels of M and HN proteins in IFN treated cells. Whether the residual virus transcription that occurs in cells in an IFN-induced antiviral state occurs within these inclusion bodies is not known.

The addition of IFN to cells infected with CPI+ (an SV5 strain that blocks IFN signalling) had no effect on the distribution of the virus proteins, which was expected given that IFN treatment did not alter the pattern of CPI+ protein synthesis (Figure 3.10, Panel A). However, IFN treatment did limit the replication of strains of SV5 (CPI+) that block IFN signalling and enhanced the formation of inclusion bodies if cells were treated with IFN prior to infection. Thus, the pattern of both CPI- and CPI+ protein synthesis was significantly altered following infection of cells in an IFN-induced antiviral state, such that NP and P protein synthesis was generally reduced, and very little M and no HN or L could be detected (Figure 3.15). However, such effects were not indefinitely maintained in CPI+ infected cells as the levels of virus protein synthesis increased as the infection progressed. It has been previously shown that wt SV5 targets STAT1 for degradation in cells which have entered an antiviral state (Didcock *et al.*, 1999b), and therefore most likely STAT1 was degraded in cells infected with CPI+, but not CPI-. As a consequence, CPI+ infected cells could not maintain an antiviral state indefinitely in the eventual absence of IFN signalling. Analysis of the distribution of the viral proteins revealed that IFN pre-treatment also induced the formation of inclusion bodies in CPI+ infected cells (Figure 3.16). In addition, IFN treatment, following a low m.o.i., also induced the formation of inclusion bodies in cells infected with MuV and hPIV2 (Figure 3.22, Panels A and B, respectively), indicating that this phenomenon is not specific to the CPI- virus.

4.2 Nature of inclusion bodies

Experimental examination and characterization of the SV5 inclusion bodies, including the driving forces for their formation, the role of IFN-induced cellular proteins in their formation and their composition, was also attempted in the work presented in this thesis. Findings from the experiments presented in Chapter 3 have shown that in addition to the NP and P viral proteins, the large polymerase protein is also included in the SV5 inclusion bodies (Figure 3.12).

The IFN-inducible protein MxA has a well documented role in the antiviral response against many RNA viruses (described in Introduction). Experimental

data presented in this thesis showed that, although MxA does have an effect on the growth of SV5 at late times post-infection (Figure 3.28), it is not responsible for the observed effect of IFN treatment on protein synthesis of SV5 (Figure 3.29). MxA has been shown to co-localize with the N protein of La Crosse Virus, and, by sequestering it into large perinuclear complexes, inhibits viral genome replication (Kochs *et al.*, 2002). Results presented in Chapter 3 showed that this is not the case in SV5 infected cells, and MxA does not co-localize with the SV5 inclusion bodies, neither is responsible for their formation. However, a close examination of the effect of MxA on the formation of inclusion bodies suggested that MxA may have an indirect role in their formation, although it is not the primary IFN-induced effector mechanism responsible for the inclusion bodies formation (Figure 3.30).

It has been shown for a number of viruses that the formation of viral factories, discrete perinuclear foci where viral genome replication and assembly take place, are frequently located in specific intracellular compartments where viral components are concentrated. As a consequence, the efficiency of those viral processes is increased. The mechanisms of formation of these factories often involve the recruitment and association of membranes, mitochondria, and cytoskeletal elements (Novoa *et al.*, 2005). Colocalization studies of SV5 inclusion bodies with various cellular membrane proteins showed that the viral inclusion bodies were excluded from all of the cellular compartments examined. Various attempts were made to purify the inclusion bodies formed in CPI- infected cells in the presence of IFN in order to identify putative host cell proteins that could be interacting and mediating their formation, but the results from these experiments were inconclusive (data not shown). Further investigation of the nature of the inclusion bodies and their molecular characterization should be done, as this would enable a better understanding of the role(s) of the inclusion bodies in the virus life cycle, as well as in the establishment of persistent infections by SV5 (see below).

Although it is clear that IFN treatment induces the formation of inclusion bodies, the precise driving force for their formation is poorly understood. IFN treatment

induced the relocalization of NP and P proteins into inclusion bodies, while there was a striking decrease on the levels of M and HN proteins. It is possible that the lack of these proteins forces NP and P proteins, which are being expressed at "normal" levels, into inclusion bodies. It has been suggested that towards the end of the replication cycle, M is the driving force for the assembly and release of newly virus particles, and persistent paramyxovirus infections have been reported where M is inactivated and virus budding fails (reviewed in Schmitt & Lamb, 2004). Experiments were carried out, using short interfering RNA (siRNA) for silencing the M protein, to examine whether the reduction of the levels of M protein would force the formation of inclusion bodies even in the absence of IFN. Another experimental approach was attempted, in which Vero cells were transiently transfected with a plasmid expressing the M protein, to observe whether over-expression of M would prevent the formation of NP/P inclusion bodies following IFN treatment. The results from such experiments (data not shown) did not show convincing observations that could lead to an absolute conclusion regarding a correlation between the levels of M with inclusion body formation.

4.3 SV5 persistence

A critical factor that determines the outcome of acute virus infections includes the interaction of viruses with the IFN system (Biron & Sen, 2001; Goodbourn *et al.*, 2000; Levy & Garcia-Sastre, 2001; Sen, 2001; Stark *et al.*, 1998). As described in Chapter 1, the ability of paramyxoviruses to establish persistent infections *in vivo* has been suggested to be linked to their ability, or not, to block the IFN response. Thus, for example, viruses that do not block IFN signalling, such as CPI-, may be selected *in vivo* because they are better able to establish persistent infections. The model for SV5 persistence in our laboratory proposes that as the virus becomes repressed in response to IFN, virus glycoproteins are lost from the surface of infected cells and virus nucleocapsid proteins accumulate in cytoplasmic inclusion bodies (Chatziandreou *et al.*, 2002). Indeed, as presented in Chapter 3, using the model system developed in the work presented in this thesis, CPI- was demonstrated to be significantly affected by IFN treatment in IFN-non producing

cells (Vero cells) in which IFN was added when virus replication was already established. Accordingly, as described above, whilst NP and P inclusion bodies were readily detected in the cytoplasm of those cells, the M and HN proteins were undetectable. Furthermore, as shown by the results from the experimental data, such cells (infected with CPI-), upon prolonged passaging in the presence of IFN, can become persistently infected. Immunofluorescence analysis of the passaged cells showed that the cells remained persistently infected, whilst large inclusion bodies could be observed in their cytoplasm, suggesting that the occurrence of inclusion bodies might indicate that the virus has entered a quiescent state. Interestingly, upon removal of IFN, the virus appeared to reactivate, as NP and P proteins redistribute into a more diffuse pattern and HN protein became detectable on the cell surface (Figures 3.26 and 3.27). It is of note that the lack of M protein in particular has been suggested as a mechanism of paramyxoviruses persistence, due to restriction of viral budding. In cases of SSPE, a persistent measles infection of the brain, M is either absent or fails to associate with budding structures. Moreover, in persistent SeV infections, the described change from a lytic to a persistent infection correlates with M being unstable and budding structures being absent (Lamb & Kolakofsky, 2001).

Extensive investigations have attempted to elucidate the molecular basis by which cellular proteins with antiviral activity work, and how specific viruses circumvent the IFN response. However, evidence from the results presented here highlight that virus pathogenesis may also be influenced by the specific way viruses replicate in cells that have entered an IFN-induced antiviral state. Indeed, the results presented in this thesis have demonstrated that the replication of strains of SV5 that block IFN signalling is limited if cells are pre-treated with IFN prior to infection. It is therefore suggested that if cytoplasmic inclusion bodies do have a role to play in virus pathogenesis, then they may also be formed when viruses that can block IFN signalling infect cells already in an IFN-induced antiviral state (Figure 3.16). Indeed, cytoplasmic inclusion bodies may be a virus defence mechanism in which the virus can "hide" both from intracellular antiviral responses and adaptive immune responses (Chatziandreou *et al.*, 2002; Fearns *et al.*, 1994). If this is the case, it is plausible to speculate that viruses such as SV5,

which change their pattern of virus transcription and protein synthesis in response to the programmed IFN response, may have been selected for during virus evolution.

It is also noteworthy that a number of paramyxoviruses have been shown to be sensitive to IFN action. For example, IFN was shown to restrict MuV replication in mouse L929 cells (Yamada *et al.*, 1984) and to play a role in the establishment of MuV persistence in these cells (Ito *et al.*, 1986). Transmission electron micrographs of MuV infected cells revealed the presence of large cytoplasmic areas of viral aggregates that included nucleocapsid-like material (Afzal *et al.*, 2005). Moreover, IFN appears to be critical in the establishment of MeV infection of human lymphocytes (Jacobson & McFarland, 1982). Furthermore, SSPE and measles inclusion body encephalitis have been associated with persistent measles infections, as this virus is able to establish asymptomatic persistent infections in humans, without symptoms for the time between the acute infection and the start of clinical symptoms (reviewed in Rima & Duprex, 2005). Electron microscopy studies have helped identify the link between SSPE and MeV infection (Connolly *et al.*, 1967; Payne *et al.*, 1969; Tellez-Nagel & Harter, 1966) and various studies have contributed to show that SSPE is caused by persistent MeV infection (Connolly *et al.*, 1967; Connolly *et al.*, 1971), along with the presence of MeV-specific inclusions in infected cells (McQuaid *et al.*, 1993). Moreover, the nuclei and cytoplasm of osteoclasts in tissue affected by Paget's disease of bone are marked by the presence of microcylindrical inclusions, which are morphologically similar to viral structures. Immunocytological techniques have revealed the presence of paramyxovirus antigens of MeV, SV5, human parainfluenza virus, and RSV in pagetic osteoclasts, which suggests that paramyxoviruses may play a role in the aetiology of the bone disease (Basle *et al.*, 1987; Basle *et al.*, 1985). However, the role of paramyxoviruses in chronic human disease such Paget's bone disease (Bender, 2003; Rall, 2003; Rima *et al.*, 2002) remains highly controversial. A greater understanding of the effect of these viruses on the IFN response and the role of IFN in the control of normal cellular function, such as osteoclast function (Takayanagi *et al.*, 2002), may lead to a more

rational basis for their involvement in chronic disease and this is an area worthy of further study.

As mentioned above, investigation including the nature of the inclusion bodies and their molecular characterization was attempted in the work presented in this thesis. In addition, unsuccessful experiments were carried out to examine whether it would be possible to rescue infectious virus particles from the SV5 inclusion bodies. Given the presumable importance of the inclusion bodies, further investigation of the SV5 inclusion bodies, including electron microscopy studies, as well as examination of their nature and the molecular basis for their formation would be essential to define their role, if any, in the maintenance of persistent infections.

Conclusions

From the results presented in this thesis it is clear that cells in an IFN-induced antiviral state severely restrict SV5 replication. However, if cells in an IFN-induced antiviral state are infected with strains of SV5, such as CPI+, which block IFN signalling by targeting STAT1 for degradation, they cannot maintain the antiviral state once STAT1 has been degraded, thereby allowing the virus to replicate normally and to spread from an initial focus of infection. The mode by which IFN inhibits the spread of SV5 was examined. The results from such studies demonstrated that IFN induces an antiviral response in cells, which changes the pattern of SV5 transcription and protein synthesis. Furthermore, IFN treatment was shown to result in an altered distribution of virus proteins, such that following IFN treatment of CPI- infected Vero cells, the NP and P proteins rapidly became localized in cytoplasmic inclusion bodies. A similar delay of virus replication and formation of virus cytoplasmic inclusion bodies was also observed when cells in an IFN-induced antiviral state were subsequently infected with strains of SV5 (CPI+), hPIV2 and MuV that block IFN signalling, showing that this phenomenon is not specific to the CPI- virus. These observations highlight that virus pathogenesis may be influenced by the specific way viruses replicate in cells that have entered an IFN-induced antiviral state. The nature of the inclusion bodies, as well as the molecular events that underlie their formation (the role of IFN-induced cellular proteins, e.g. MxA) was investigated, as was the relevance of viral inclusion bodies to SV5 persistence. The results presented in this thesis suggest that if cytoplasmic inclusion bodies do have a role to play in virus pathogenesis, then they may also be formed when viruses that can block IFN signalling infect cells already in an IFN-induced antiviral state. Indeed, cytoplasmic inclusion bodies may be a virus defence mechanism in which the virus can hide both from intracellular antiviral responses and adaptive immune responses (Chatziandreou *et al.*, 2002; Fearns *et al.*, 1994). If this is the case, then it opens up the question as to whether the way in which viruses such as SV5 change their pattern of virus transcription and protein synthesis in response to the programmed IFN response may have been selected for during virus evolution.

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Appendix 1: Primer sequences and positions of binding

Name	Annealing sequences (5'→ 3')	Viral genome binding position
Actin For	CCCTGTACGCCTCTGGCCG	-
Actin Rev	GCCACAGGACTCCATGCCC	-
F For	CCTACTGGGGAGTACCTTGCCG	5207-5228
F Rev	CCCAACCACTGGAGAGAAGGTGC	5596-5618
HN For	GCCACGACACCAGAGGGCTGC	7046-7066
HN Rev	CCCCGCTTCCTGTTCCTGGGTTC	7444-7466
M For	CCCTTGGATGTGGGCCTAATCTACC	3409-3433
M Rev	CCCTCCTTGTCAGGGGTATGGACC	3797-3820
NP For	GGCTTTGAGGAGGGATCATTCCGC	467-490
NP Rev	GTCAAAAAGCCTCCCATTCC	944-966
P For	CCCCCATCGATTTTAAGAGGGGGG	2319-2340
P Rev	GGTTACTTAGTGTCTTGCGTACATCTTCCAC	2706-2736

Appendix 2: Publications

Andrejeva, J., Childs, K. S., Young, D. F., **Carlos, T. S.**, Stock, N., Goodbourn, S. and Randall, R. E. (2004). The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc Natl Acad Sci U S A* **101**, 17264-9.

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