Interfering with interferon: developing a reporter system to study the interaction between hepatitus C viral proteins and the interferon signalling pathway

Jones, Meleri

INTERFERING WITH INTERFERON:

DEVELOPING A REPORTER SYSTEM TO STUDY THE INTERACTION BETWEEN HEPATITIS C VIRAL PROTEINS AND THE INTERFERON SIGNALLING PATHWAY.

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"Insanity: doing the same thing over and over again and expecting different results."

A. Einstein

Abstract

The aim of the project was to investigate the mechanism by which HCV evades therapeutic IFN treatment. This involved the development of novel testing systems and their application to patient samples. Initial experiments focused on flavivirus replicons and novel observations on effects of one of these replicons (dengue virus) on interferon signalling were made. The dengue replicon system was demonstrated to inhibit IFNα signalling by reducing the expression of STAT2, an essential component of the type I IFN signalling pathway. This phenomenom was then further examined in dengue virus infected human cells and again it was observed that the expression of STAT2 was reduced. The mechanism of STAT2 degradation was further explored and STAT2 expression was found to be restored using a proteasomal inhibitor.

A second flavivirus replicon system involving BVDV was also developed as a reporter system, again with novel observations. The BVDV replicon system was shown to be sensitive to the antiviral effects of IFNα and was not shown to inhibit the IFNα signalling pathway. The BVDV replicon was tested as a reporter system using a well-known viral inhibitor of IFNα. The viral inhibitor, inhibited the antiviral action of IFNα on the BVDV reporter. Having developed and validated this system, the effects of a small number of patient derived samples were assessed and it was demonstrated that NS5a derived from a patient who failed to respond to IFNα treatment inhibited the effects of IFNα on the BVDV reporter. To increase the senstitivity of the assay the reporter cassette was then changed to a destabilised GFP for use in a FACS based assay.

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PAPERS

Dengue Virus Inhibits Alpha Interferon Signalling by reducing STAT2 Expression. Jones M., Davidson A., Hibbert L., Gruenwald P., Schlaak J., Ball S., Foster GR., Jacobs M. J. Virol. 2005 79; 5414-5420.

POSTERS

Jones M., Hibbert L., Davidson A., Ball S., Schlaak J., Foster GR., Jacobs M. Dengue virus inhibits early events in interferon signalling – ISICR Puerto Rico 2004, Single stranded RNA virus conference, San Francisco 2004, Medical Research Society, London 2004, ICMS Institute day 2005 – 1st prize, UCL Research day 2005 – 1st prize.

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TALKS

Dengue virus inhibits alpha interferon signalling by reducing STAT2 Expression. CDC Puerto Rico 2004 and British Infection Society, London 2005.

TABLE OF CONTENTS

Table of Figures

Table of tables

ABBREVIATIONS USED

aa	Amino Acid
ALT	alanine aminotransferase
Amp	ampicillin
APS	Ammonium persulphate
bp	base pair
BSA	bovine serum albumin
BVDV	Bovine viral diarrhoea virus
C	core protein of HCV
cDNA	complementary DNA
CMV	Cytomegalovirus
CO2	Carbon Dioxide
CR	Complete Responder to IFN treatment
CTL	cytotoxic T lymphocytes
Den	Dengue (virus)
DEPC	Diethyl pyrocarbonate
dH2O	distilled water
DMF	Dimethylformamide
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
ds	double stranded
dsRNA	double stranded RNA
E1, E2	envelope proteins of HCV
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetra acetic acid
eIF	eukaryotic Initiation Factor
ELISA	Enzyme linked Immunosorbant assay

EMCV	Encephalomyocarditis virus
EtBr	Ethidium Bromide
FACS	Fluorescent activated cell sorter
GFP	Green Fluorescent Protein
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HSV	herpes simplex virus
HVR	Hyper variable region
IFN	Interferon
IFNAR	Interferon Alpha Receptor
IL	Interleukin
IP10	IFNγ inducible protein 10
IPTG	isopropylthio-β-D-galactoside
IRES	Internal ribosomal entry site
IRF	Interferon Regulatory Factor
ISDR	Interferon Sensitivity determining region
ISG	Interferon Stimulated Gene
ISGF	IFN-Stimulated gene factor
IU	International unit
JAK	Janus kinase (Just another kinase)
JEV	Japanese encaphilitis virus
kb	kilo base
kDa	kilo Dalton
LB	Luria Bertani
LB	LB Agar
LFT	Liver function tests
MCS	Multiple cloning site

MHC	Major histocompatibility complex
NANB	Non-A, Non-B (Hepatitis)
NDV	Newcastle Disease Virus
NR	Non-responder (to IFN treatment)
NS	Non-structural proteins
nt	nucleotide
ORF	Open reading frame
PBS	phospho-buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
PFA	paraformaldehyde
PIAS	Protein inhibitor of activated STAT
RdRp	RNA-dependent, RNA-polymerase
RFP	Red Fluorescent Protein
RIG-I	Retinoic Acid Inducible Gene-I
rpm	revolutions per minute
RT	room temperature
SDS	sodium-dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SeV	Sendai virus
ss	single stranded
STAT	Signal transducer and activator of transcription
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TBE	Tick-borne encephalitis (virus)
TBS	tris-buffered saline
TEMED	N,N,N'N'-tetramethylethylenediamine
TLR	Toll-like receptor

TNF-α	Tumour Necrosis Factor alpha
TRAM	Toll receptor adaptor molecule
TRIF	Toll/IL-1 receptor domain-containing adaptor inducing IFN-β
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
TYK	Tyrosine Kinase
SV5	Simian virus 5
UTR	Untranslated region
UV	Ultraviolet
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactosidase
YF	yellow fever (virus)

1. INTRODUCTION

Although the focus of this thesis is the effects of HCV derived patient proteins on the interferon (IFN) system, to analyse the effects, various reporter systems were developed. Background information for the individual reporter assays will be given in the relevant result chapters and here in the introduction the emphasis shall be put upon the interferon system, hepatitis C virus and their interactions.

1.1.1 Interferons

The Interferons are a large family of multifunctional secreted proteins that are involved in antiviral defence, cell growth regulation and immune activation (Pestka S, 1987). They were first discovered in 1957; Isaacs and Lindenmann reported that chick cells infected with heat inactivated influenza virus produced a soluble factor, which rendered new cells resistant to infection by live influenza virus (Lindenmann *et al*, 1957). This factor could not be sedimented by centrifugation at 100,000g for 15 minutes, was non-dialyzable and was stable over the pH range of 1-10. They called it IFN as it interferes with viral replication (Isaacs *et al*, 1957).

The IFN family is separated into two types, I and II and even though there is no structural similarity between the two families, there is a broad overlap in the induced gene expression. Both families act in a functionally distinct manner; type I induce an antiviral state in target cells, whilst type II IFNs are predominately immunomodulatory (Pestka *et al*, 1987).

1.1.2 Overview of Type I Interferons

Type I IFNs consist of alpha, which is subdivided into 13 subtypes, clustered on the short arm of human chromosome 9 (9p21.3) (Diaz *et al*, 1993), interferon beta (β) and interferon omega (ω). Evolutionary the IFNα and IFNβ genes separated about

800 million years ago, the IFNω separated 700 million years later and the multi IFNα subtypes appeared approximately 80 million years ago (Roberts *et al*, 1998). IFNα is produced primarily by leukocytes and IFNβ by fibroblasts, but production of type I IFNs is transiently induced in most nucleated cells in response to virus infection, dsRNA and bacterial infections.

The IFNα subtypes are intronless genes that share about 60% amino acid homology within the coding region; they contain four conserved cysteine residues that form two intramolecular disulphide bonds. It is not known why there are so many different interferon α subtypes, whether it is dependent upon the type of viral infection or whether they stimulate different antiviral genes.

1.1.3 Overview of Type II interferon

IFNγ is the only member of the type II interferon family. It has no sequence homology with the type I interferons, but is closely homologous to IL-10 and is found on the long arm of chromosome 12 in humans. IFNγ plays an important role in both innate and adaptive immunity and antigen-specific stimulation results in it's secretion by NK cells and T lymphocytes. It can enhance MHC class I and II expression, activate macrophages resulting in increased phagocytosis, upregulate IL-2 receptor expression on cytotoxic T cells and promote B cell differentiation.

IFNγ exerts its effects by binding to the IFNγ receptor, which is composed of two receptor chains IFNγR1 and IFNγR2 which are present on most cells. The IFNγ receptor is associated with the Janus tyrosine kinases, Jak1 and Jak2 which become activated upon ligand binding and are phosphorylated leading to the dimerisation and nuclear translocation of STAT1 (Signal transducers and activators of transcription) [reviewed in (Platanias, 2005)]. Mice with a deficiency in IFNγ, the

receptors or STAT1 are more susceptible to microbial pathogens and some viruses. Also humans with mutations in the IFNγ pathway are more susceptible to mycobacterium infections with some patients dying in early childhood (Dupuis *et al*, 2001).

1.1.4 Interferon α/β Signalling Pathway

The type I interferon system is one of the best-studied signalling pathways; the JAK-STAT pathways were discovered through the analysis of the mechanisms used by the IFNα/β. All type I IFNs bind to the same receptor on the cell surface, but with varying affinities, IFNα1 shows a much lower binding affinity for human membrane receptors than IFNα2 or α8 (Zoon *et al*, 1982). The interferon receptor is composed of two chains; IFNAR1 and IFNAR2, both of which have been cloned (Uze *et al*, 1990) and shown to be essential for the high affinity binding and biological activity of the ligands (Lutfalla *et al*, 1995) , IFNAR2 being the major ligand binding component of the complex. The intracellular domains of the receptor chains are associated with the tyrosine kinases, JAK1 and Tyk2. Ligand binding to IFNAR results in the activation of Jak1 and Tyk2, phosphorylating IFNAR1 at residue Y455, triggering a chain of events leading to the phosphorylation of the STATs. STATs form specific multimeric complexes, in this case with a member of the IRF (Interferon regulatory factor) family. The complex, consisting of STAT1, STAT2 and IRF9, called ISGF3 trans-locates to the nucleus and activates transcription from IFN type I gene promoters containing the Interferon stimulated-response element (ISRE) sequence, a highly homologous DNA element of 14 base pairs found in all Interferon stimulated genes (ISGs) (Porter *et al*, 1988).

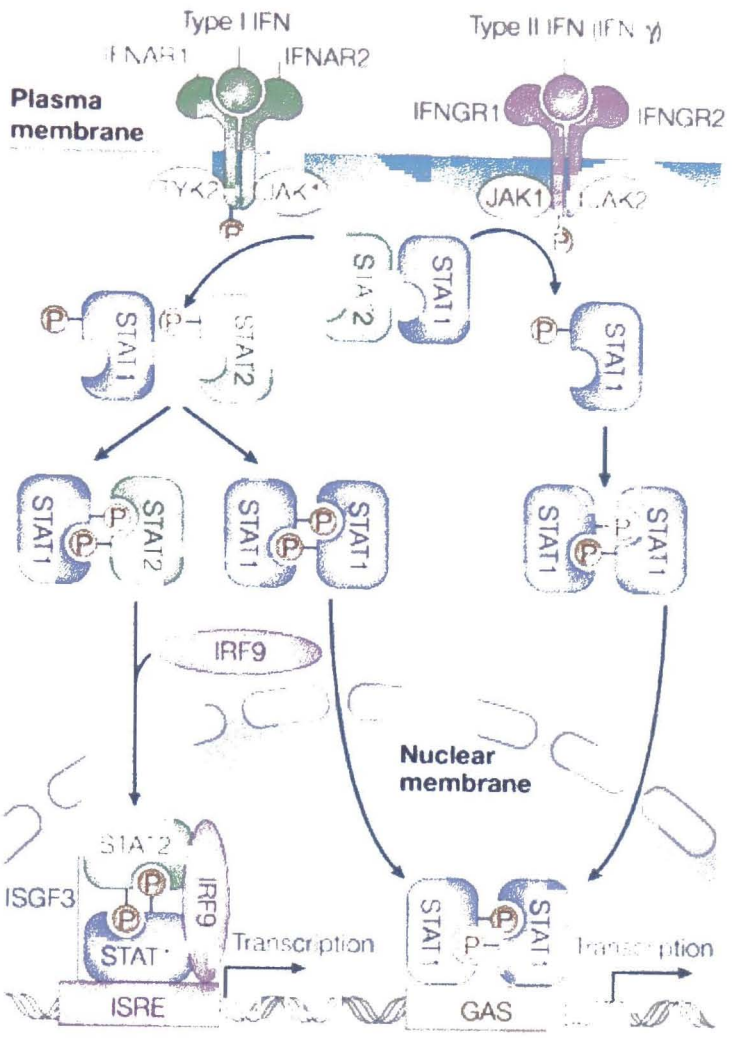


Fig1.1 The Interferon signalling pathway. Type I-Binding of the type I IFN to the extracellular domain of the receptor chains causes the activation of Jak1 and Tyk2 resulting in the phosphorylation of the STAT1-2 heterodimer forming ISGF3 with IRF-9. ISGF3 translocates to the nucleus and binds to the ISRE elements of the promoters in type I IFN inducible genes to activate their transcription. Type II-The IFNγ binds to the receptor causing the activation of STAT1 via the Jaks. STAT1 homodimers translocate to the nucleus and bind to the IFNγ activated site (GAS) promoter sequences to stimulate the appropriate gene transcription.(Decker *et al*, 2005)

1.1.5 Virus Induction of Type I Interferon

1.1.5.i Feedback loop

A wide range of interferon inducers are known, such as natural and synthetic dsRNA, viral infection, intracellular bacteria, microbial products e.g. LPS and low molecular weight substances e.g. cyclohexamide. Although these share no structural similarity, recent advances have shown that they induce the type I interferon response via different mechanisms but that the pathways eventually converge to activate the same transcription factors, IRF3, NFκB and AP-1. IRF3 is a constitutively expressed protein found in the cytoplasm (Lin *et al*, 1998), viral infection or the presence of dsRNA leads to it's phosphorylation by TBK1 and IKK-*i* and it is then rapidly translocated to the nucleus where it binds specifically to IFNβ IRF-E (PRDI element) resulting in the weak activation of IFNβ (Schafer *et al*, 1998). This leads to a positive feedback induction loop with the initially produced IFNβ inducing IRF7 expression (Marie *et al*, 1998). IRF7 is not constitutively expressed in cells and is induced by

IFN signalling via the JAK-STAT pathway through the activation of ISGF3. The transcription of IFNα/β is controlled primarily through the activation of the IRFs, especially IRF3 and IRF7; IRF7 deficient mice are incapable of mounting a credible antiviral response (Honda *et al*, 2005).

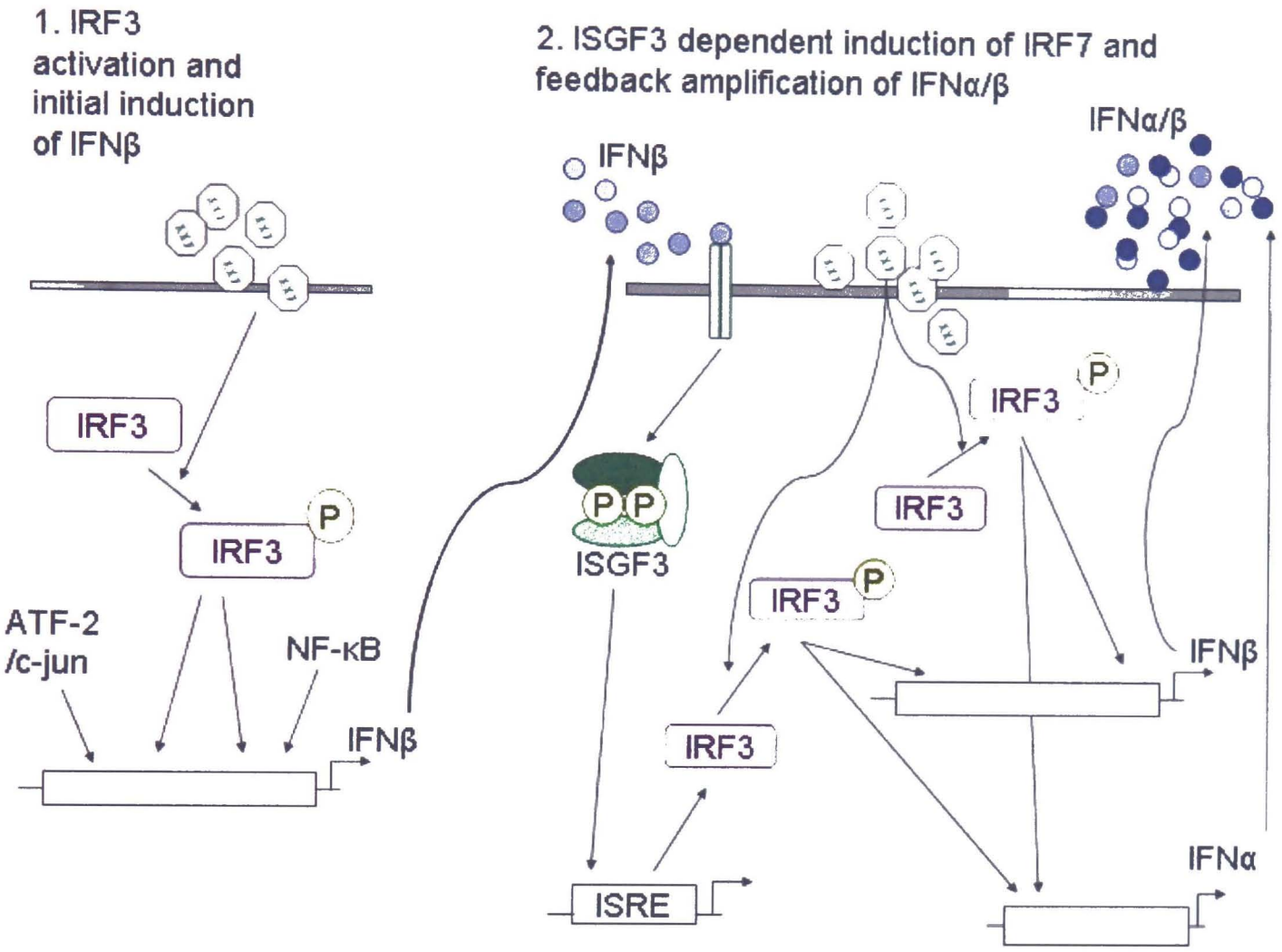


Fig 1.2. IFNα/β induction and feedback loop. 1. Virus infection stimulates a phosphorylation cascade, leading to the activation of at least three families of transcription factors, including NF-κB, AP-1 and IRF3. Activation of the IFN-β promoter requires all three transcription factors. **2.** IRF7 induction phase. Secretion of early IFNβ produces an autocrine response through stimulation of the JAK-STAT pathway by the IFN receptor system, leading to activation of the transcription factor complex, ISGF3. Among the genes activated by ISGF3 is IRF7, leading to accumulation of this protein only in response to IFN production. The continued presence of viral infection also leads to IRF7 phosphorylation and subsequent activation. Many members of the IFN-α gene family possess promoter binding sites for activated IRF7 and become transcriptionally active, augmenting the IFN response. (Taniguchi & Takaoka, 2002)

1.1.5.ii Toll like receptors

Until recently it was thought that the interferon response to viral factors occurred when dsRNA was detected, and directly activated the transcription factor IRF3. We now know that there are other ways to induce the IFN. Since the discovery of the Toll

like receptor (TLR) family, our understanding of how the innate immunity recognises pathogens and mounts and effective response has grown. Toll-like receptors were originally discovered in Drosophilia as receptors responsible for anti-fungal immunity (Lemaitre *et al*, 1996). Since then several mammalian equivalents were identified. There are now 11 members of the human TLR family all of which are transmembrane receptors that recognise different pathogen associated molecular patterns (PAMPs). Each TLR has a number of potential ligands and the ability to form hetero- and homo- dimers, suggesting that the TLRs contain a multitude of potential binding sites.

Table 1.1 Principle ligands of the human toll-like receptors(Smith *et al*, 2005)

Toll-like receptors	**Ligands**
TLR1 and 2	Bacterial or spirochetal lipoproteins
2 and 6	Bacterial lipoproteins
2	Peptidoglycan
3	Double stranded RNA
4	Lipopolysaccharide
5	Bacterial Flagellin
7	SsRNA
8	SsRNA
9	CpG DNA
10	Unknown
11	Uropathogenic Bacteria (In mice)

Signalling pathways via TLRs originate from the conserved TIR (Toll-IL1 receptor) domains via interactions with adaptor molecules such as myeloid differentiation factor 88 (Myd88), TIR associated protein (TIRAP), Toll receptor associated

molecule (TRAM) and Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF). Both TLR 7 and 9 have been shown to use the MyD88 adaptor molecule which can directly associate with IRF7, whilst TLR 3 and 4 transmit signals via TRIF leading to the activation of IRF3 which induces the transcription of IFNβ, which then activates the feedback loop to induce the transcription of the other type I IFNs. Although the idea of TLRs as viral sensors is an attractive mechanism, it has a few flaws that can only be accounted for by the presence of another viral alarm system such as the cytoplasmic recognition of dsRNA by RNA helicases.

1.1.5.iii Retinoic acid inducible gene-I

As TLRs are transmembrane proteins that exist either on the plasma membranes or in endosomes, they can only detect PAMPS in the extracellular space or when viruses enter the cells via a vesicular pathway. This does not account for all viruses; several RNA viruses which have been shown to be potent inducers of type I IFN enter the cytoplasm by fusing with the cell membrane or by directly injecting their nucleic acid; they do not encounter the TLRs. $MyD88^{-/-}TRIF^{-/-}$ mice have been shown to be unresponsive to stimulation with all known TLR ligands, (Yamamoto *et al*, 2003). However when $MyD88^{-/-}TRIF^{-/-}$ embryo fibroblasts were infected with Newcastle disease virus (NDV), IFNβ was still induced to the same extent as the wild type cells (Kato *et al*, 2005a).

The gene that was recently identified using an expression library, responsible for this IFNα/β induction in the absence of the TLR pathway was the retinoic acid inducible gene-I (RIG-I) (Yoneyama *et al*, 2004), the overexpression of which was also shown to inhibit both VSV and EMCV-induced infectivity. RIG-I is a DExD/D-box helicase that also contains two caspase recruitment domains (CARD). In the absence of any PAMPS, the RIG-I helicase domain suppresses the signalling actions of the CARD

domains, but the binding of the viral RNA allows the CARD domain of RIG-I to recruit the CARD adaptor inducing IFNβ (Cardif) protein. The mitrochondrial bound cardif then recruits IκK to activate NF-κB and IRF3. The importance of RIG-I in antiviral defense was demonstrated with RIG-I deficient mice (Kato *et al*, 2005b); RIG-I defends the host against NDV, VSV and SeV infections within the cytoplasm of mouse embryonic fibroblasts (MEFs) and DCs whereas the TLR antiviral defense recognised nucleic acids within endosomal compartments within pDCs.

Since the discovery of RIG-I, other closely related proteins which also lead to NFκB and IRF3 activation and also transmit antiviral responses through the cardif adaptor have been found, one such example is MDA5. Similar to RIG-I, MDA5 also senses cytoplasmic dsRNA. Taken together with the TLR pathways, these sensors all lead to the stimulation of the IFNβ gene which will then lead onto the feedback loop allowing an appropriate IFNα/β anitiviral response to occur.

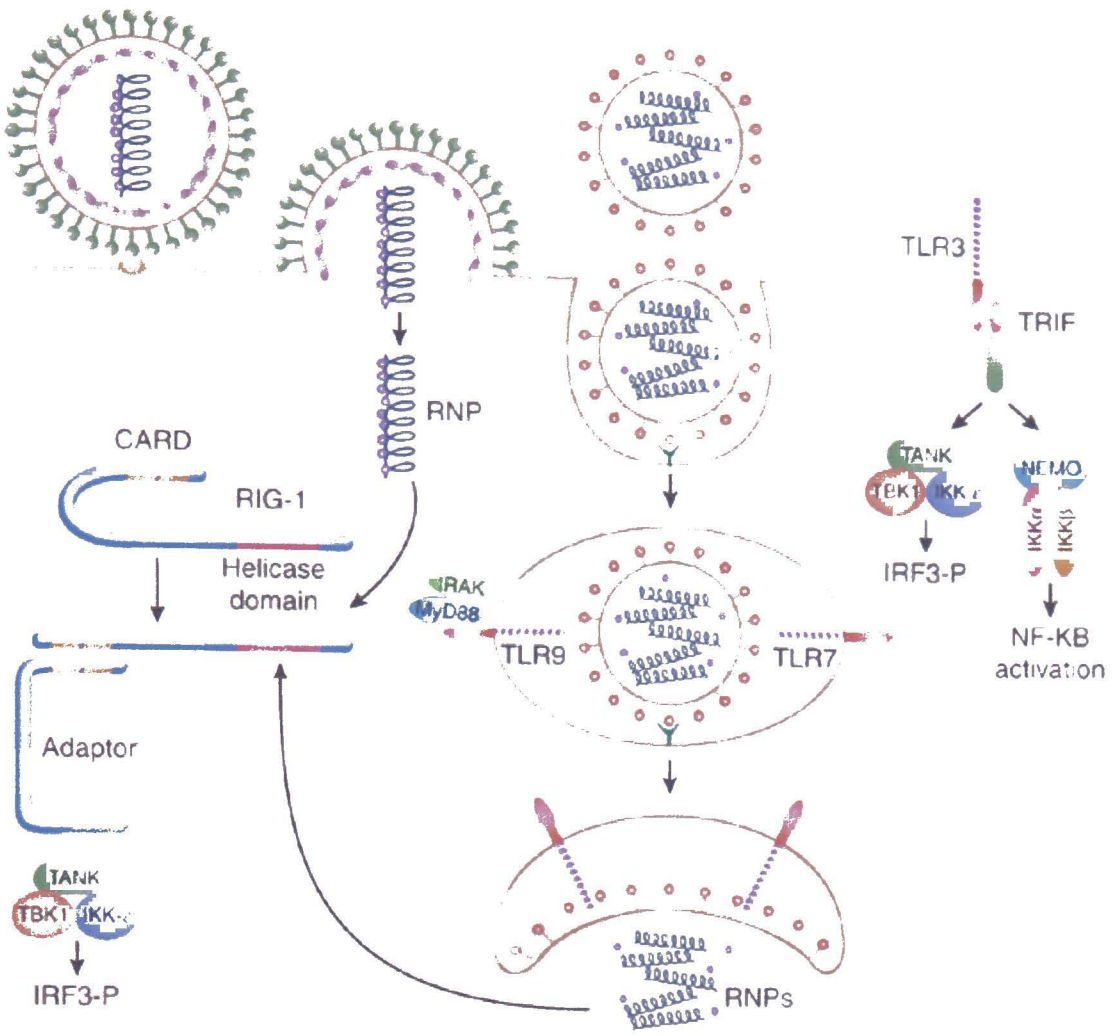


Fig1.3 Pathways of type I IFN induction. Most negative-strand RNA viruses enter cells by membrane fusion, either at the plasma membrane (left) or through an endocytic process (middle), and release their nucleic acid into the cytoplasm. TLR3 binds extracellular dsRNA and signals through adaptor protein association involving the TIR domain protein TRIF, which is required for signalling to two protein kinase complexes, TBK1-IKKε-and IKKα-IKKβ, leading to activation of IRF3 and NF-κB. TLR3 is found both at the cell surface as well as on endomembranes, along with the other nucleic acid sensors TLR7 and TLR9, which signal through the adaptor molecule MyD88 and the kinase IRAK. Whether these receptors ever 'see' viral nucleic acid components during primary infections is unknown. RIG-I processes viral RNPs with its helicase domain in the cytoplasm, resulting in signalling through its CARDs. Signal propagation presumably involves a CARD-containing adaptor protein that relays the signal to TBK1-IKKε kinase complexes to phosphorylate IRF3, and possibly to IKKα-IKKβ to activate NF-κB as well. (Levy & Marie, 2004)

1.1.6 Antiviral properties of Type I IFNs

The original definition of the interferons was based upon their antiviral abilities and it has since been shown that this role is vital to the survival of higher vertebrates. This early line of defence kicks in hours to days before the immune system can react. Mice lacking the IFN receptors (α,β and γ) show reduced resistance to viral infection.

Type I IFNs activate a cascade of intracellular signalling pathways leading to upregulation of more than a thousand IFN-stimulated genes (ISGs) within the cell. The function of some of the IFNα/β-induced proteins is well described, whereas

others remain poorly characterized. IN combination the upregulation of these ISGs confer an antiviral state, which limits the ability of the virus to infect neighbouring cells. The best studied genes are known to be involved in the antiviral mechanism of IFNα/β action such as PKR (Protein Kinase R), MxA and 2'5'OAS (2' 5' Oligo Adenylate Synthetase).

1.1.7 PKR System

Several different IFNα/β inducible proteins degrade viral RNA and inhibit protein synthesis, perhaps the most significant protein being PKR. PKR is a 68KDa protein that has a conserved domain characteristic of a serine threonine kinase and a domain that binds dsRNA (Meurs *et al*, 1990;Meurs E, 1990). It is ubiquitously expressed at low levels in cells; IFN causes an increase in its expression and it is activated by the binding of dsRNA which is generated during the replication of RNA and DNA viruses.

The PKR binds dsRNA, autophosphorylates and dimerises and is then capable of phosphorylating eIF-2a. PKR phosphorylates a serine residue on the alpha subunit of the eIF-2α leading to obstruction of protein synthesis. The factor eIF-2B mediates the guanine nucleotide exchange step required to regenerate active eIF-2GTP from the inactive eIF-2GDP that is produced after each round of initiation.

PKR also activates the NF-kB transcription factor which is required for IFNβ synthesis, enhances cytokine production and MHC I presentation. In virus-infected cells, PKR promotes cellular apoptosis through a Fas-dependent pathway, thereby preventing the spread of the virus to neighbouring cells. PKR also plays a role in modulating cell proliferation and growth; it has been implicated in the signal transduction pathway of IL-3, PDGF and IFNγ. Although activated by a low

concentration of dsRNA, high levels have been shown to inhibit the activity of PKR. This has lead to different models of activation for PKR (Proud, 1995).

1.1.8 The MxA System

The Mx proteins were originally discovered as IFNα/β-induced proteins that are capable of blocking the replication of influenza in a mouse model (Horisberger *et al*, 1983). Other members of the family were later shown to have an antiviral action against other RNA viruses. They are IFN-induced 70-80KDa GTPases of the dynamin family that have a tendency to self assemble, a relatively low affinity for GTP and a high rate of GTP hydrolysis.

Another family of the large GTP-binding proteins include the human guanylate-binding proteins (hGBP-1 and hGBP-2), human dynamin1 (hDyn1), the yeast dynamin homolog VPS1 (yVPS1) and human dynamin-related protein1 (hDrp1). The hGBPs are induced mainly by IFNγ; indeed GBP-1 is one of the most abundant antiviral proteins induced by IFNγ and has been shown to inhibit the replication of both EMCV and VSV (Vesticular stomatitis virus) (Anderson *et al*, 1999).

The Mx proteins act by modifying cellular functions needed for viruses to replicate (Nakayama *et al*, 1993;Nakayama M, 1993), thereby protecting the cells. IFNAR-/- mice expressing the human MxA gene, survived a lethal dose of Le Crosse virus in comparison to mice not expressing the human MxA gene (Hefti *et al*, 1999).

In humans there are two distinct Mx GTPases; MxA and MxB, which are encoded on chromosome 21. Only MxA has detectable antiviral activity, shown against a wide spectrum of viruses (including bunya-, orthomyxo-, paramyxo-, rhabdo-, toga-, picorna- and hepatitis B viruses). Murine Mx1, in comparison appears to be specific for orthomyxoviruses, interfering with the influenza viral replication at the primary

transcription level. The difference in cellular localisation between the Mx proteins appears to give rise to their specificity; Mx1 appears to be specific for viruses that accumulate in the nucleus whereas, MxA accumulates in the cytoplasm of interferon treated cells and is partly associated with the ER where it forms tight oligomeric complexes (Richter *et al*, 1995).

Although the molecular mechanism of Mx action is not yet well understood, the GTPase activity seems to be essential for the antiviral function (Pitossi *et al*, 1993). MxA has been shown to retain the nucleocapsid of Thogoto virus in the cytoplasm thereby preventing the virus from entering the nucleus and transcribing its genome, which can be reversed with specific MxA antibodies (Haller & Kochs, 2002). Conversely MxA has been show to exert it's antiviral effect against Semliki Forest Virus without the presence of the viral structural genes (Landis *et al*, 1998).

1.1.9 The 2'5' OAS system

PKR and the Mx proteins are preferentially induced by type I IFNs, other antiviral systems such as 2'5'OAS synthetase can be induced by both types. The 2'5'OAS synthetase is a family of enzymes that are activated by dsRNA to synthesize 2'-5'-linked oligoadenylates. The number of adenosine bases varies; one form dimerises inactive RNaseL leading to the degradation of mRNA and another has been shown to bind to the bcl-2 family of proteins that has been associated with apoptosis. The different forms of the 2'5'OAS synthetase have different IFN and dsRNA dose response relationships and are active in different subcellular locations. The 2'5'OAS system has been shown to inhibit the replication of picornaviruses such as EMCV, which is thought to occur mainly in the cytoplasm (Williams & Kerr, 1978), but other viruses such as VSV are thought to avoid the attention of the RNaseL as their replication occurs in the nucleus (Chebath *et al*, 1987).

1.1.10 The ADAR system

Another way of inhibiting the virus by means of the type I interferon system is to disrupt its coding sequence. ADAR adenosine diaminase edits adenosine to inosine, which provides a mechanism to alter the functional activity of viral and cellular RNA. This usually occurs on viral RNA in particular negative strand RNA genomes resulting in mutations within the viral RNA (Bass BL, 1997).

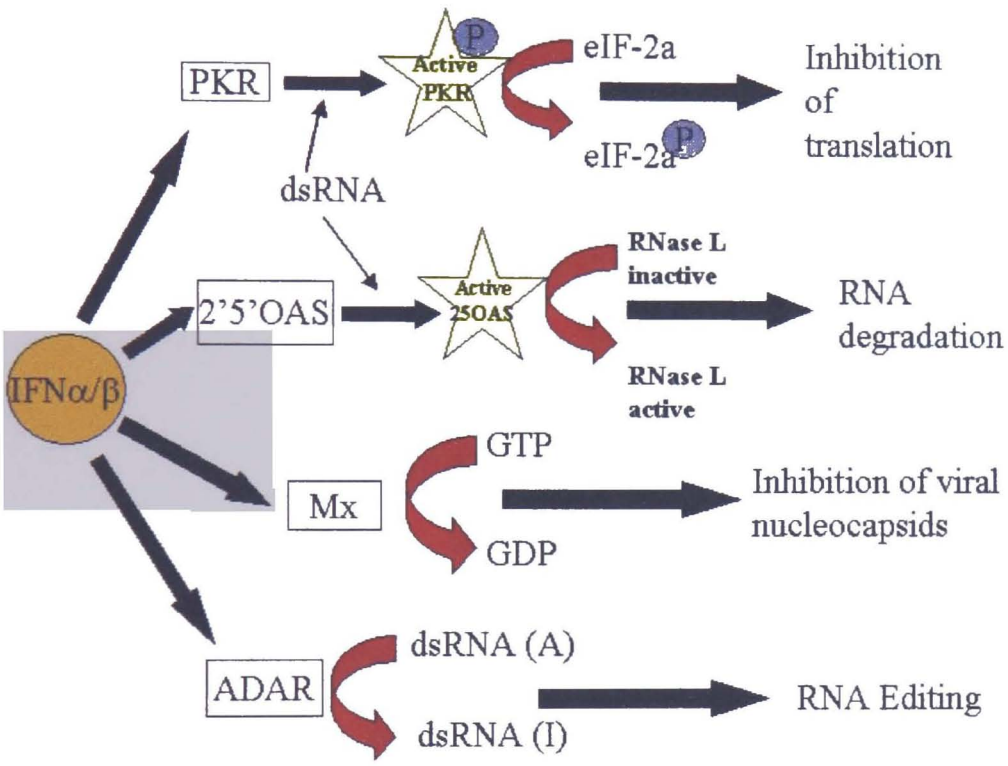


Fig 1.4 Antiviral mechanisms of IFN action. Several proteins within the cells inhibit viral replication. PKR inhibits translation initiation through the phosphorylation of protein synthesis initiation factor eIF-2a; 2'5'OAS family mediates RNA degradation; the Mx protein GTPases target viral nucleocapsids and inhibits viral RNA synthesis; and ADAR edits dsRNA by deamination of adenosine to inosine.

However, as with all things, a state of equilibrium exists, as the immune system via the interferon pathway fights the virus, most viruses have evolved ingenious strategies to interfere with the IFNα/β pathway. These include blocking the IFNα/β induction/expression, intercepting receptor binding of IFNs through viral decoy type I IFN receptors, perturbation of the intracellular IFNα/β signalling pathway and directly down- regulating the level of expression of ISGs.

1.2.1 The viruses fight back

Different viruses have evolved different mechanisms to evade the antiviral effects of IFNα/β. Broadly, these fall into three categories, inhibition of: 1. IFNα/β induction, 2. JAKSTAT pathway and 3. antiviral gene product synthesis. Both RNA and DNA viruses have been shown to inhibit the function of the antiviral gene products and suppress IFN induction and some viruses have developed a combination of strategies to avoid the antiviral effect of IFNα/β.

Table 1.2 How different viruses evade the type I IFN response

Mechanism	Viral Proteins
IFN induction	Influenza NS1
	Ebola VP35
	Bunyavirus NS1
JAK/STAT	Mumps V protein
	Adenovirus E1A
	Ebola VP35
	Simian Virus 5 protein
Antiviral gene product synthesis	HIV TAT
	Adenovirus VA1
	Influenza NS1

1.2.2 Inhibition of Interferon induction

Viruses have developed mechanisms to avoid detection e.g. 1) negative strand RNA viruses encapsidate both their genomic and antigenomic RNA, 2) retroviruses integrate their viral genome into the host's and use the host cell machinery for their transcription and replication, 3) positive strand RNA viruses replicate within

intracellular membrane vesicles and 4) some viruses minimise the production of dsRNA to prevent the induction of IFNα/β. Ebola virus infection has been shown to block host cell responses to IFNs, inhibit induction of antiviral gene expression, and block IFNα/β production from infected cells. Expression of the Ebola virus protein VP35 complemented the growth of the IFN-sensitive influenza virus and also prevented the transcriptional activation of both the beta IFN (IFN-ß) promoter and the IFN-stimulated ISG54 promoter (Basler *et al*, 2000). VP35 was later shown to block IFNβ production by inhibiting IRF3 phosphorylation and thereby blocking its subsequent nuclear transportation (Basler *et al*, 2003). Several other viruses have been reported to target IRF3 activation, including the influenza virus, which uses the dsRNA binding ability of its NS1 protein (Talon *et al*, 2000) and the E3L protein of the vaccinia virus which uses a similar mechanism (Smith *et al*, 2001).

Hepatitis A virus (HAV) is a picornavirus, with a positive-stranded RNA genome. Although HAV targets the liver, it is transmitted by the faecal-oral route but although in cultured cells it leads to a persistant, non-cytopathic infection. Within its natural host it does not lead to chronic disease. It has been shown that HAV infection does not induce IFNα in lymphocytes or IFNβ in fibroblasts due to the ability of the virus to inhibit the ds-RNA induction of interferon (Brack *et al*, 2002). This was later narrowed down to an inhibition of IRF3 phosphorylation by preventing RIG-I signalling to the IKKε/TBK1 kinases which activate IRF3 (Fensterl *et al*, 2005).

As HAV replication occurs in association with intracellular vesicles, IFNβ induction could be stimulated via TLR3, but HAV has also been shown to inhibit this pathway. Although the viral protein responsible for this inhibition has yet to be found, the inhibition of IRF3 by HAV is a allows the virus to establish a persistent infection in cell cultures and evade the antiviral effects of type I IFN.

1.2.3 Inhibition of the STAT proteins

The STATs are essential mediators of interferon signalling and numerous viruses have found ways to stop their action, e.g. several members of the *Paramyxoviridae* have been shown to inactivate the STAT proteins, mainly through the action of the V protein. The paramyxoviruses are a large family of enveloped, negative strand RNA viruses that cause a multitude of diseases. Within the family several genera exist such as the *Rubulavirus, Respirovirus and Morbillivirus*. The V protein of the Paramyxoviridae have been implicated in many mechanisms, including IFN signalling inhibition, inhibition of dsRNA signalling and direct interaction with STAT proteins. The V protein contains a highly conserved cysteine rich region which has been shown to be responsible for the attenuation of the antiviral effects of interferon. STAT1 was first found to be reduced in cells infected with the simian virus 5 (SV5) and this was due to the expression of the V protein, and the ability of the SV5-V protein to target the STAT1 protein for polyubiquitination and proteasomal degradation (Didcock *et al*, 1999). The ubiquitination of STAT1 was later shown to require the presence of STAT2 (Parisien *et al*, 2002), whilst the polyubiquitination of STAT3 that occurs with the Mumps virus was STAT2 independent (Ulane *et al*, 2003).

Newly emerged paramyxoviruses e.g. Nipah and Hendra viruses also use their V protein to evade the IFN response through a different mechanism; they sequester STAT1 and STAT2 in high molecular weight cytoplasmic complexes without inducing their polyubiquitination (Rodriguez *et al*, 2002). They can also transport activated STAT1 from the nucleus back to the cytoplasm. A similar mechanism is used by the Measles virus, which blocks IFN induced STAT1 and STAT2 nuclear transport (Palosaari *et al*, 2003).

1.2.4 Inhibition of the function of the antiviral gene products

Several viruses have developed more than one way to evade the interferon response, such as the influenza virus. Influenza is a member of the orthomyxovirus family that has a segmented negative strand genome which encodes 10 proteins. With regards to IFN response the only influenza protein that appears to function as an IFN antagonist is the NS1 protein. The NS1 protein is an RNA-binding protein; it was recognised as an important antagonist of IFNα when an NS1 knockout influenza virus was generated, although the virus was still viable, it was highly sensitive to the antiviral effects of IFNα and could only replicate efficiently in cells where various arms of the interferon antiviral pathway are knocked out. Using micro-array studies the NS1 deleted viruses induced a higher expression of ISGs and NFκB in comparison to the wild type virus. In addition, NS1 has also been shown to directly interact with PKR and inhibit its kinase function.

Another virus which has been shown to interact with the PKR system is the vaccinia virus. Vaccinia virus (VV) is a member of the Orthopoxvirus genus; it has a large complex virion and a dsDNA genome that encodes approximately 200 proteins, several of which are specifically employed to avoid the actions of interferon. Proteins E3L and K3L have been shown to act intracellularly against PKR.

The E3L gene encodes two proteins; p20 and p25 that bind cytoplasmic dsRNA sequestering them away from PKR, thereby causing their inactivation (Chang *et al*, 1992). The K3L protein exhibits some amino acid homology to eIF2α such that K3L can competitively inhibit PKR phosphorylation (Beattie *et al*, 1991). Deletion of either E3L or K3L leads to a virus with enhanced sensitivity to the antiviral effects of IFN (Beattie *et al*, 1995). The E3L protein has also been shown to block the antiviral mechanisms of 2'5'OAS (Rivas *et al*, 1998) and ADAR (Liu *et al*, 2001) as well as

blocking IFN induction through IRF3 and IRF7. Yet another protein, VH1, has been shown to bind and dephosphorylate STAT1, which could be another mechanism to downregulate the IFN antiviral response.

The othopoxviruses also encode IFNα/β receptor homologues to evade the antiviral responses of interferon (Symons *et al*, 1995). The B18R gene of the Western Reserve strain of VV encodes a protein that can function as a soluble receptor that binds type I IFN with high affinity despite the lack of homology to IFNAR. It can also bind to the surface of cells and inhibit IFN activity (Alcami *et al*, 2000).

1.2.5 Other inhibitory methods

Although past work has focused upon the inhibitory mechanisms that viruses use to perturb the interferon system, due to the large volume of research in the last few years into other viral sensors, evasion mechanisms have also been described against the TLR and the helicase systems.

A vaccinia encoded protein (A46R) contains a TIR domain which it uses to inhibit TRIF mediated IRF3 phosphorylation but A46R can also binds to MyD88, TIRAP and TRAM thereby also interferrring with NF-κB and MAP kinase activation (Stack *et al*, 2005) . The V proteins of paramyxoviruses have been reported to specifically bind to and inhibit MDA5-dependent type I IFN production but to have no effect on the RIG-I dependent IFN signalling (Yoneyama *et al*, 2005); (Childs *et al*, 2007).

However, HCV has been shown to inhibit both the RIG-I and TLR3, but due to the importance of IFNα treatment in patients with Hepatitis C infection, the inhibition of IFN by this Flaviviridae is a much researched field and is the subject of this thesis.

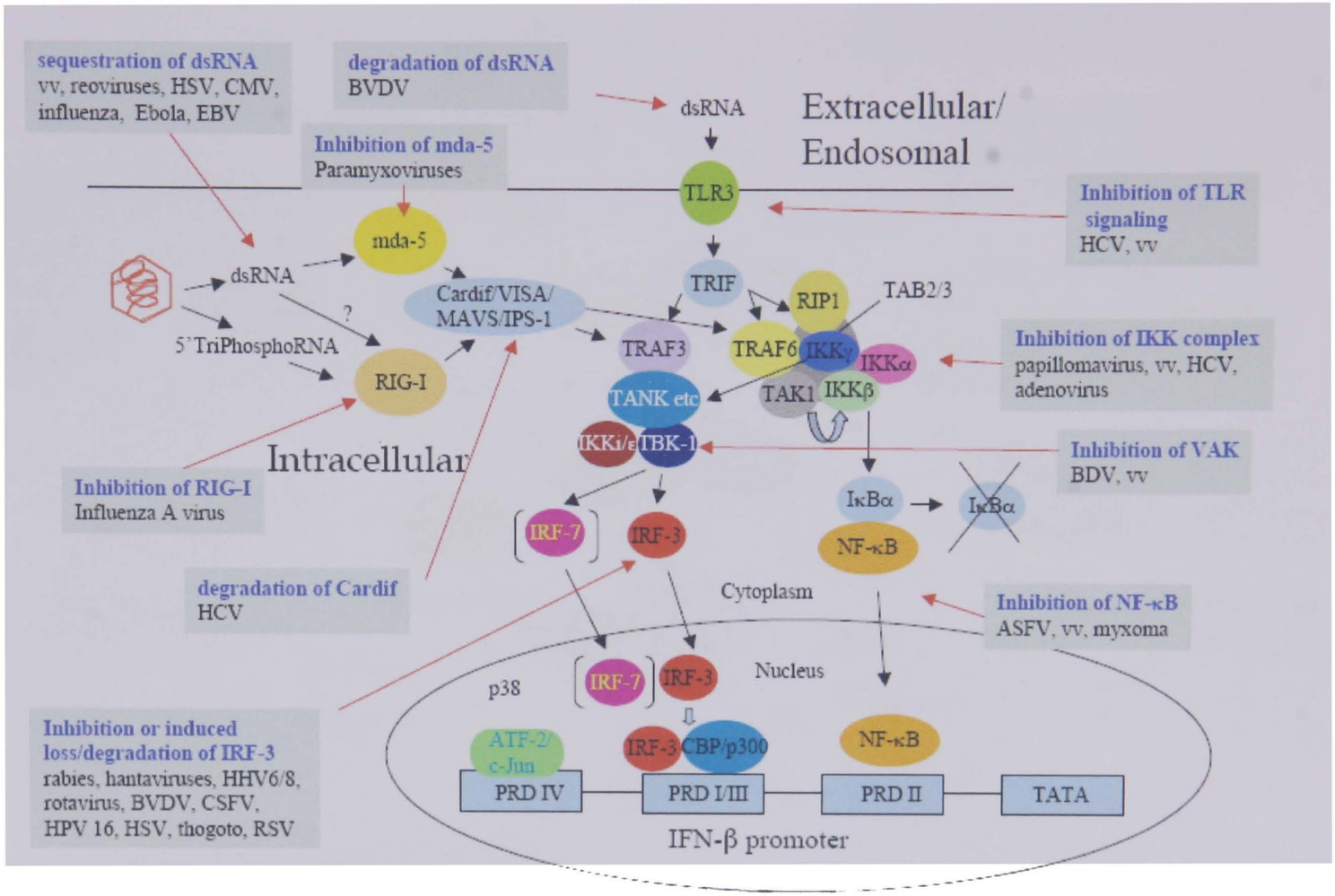


Fig 1.5 Viral antagonists of IFNα/β production

Examples of viral IFN antagonists that specifically block/limit the production of IFNα/β from virally infected cells. (Randall R. In press)

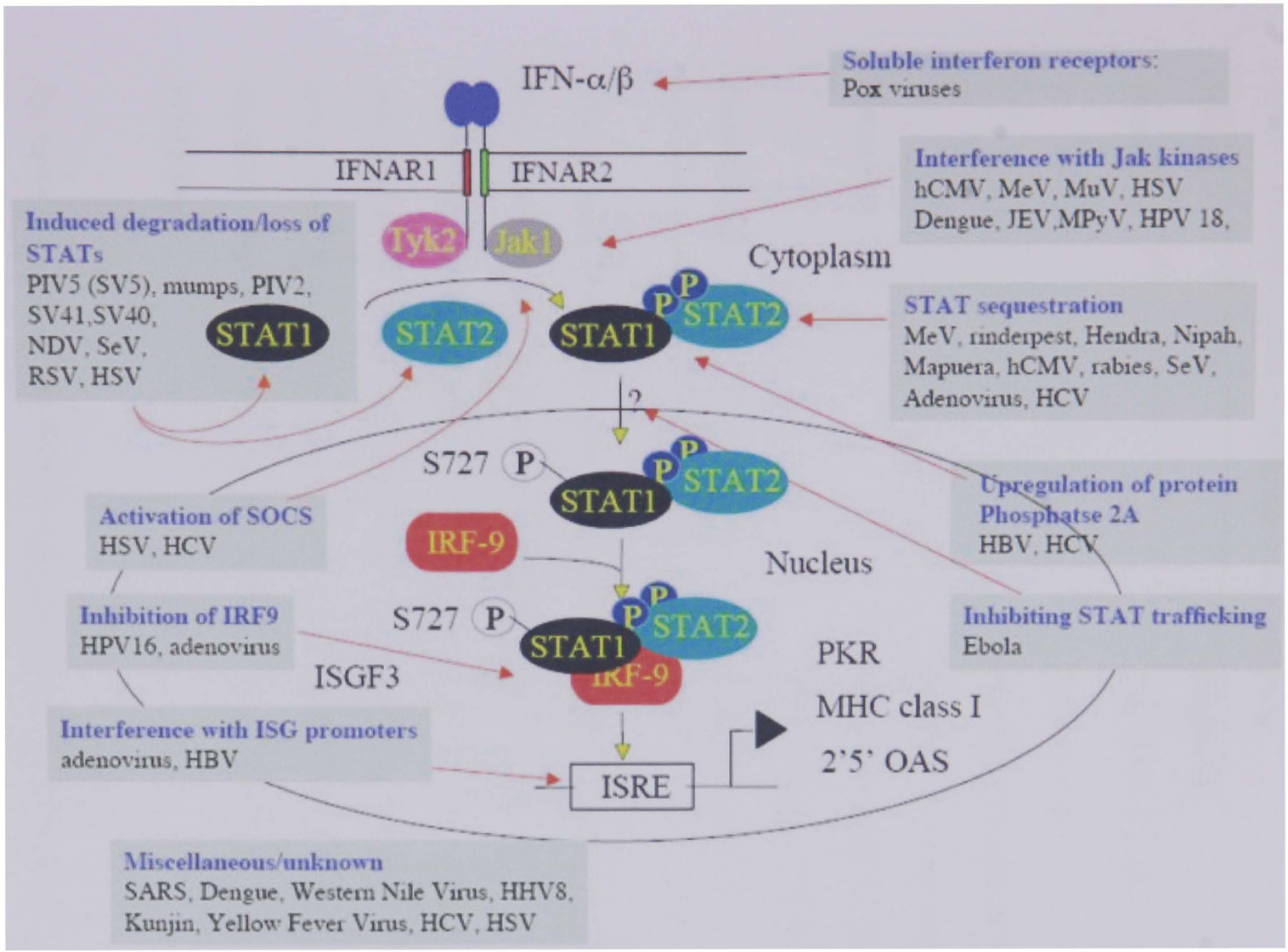


Fig 1. 6 Examples of viral antagonists that specifically inhibit IFNα/β signalling within virally infected cells. (Randall R. In press)

1.3.1 *Flaviviridae*

HCV is a member of the *Flaviviridae* family which is composed of three genera; Flavivirus, Pestivirus and Hepacivirus of which HCV falls into the latter group.

Table 1.3 Examples of the classification of the *Flaviviridae*.

Genus	Examples
Flavivirus	Yellow fever virus
	Dengue virus
	Japanese encephalitis virus
	Tick-borne encephalitis virus
Pestivirus	Bovine viral diarrhoea virus
	Classical swine fever virus
Hepacivirus	Hepatitis C Virus

The viruses of the *Flaviviridae* are defined by their positive-sense single stranded RNA genome and a single polypeptide protein produced by translation of a single large open reading frame (ORF) which is subsequently cleaved by host and viral proteases. Both the Pestivirus and the Hepacivirus genus contain internal ribosomal entry sites (IRES) that provide a site of translation initiation for host ribosomes. In contrast, the Flavivirus genus uses ribosomal scanning to commence protein synthesis.

An estimated 3% of the World's population are infected with HCV, an infection that can lead to cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC). Although the amino acid and nucleotide homology between HCV and other viruses in the *Flaviviridae* is poor, several common features have been discovered.

1.3.2 The discovery of Hepatitis C virus

Hepatitis C is a viral infection of the liver which was originally referred to as 'non-A, non-B' hepatitis until identification of the causative agent was achieved in 1989. HCV was discovered by cloning a fragment of viral cDNA from a chimp infected with Non-A, Non-B hepatitis. Subsequently, the entire genome was cloned which was shown to be infectious when injected intrahepatically into a chimpanzee(Kolykhalov *et al*, 1997).

HCV has always been known as a difficult virus to work with and until recently, research was hampered by the lack of an infectious molecular clone of HCV and the inability to propagate the virus in tissue culture. With the development of infectious clones, HCV replicon system(Lohmann *et al*, 1999b) and a transgenic mouse model for HCV infection, the field has moved on drastically.

1.3.3 Transmission

HCV is a major human pathogen, chronically infecting about 170 million people worldwide, causing cirrhosis and hepatocellular carcinoma (HCC). Factors most strongly associated with HCV infection are illicit drug use and receipt of blood or blood products prior to an adequate screening program. In the UK, virtually every haemophiliac (~3,500) who received pooled factor 8 in the early 1980s became HCV positive (Watson *et al*, 1992), but the risk of acquiring HCV from transfusions has since been diminished. Other risk factors include; lack or inadequately sterilised medical equipment, high risk sexual behaviours and social or cultural practices such as circumcision and tattooing. Although rare, perinatal infection can occur; approximatley 5% with a higher incidence seen in mothers with HIV co-infection.

1.3.4 Diagnosis

The biological markers of liver disease are serum alanine aminotransferase activity (ALT) and aspartate aminotransferase activity (AST), both of which are normally found in hepatocytes but are released into the serum as a result of liver damage. Upon HCV infection their levels increase about ten-fold, but they are a poor guide to the extent of hepatic inflammation and are not specific to HCV liver damage. Liver biopsies, normally carried out pre-treatment are used to determine the degree of fibrosis and liver damage.

Initial diagnosis of HCV is based on serological antibody tests, either enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA) which detect a mixture of antibodies directed against various HCV epitopes. The latest generations of tests are highly reliable and have an accuracy rate of up to 99%. To detect HCV RNA, a marker of active HCV replication, a qualitative PCR is used. HCV RNA is detectable in the serum as early as 1-2 weeks after infection, which generally decreases and disappears in acute infections but in chronic infections the HCV RNA levels, gradually decline before reaching a plateau. For chronic HCV, quantitative PCR is used to monitor the efficiency of antiviral treatments and HCV clearance, which is usually bi-phasic; a rapid initial decline followed by a more prolonged response.

1.3.5 Natural History of HCV Infection

Acute hepatitis C infection is usually sub-clinical. After an incubation period of about 4-20 weeks, about 20 % of patients with acute hepatitis C become jaundiced; often accompanied with fatigue, muscular pain, low-grade fever, nausea and vomiting. Around 20% of patients infected will effectively clear the virus, the majority will develop a persistent infection and of these a high proportion will develop chronic liver damage. At least 20% of patients with chronic hepatitis C develop cirrhosis,

characterised by regenerating nodules and a build up of fibrous tissue, a process that takes at least 10 to 20 years. After 20 to 40 years, a smaller percentage of patients with chronic disease develop hepatocellular carcinoma (HCC). Liver failure from chronic hepatitis C is one of the most common reasons for liver transplants in the United States and hepatitis C infection is currently the cause of about half of cases of primary liver cancer in the developed world.

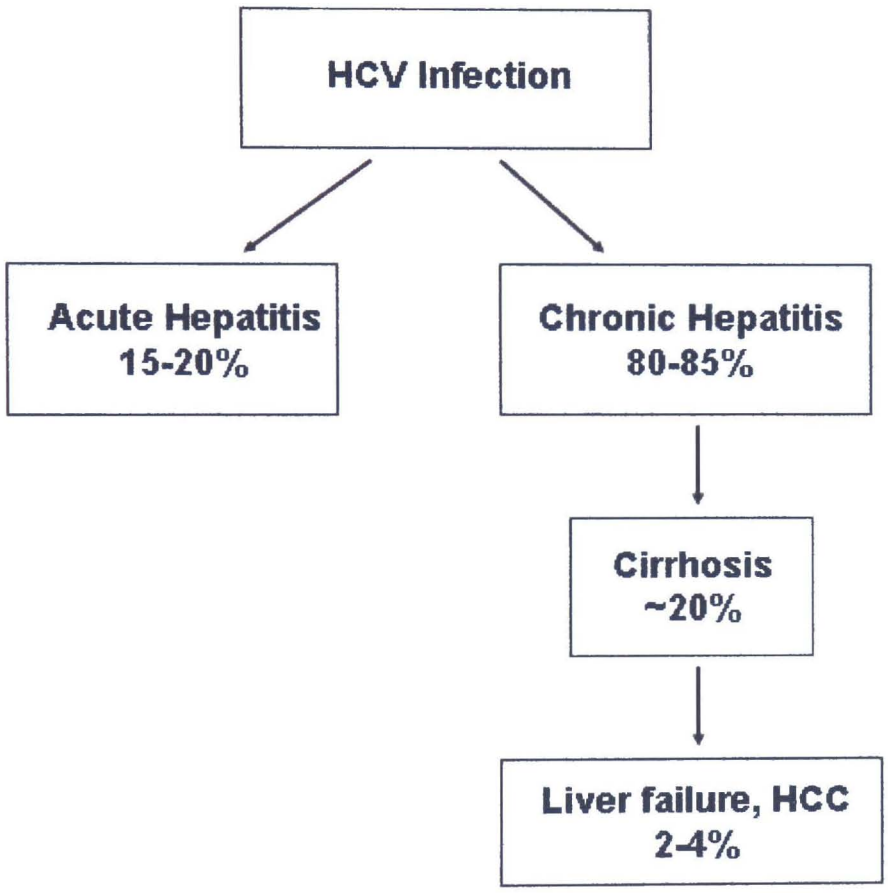


Fig 1.7 Natural history of Hepatitis C infection. HCV is a hepatotrophic virus which causes an acute infection in 20% of patients. 80-85% of patients develop a chronic disease with life threatening consequences such as liver failure and hepatocellular carcinoma.

1.3.6 HCV Molecular Biology

HCV has a positive stranded RNA genome that contains a single open reading frame (ORF) encoding a single polyprotein of 3011a.a in length which is processed by host cell and viral proteases into 3 major structural proteins and several non-structural proteins, necessary for viral replication (Choo *et al*, 1989;Kato *et al*, 1990)

1.3.7 The HCV virion

Due to difficulties of replicating the virus in tissue culture, the elucidation of the structure of the HCV virion has been hampered. It is known that HCV is a spherical

enveloped virus (Feinstone *et al*, 1983) of approximately 50nm in diameter (Bradley, 1985). Potential HCV particles have been observed in human plasma, chimp liver and experimentally infected or transfected cell lines.

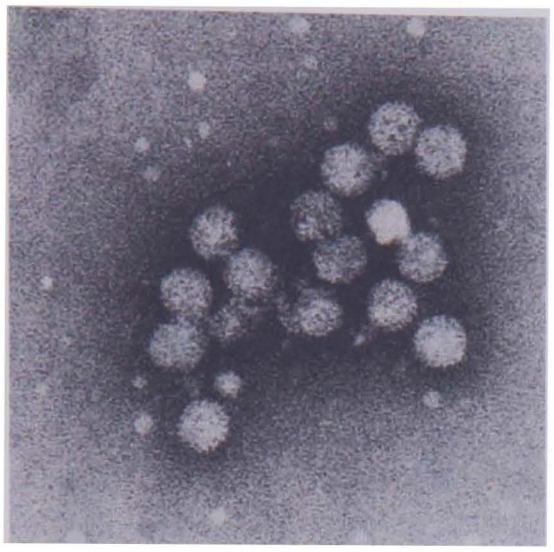


Fig 1.8 Electron microscopy of the HCV virion. (Taken from www.epidemic.org/theFacts/ hepatitisC/hepatitisC.html)

1.3.8 HCV genome structure

The HCV genome is translated as a large polyprotein which is cleaved into individual proteins during and following translation. The HCV proteins were identified from mammalian and insect cells transfected with HCV cDNA. The location of the cleavage sites have been identified using amino acid N-terminal sequence analysis of the resulting protein products.

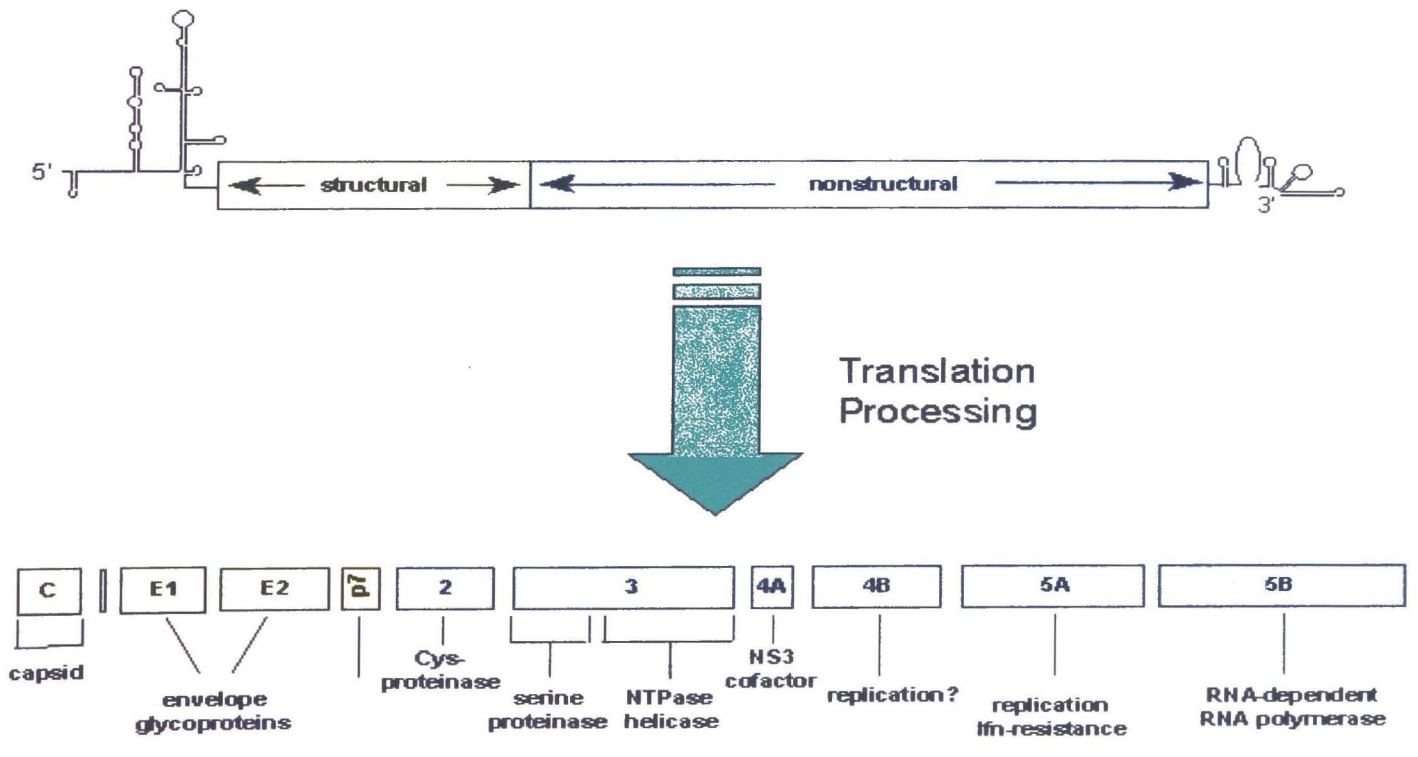


Fig. 1.9 Genomic organisation of HCV. A schematic representation of the HCV genome and its translation products. The boxed area corresponds to the single open reading frame of the hepatitis C virus (HCV) genome. The stem–loop structures represent the 5' and 3' non-translated (NTR) regions, including the internal ribosome-entry site (IRES).

The proteins have been classified into structural and non-structural. The structural proteins (core, E1, E2 and p7) form the nucleocapsid and the envelope of the virus i.e. the virion, whilst the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) encode the proteinase, helicase, RNA polymerase and co-factors necessary for viral replication.

Table 1.4 Putative functions of the HCV proteins. Numbering based on the HCV-H strain. gp=glycoproteins

Protein	Nucleotide Location	Amino Acid Location	Mol Wt. (kDa)	Function
5'UTR	1-341			Initiation of translation
Core	342-857	1-191	p21	Nucleocapsid of virion
E1	915-1490	192-383	gp31	Envelope glycoprotein
E2	1491-2579	384-746	gp70	Envelope glycoprotein
p7	2580-2768	384-809	p7	Viroporin – ion channel
NS2	2769-3419	810-1026	p21	Proteinase
NS3	3420-5312	1027-1657	p70	Proteinase & NTPase/Helicase
NS4a	5313-5476	1658-1711	p6	NS3 Co-factor
NS4b	5477-6257	1712-1972	p27	Replicase component
NS5a	6258-7600	1973-2420	p58	Replicase component, ISDR
NS5b	7601-9374	2421-3011	p68	RNA polymerase
3'UTR	9375-9621			Replication & Packaging

The non-structural proteins are involved in the polyprotein processing and RNA replication of the virus. The processing of the polyprotein requires the presence of two distinct proteinases, the NS2 protein and the N-terminal region of the NS3 form one, a zinc-dependent metalloproteinase and the N-terminal region of the NS3 protein form the other, a serine protease. The NS2-NS3 proteinase cleaves at the

NS2/NS3 site and the remaining NS proteins are released by the NS3 serine proteinase in association with its co-factor NS4a. (Hijikata *et al*, 1993).

1.3.9 5'UTR

The structural proteins are encoded by the N-terminal end of the polyprotein ORF but are preceded by a 5' untranslated region (UTR) which consists of 341 nucleotides (Choo *et al*, 1991). Although the nucleotide sequence of HCV is highly variable as the viral polymerase has no proof-reading ability, the 5'UTR is a highly conserved hairpin structure, closely related to the 5'UTR of the pestiviruses (Bukh *et al*, 1992). The 5'UTR contains 4 highly strucutured domains, numbered I to IV. Domains II, III and IV together with the first few nucleotides of the core gene make up the regulatory elements important for viral gene expression namely, the internal ribosomal entry site (IRES). The IRES allows the expression of the viral genome in a cap independent manner. The IRES of HCV is bound specifically by the host cell 40s ribosomal subunit and the eukaryotic initiation factor eIF3. These interactions allow for the formation of the ribosome complex that leads to the initiation of protein synthesis initiated in a manner distinct from the host (Lytle *et al*, 2001).

1.3.10 Core

The core protein is an RNA-binding polypeptide that binds to the HCV genome to form the viral nucleocapsid. It is derived from the N-terminal 191 amino acids of the polyprotein and is well conserved among all isolates (Bukh *et al*, 1994). The core protein is mostly cytoplasmic, but three possible nuclear localisation signals have been identified in the N-terminal region of the protein, making it possible for a truncated version of core to be translocated to the nucleus (Suzuki *et al*, 1995) . Core has been shown to bind to a number of cellular proteins including apolipoprotein AII, a cellular helicase, a heterogeneous nuclear ribonuclearprotein K and a bZIP

transcription factor. It can transactivate or repress a number of cellular promoters and interacts with c-JNK, ErK and MAPK signalling. Core has been shown to interact with the lymphotoxin-β receptor (LTβR) which is involved in the developmental regulation of lymphoid organs, lymph node development and apoptopic signalling thereby the binding of core to LTβR could lead to HCV persistence.

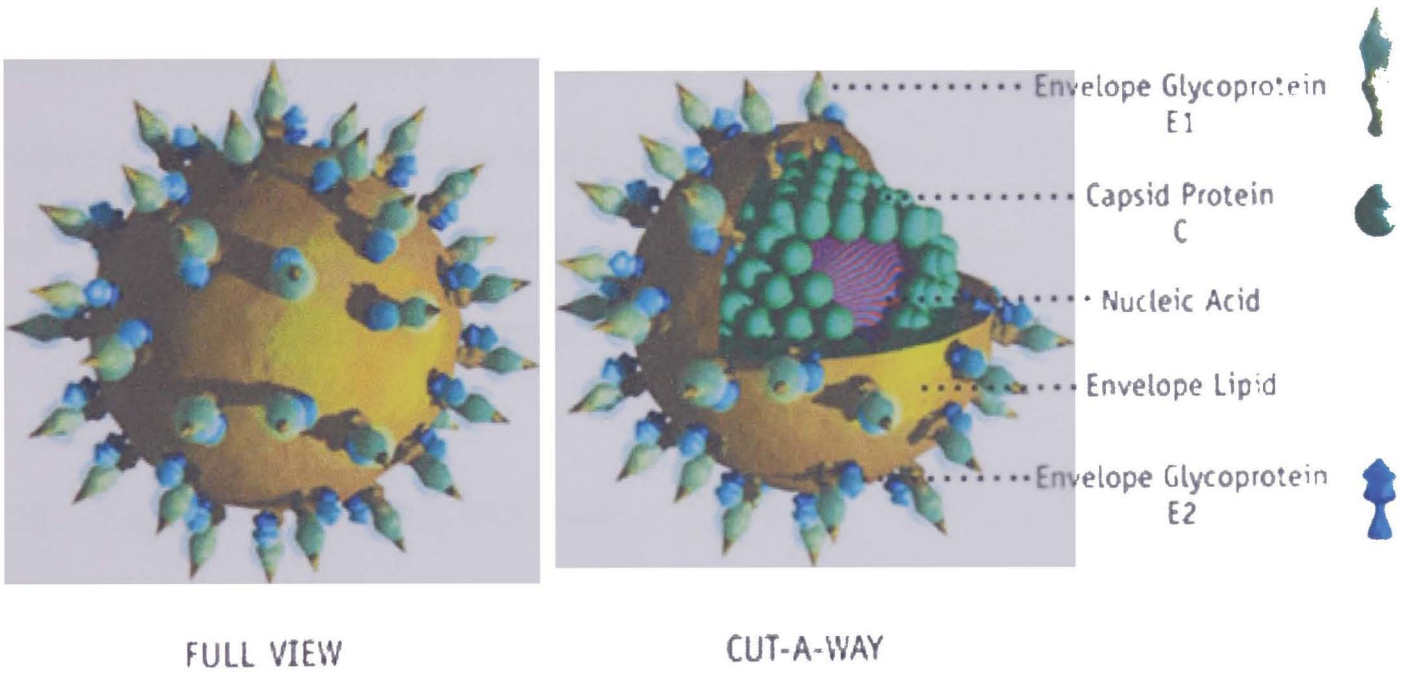
1.3.11 The Envelope proteins (E1, E2 and p7)

The viral envelope proteins, E1 and E2, located in the N-terminal region of the polyprotein, are highly glycosylated, transmembrane proteins that form stable non-covalent heterodimers (Deleersnyder *et al*, 1997). Also, E1 has been shown to associate through its carboxy terminus with the core protein (Lo *et al*, 1995) and E2 associates with the NS2 protein (Selby *et al*, 1994). Expression of cloned HCV cDNA in baculovirus has indicated that an internal signal sequence, upstream of the E1 and E2 proteins direct the precursor polyprotein to the endoplasmic reticulum (ER), where translocation into the lumen and signal sequence cleavage takes place by host signal peptidases (Hijikata *et al*, 1991). A high degree of variation occurs in the N-terminus of the E2 protein (aa 1-27), which is referred to as the hyper-variable region 1(HVR-1). The HVR-1 epitopes are probably presented on the surface of the virion as part of the viral envelope, and contains a neutralizing epitope, which is susceptible to immune pressure and the selection of escape mutants. There have been several contradictory studies on the mutations of the HVR-1 region; it may alter B cell or T cell epitopes and favour viral persistence. The E2 protein contains a region that can compete with eIF-2α for binding to PKR, thereby preventing the inactivation of translation by PKR (Taylor DR, 1999). This region is highly variable between HCV isolates and differences have also been observed between the HCV genotypes.

The p7 protein is something of an enigma. It is not necessary for RNA replication and therefore does not fit in with the non-structural proteins but it is not known whether it is a virion component either. It is a small (63 a.a), highly hydrophobic protein which has similar characteristics to viroporins, proteins that form ion channels, most often involved in virus assembly or entry/exit. It has been shown that amantadine specifically inhibits the ion channel activity of the p7 protein of HCV on artificial membranes (Griffin *et al*, 2003). Together the HCV structural proteins assemble to form a protective structure to surround the viral nucleic acid. (Fig 1.9)

Fig 1.10 Model of the assembled structure of hepatitis C virus.

(Taken from http://www.prn.org/images)



1.3.12 The Non-Structural proteins.

The non-structural proteins of HCV are involved in polyprotein processing and RNA replication.

1.3.13 NS2

The NS2 is the first of the non-structural HCV proteins. It encodes a protein of approximately 21-23KDa. NS2 is a transmembrane protein downstream of p7 with the C-terminus located in the lumen of the ER and the N-terminus in the cytosol. The NS2 is a metalo-proteinase, specific for the NS2-NS3 cleavage. The active domain responsible for the cleavage encompasses the C terminus of NS2 as well as the N terminus of NS3 (Hijikata *et al*, 1993). As soon as the NS2-NS3 proteinase cleavage occurs the activity is lost.

1.3.14 NS3

The NS3 protein is approximately 70KDa and has been shown to posses three different catalytic activities; a protease, helicase and an ATPase function. The C-terminal domain contains the NTPase/helicase function and the N-terminal domain contains the serine protease activity. The amino acid sequence of the NS3 is fairly well conserved across the HCV genotypes and the functional motifs of NS3 are conserved across the *Flaviviridae*.

The N-terminal contains the serine proteinase, which cleaves at the NS3/4A, NS4A/B, NS4B/NS5A and NS5A/B sites (Bartenschlager *et al*, 1993). Cleavage at these positions was abolished when mutations were introduced to the predicted residues of the serine protease catalytic triad His^{1083}, Asp^{1107} and Ser^{1165} (Bartenschlager *et al*, 1993), (Tomei *et al*, 1993).

Recent knockout experiments show that the NS3 helicase activity is essential for virus replication (Gu *et al*, 2000). The negative-strand RNA intermediate is used as a template for the synthesis of the positive-strand progeny RNA. As the strands are complementary, the NS3 helicase is necessary for strand separation.

A role for NS3 has also been suggested in host cell transformation, which may be involved in hepatic cancer and liver cirrhosis associated with HCV infection (Sakamuro *et al*, 1995). The N-terminal domain (protease) of the NS3 protein may have a suppressive effect on apoptosis by decreasing the amount of cellular p53 (Fujita *et al*, 1996), p53 has been found to enhance the nuclear localisation of NS3 (Ishido *et al*, 1997).

1.3.15 NS4a

The NS4a is a small protein of 54a.a, which forms a stable complex with the NS3 protein and acts as an essential cofactor of the NS3 proteinase. It is essential for the cleavage of the NS4B/NS5A site and plays an important role in the NS3-mediated cleavage of other sites (Tomei *et al*, 1993). Stable NS3-NS4a complex formation requires the 22a.a. residues at the N-terminal of the NS3 protein (Failla *et al*, 1995) and further studies have shown that the central region of NS4a is important for the NS4a dependent activation of NS3.

1.3.16 NS4b

The NS4b protein of HCV is a 27KDa protein for which no structure or function has yet been published, although recently it has been shown to induce a morphological change in the ER membrane that might have a direct role in viral RNA replication (Gretton *et al*, 2005). NS4b has also been implicated in the pathogenesis of HCV; expression of 90 genes encoding oncoproteins, tumour receptors and adhesion molecules were affected in HeLa cells stably transfected with NS4b (Zheng *et al*, 2005)

1.3.17 NS5a

The NS5A protein is a complex protein whose amino acid sequence can be divided into three domains. The first domain consists of a helix which tethers the protein to membranes, chelates zinc and allows the protein to form homo- and hetero-dimers but the function of the other two domains are so far unknown.

As well as the three domains, the NS5A protein exists in two forms; a phosphorylated 56KDa (p56) and a hyperphosphorylated 58KDa (p58) form. It was proposed that phosphorylation took place in two phases: firstly basal-phosphorylation converts p49 to p56 and then hyper-phosphorylation converts p56 to p58. Conflicting data has been presented on the requirement of NS4A for these phosphorylation events, Kaneko *et al.* showed that the p58 form of NS5A was enhanced by co-expression of NS4A (Kaneko *et al*, 1994b), but other groups using different strains of the virus could not show an association (Reed *et al*, 1997).

Although the function of NS5a is so far unknown (at a good guess it is part of the RNA replication complex) it has been widely studied with regards to the Interferon sensitivity determining region (ISDR) it contains; a region of the HCV that is known to have a high mutation rate. This region is capable of binding to and inhibiting dsRNA induced PKR. The NS5a from patients infected with genotype 1 of the virus, specifically strains 1a and 1b, has been shown to bind PKR. Mutations in this region are thought to confer inhibition to IFN through its PKR binding ability, although this has only been found in patients with HCV-1b (Enomoto N, 1995) and may depend upon the treatment that the patient was receiving. It also has been shown that the inhibition of IFN by NS5a in the lab does not correlate with patient out-come after IFNα treatment (Paterson *et al*, 1999) as the levels of the protein expressed are so high that they could in no way physiologically represent the *in vivo* situation.

IFN normally acts by inducing the transcription of several antiviral genes, including PKR. PKR is responsible for the phosphorylation of eIF-2a that blocks protein synthesis and therefore inhibits viral replication. Several viruses are known to overcome the antiviral action of PKR. NS5a is thought to target the PKR dimerisation required for its catalytic activity. This in turn blocks the repression of mRNA translation and viral replication induced by cellular exposure to IFN, thus allowing HCV to resist the antiviral effects of IFN. Mutations in the ISDR region make it less capable of binding to the PKR and disrupting its dimerisation and thus making it more sensitive to IFN.

Other possible roles for NS5A include transcriptional activation, the acidic and proline-rich amino acids found in the C-terminus of NS5A are characteristic of eukaryotic transcription activators. Kato *et al* showed that the NS5A protein when fused with the GAL4 DNA-binding domain, could stimulate transcription in yeast and in a hepatoma cell line (Kato *et al*, 1997).

NS5A has also been found to bind to the growth factor receptor-bound protein 2 adaptor proteins (Grb2). Grb2 is a protein that is involved in growth factor induced signalling by coupling the membrane receptor tyrosine kinase (RTK) to the Ras-MAPK (mitogen-activating protein kinase). The proline rich sequences of NS5A resemble the SH3-binding sites found in cellular signalling molecules and NS5A could bind to Grb2 (via the SH3 domains of Grb2) to perturb the mitogenic signalling. Several mitogen activated kinases have been shown to interact with the JAK/STAT pathway. Therefore, NS5A inhibition of Grb2 may be another mechanism by which NS5A intereferes with the interferon signalling pathway (Tan *et al*, 1999).

1.3.18 NS5b

NS5b is a 65KDa protein that encodes the RNA-dependent RNA polymerase (RdRp) of the HCV, whose amino acid sequence shows significant homology to the RdRp of other flaviviruses. NS5b plays a central role in HCV replication and viral diversity because of the lack of proof-reading activity.

Recombinant NS5b, expressed in insect SF9 cells using baculovirus, was shown to have RdRp activity (Behrens *et al*, 1996). Characterisation of the RdRp indicated that it was able to prime at the 3' end of the template RNA and to copy the complementary strand with a hairpin loop connecting the two strands using the 'copy-back' mechanism. By using mutational analysis, 4a.a sequence motifs, designated A to D were identified that are crucial for the RdRp activity (Poch *et al*, 1989).

1.3.19 The 3' Untranslated region

The 3' UTR of the HCV genome consists of an untranslated region located downstream of the stop codon at the end of the ORF encoding the viral polyprotein. The 3' UTR consists of four sequence elements; i) a short sequence of about 40 nt which appears to vary considerably between genotypes, ii) a homopolymeric U tract, iii) a polypyrimidine stretch and iv) a novel 98 nucleotide sequence which terminates in a U residue (Yanagi *et al*, 1999). Each of these regions has been found to be important for HCV RNA replication and may play a further role in HCV RNA translation.

1.3.20 HCV Life cycle

HCV is predominantly a hepatotropic virus and therefore its life cycle must start with attachment and entry into a liver cell. Until the last decade, HCV research was

hampered by lack of infectious clones and an inability to propagate the virus in cultured liver cells. Introduction of the HCV replicon system (a self-replicating subgenomic construct) (discussed later) allowed studies of viral replication, but unraveling molecular details of HCV attachment and entry into hepatocytes required the development of additional tools. These encompass reconstituted HCV envelope glycoproteins, E1 and E2 (Deleersnyder *et al*, 1997;Lambot *et al*, 2002) , HCV pseudoparticles (HCVpp) in which E1 and E2 are displayed on retroviral core particles (Bartosch *et al*, 2003a) and, more recently, HCVcc, infectious viral particles released in cell culture following transfection with a full-length HCV genome (Lindenbach *et al*, 2005;Wakita *et al*, 2005;Zhong *et al*, 2005). These systems identified several cell-surface molecules which interact with HCV, including glycosaminoglycans (GAGs), CD81, claudin-1 and SR-BI (Helle & Dubuisson, 2007;Cocquerel *et al*, 2006;Evans *et al*, 2007). These putative receptors are by themselves insufficient for HCV binding and cell entry; current studies focus on their interplay. Initial attachment via GAGs is likely, as recombinant E2 binds strongly to heparan sulphates (Barth H & et al., 2003) but these have wide tissue expression and thus cannot confer HCV hepatotropism; additional hepatocyte-specific surface molecules are required.

One vital protein is CD81, a tetraspanin with two extracellular domains: it binds glycoprotein E2 (Pileri *et al*, 1998). Infection of Huh-7.5 hepatoma cells is inhibited by CD81 antibodies or by silencing CD81 gene expression. HepG2 cells which resist HCVpp infection become permissive if transfected to express CD81 (Zhang *et al*, 2004). However, expressing CD81 in non-hepatic cells does not allow HCVpp entry, suggesting a co-receptor is involved – strong experimental evidence implicates SR-BI, though reports that CD81 has a suppressive partner EW1-2wint (Helle & Dubuisson, 2007) and that the tight-junction protein claudin-1, and also claudins 6

and 9 (Zheng *et al*, 2007) act late in the HCV entry pathway, emphasizes that the overall process is complex and multi-step

SR-BI, an ~82-kDa glycoprotein with a large extracellular loop and membrane-spanning N- and C- cytoplasmic termini, is highly expressed in hepatocytes, and steroidogenic cells, where it facilitates bidirectional cholesterol transport. Though able to bind oxidized or chemically-modified low-density lipoprotein (LDL), SR-BI is a physiological receptor for high-density lipoproteins (HDL) and functions to selectively extract cholesteryl esters (Acton *et al*, 1996;Owen & Mulcahy, 2002) Intriguingly, SR-BI also binds apolipoprotein E (Bultel-Brienne *et al*, 2002) a 34-kDa polymorphic constituent of various plasma lipoproteins which has multiple roles in HCV virion assembly and/or infectivity (Chang *et al*, 2007) including effects on the persistence of infection (Price *et al*, 2006). As with HCV-CD81 interactions, SR-BI binds the HCV envelope glycoprotein E2 (Scarselli *et al*, 2002) and infectivity of HCVpp and HCVcc is stimulated by SR-BI overexpression in Huh-7.5 cells or, conversely, is decreased by SR-BI gene silencing or by addition of anti-SR-BI antibodies (Bartosch *et al*, 2003b) Noteworthy, but still ill-understood (Bultel-Brienne *et al*, 2002) is the association of HCV in human plasma with lipoprotein particles and, while oxidized LDL inhibits HCV cell entry *in vitro* (von *et al*, 2006) HDL stimulates infectivity and protects HCVpp from neutralizing antibodies (Voisset *et al*, 2005;Dreux & Cosset, 2007) Thus, although the finer details of HCV cell binding and entry remain elusive, the HCVpp and HCVcc models verify the interplay of CD81 and SR-BI for productive infection and establish that HCV-SR-BI interactions are enhanced by HDL.

After attachment to the receptor, the virus penetrates the plasma membrane via specific receptors and clathrin-mediated endocytosis to release their genome from acidified endosomes (Helle & Dubuisson, 2007). As the genome of the virus is positive sense, the virus tricks the cell into thinking that it is a host mRNA and

therefore can be read by the host cell's ribosomes. The translated polyprotein is then cleaved and processed before being used to create a minus strand (-) RNA template which in turn serves as a template for more progeny (+) strand RNA genomes. The positive sense RNA then interacts with the structural proteins and is encapsidated. Immunostaining and electron microscopy have shown that E1 and E2, the envelope proteins, are predominantly located in the endoplasmic reticulum (ER) (Dubuisson J *et al*, 1994), suggesting that HCV buds from the ER and are released from the cells via the exocytosis pathway.

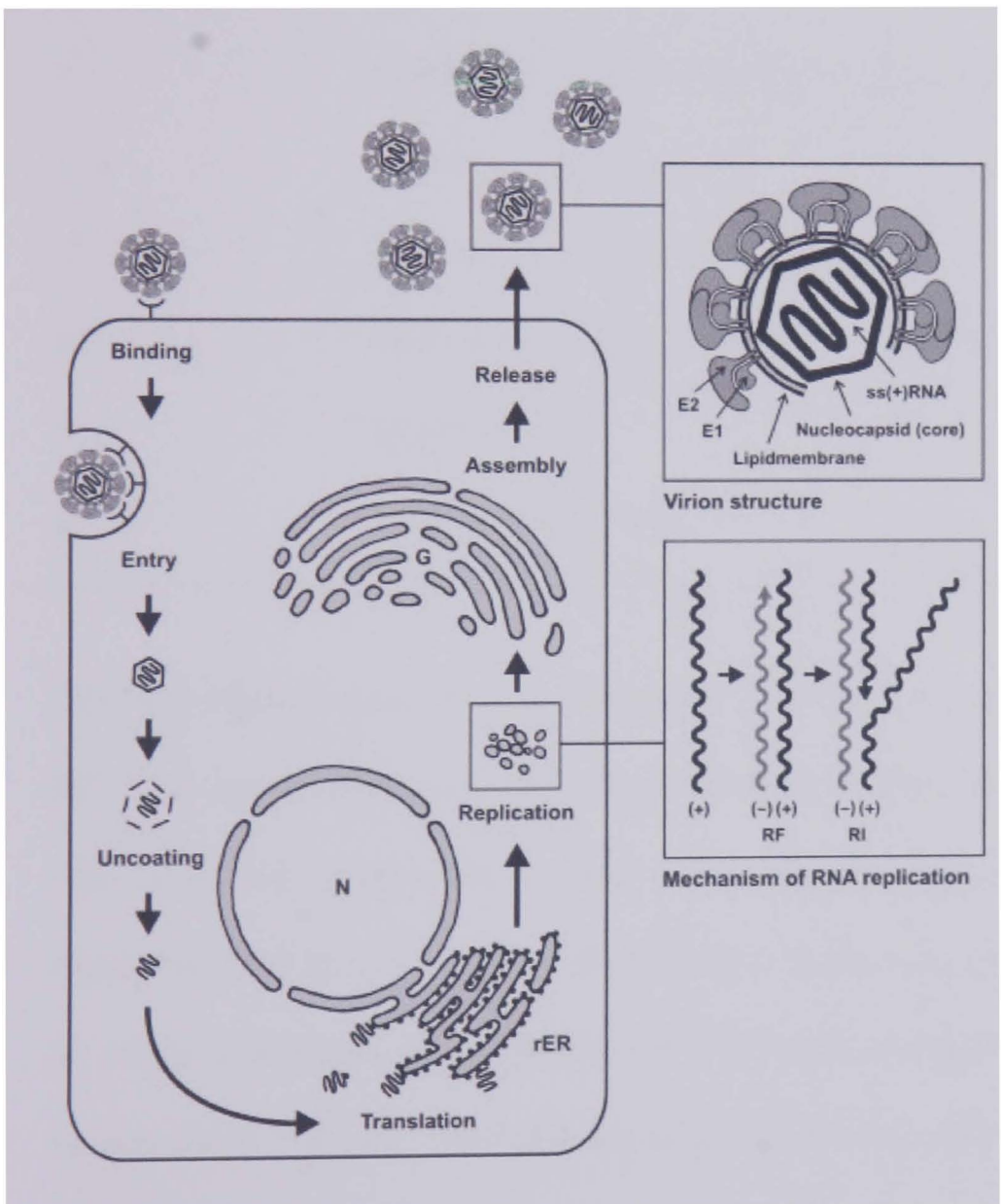


Fig. 1.11 Hypothetical HCV replication cycle. HCV particles bind to the receptor and are internalised by receptor mediated endocytosis. The viral genome is uncoated and translated on the rough ER. The genome is replicated and progeny viruses are assembled and leave the cells by the constitutive secretory pathway. The virion structures (in the top right panel) are drawn according to the structure of the TBEV envelope proteins. The lower right hand panel shows a model for the synthesis of negative strand (-) and positive strand (+) progeny RNA via a ds replicative form (RF) and replicative intermediate (RI). (Taken from http://www.med.uni-heidelberg.de/hyg/hyg5/EN/HCV.HTM)

1.3.21 HCV genotypes

HCV isolates fall into three major catagories; genotypes, subtypes and quasispecies, depending on the degree of sequence divergence. Six major HCV genotypes and several more subtypes have been identified on the basis of nucleotide sequence

similarity (Simmonds, 1999). The distribution of HCV genotype varies geographically; in the UK it is estimated that the majority of HCV infections are either genotype 1 or 3, overall in Europe the majority of infections are genotype 1, genotypes 4 and 5 are principally found in Africa and genotype 6 is primarily distributed in Asia, with genotype 3 predominant in India and Pakistan (http://hcv.lanl.gov/components/hcv-db/new_geography/geography.comp).

Table 1.5 HCV genotypes and distribution

Genotype	Distribution
1	Northern Europe, North America, Japan
2	Similar to genotype 1
3	Endemic in South East Asia
4	Middle East, Egypt and Central Africa
5	South Africa
6	Distributed in Asia

The genotypes vary in their response to Interferon alpha treatment; infection with HCV-1b associated with the lowest interferon response rate and higher chronicity rate whereas genotypes 2 and 3 are associated with a more favourable IFN response and genotypes 4, 5, and 6 are intermediate (www.nice.org.uk). Genotype 1 is more prevalent in patients with cirrhosis, suggesting that it causes a more progressive disease. The genotype of HCV may determine the patient's ability to eliminate the disease.

1.3.22 HCV Quasispecies

Not only do different genotypes and subtypes of HCV exist, but due to the lack of proof-reading ability of its RNA-dependent RNA polymerase, HCV exists as a population of different but closely related genomes known as quasispecies; multiple

clones with different nucleotide sequences have been isolated from a single patient (Martell *et al*, 1992). Different methods have been used to study HCV Quasispecies: sequencing of cloned RT-PCR products, single-stranded conformation polymorphism (SSCP), temperature-gradient gel electrophoresis (TTGE) and gel-shift analysis (GSA). These techniques have shown that quasispecies populations differ in genetic complexity (number of variants) and genetic divergence (the genetic distance between variants). Although mutations are introduced randomly during viral replication, due to constraints upon the nucleotide and/or protein sequence, the changes vary across the genome with certain areas more prone to changes. Some mutations become 'fixed' within the HCV genome whilst others are lost. The rate of fixation describes the number of substitutions per genomic site per year which can be used to compare mutation rates of different viruses, different genes within the same virus or the mutation rates of single residues. One region of the HCV genome has marked diversity among different isolates. The hypervariable region 1 (HVR 1) is situated in the amino terminus of the envelope protein E2. There has been a correlation between highly divergent HVR1 region and chronicity; patients who developed a chronic infection had a significantly higher HVR1 genetic divergence after 8-11 weeks post infection, compared to patients who eliminated the virus during the acute phase (Farci *et al*, 2000).

One of the driving forces for the evolution of the HVR quasispecies is thought to be the anti-HVR1 antibodies specific for each quasispecies. In chimps, antibodies to the HVR1 are known to be protective and when variants within the HVR1 arise, the antibodies are no longer protective and therefore the quasispecies become capable of escaping neutralisation (Farci *et al*, 1992).

The effect of the HCV quasispecies has been studied by several groups and it appears that a higher diversity in the HVR1 before the start of treatment is related to

patients who do not respond to IFN (Kanazawa *et al*, 1994). Before treatment a mixture of diverse quasispecies co-exists. During treatment the more-resistant quasispecies are selected and become predominant, while other quasispecies disappear. Although the heterogeneity of the HVR1 is important in viral evasion of antibody-mediated immune reponses, no direct role in the evasion of the IFNα treatment has been discovered. In fact, HCV quasispecies with identical HVR1 amino acid sequences have been shown to respond differently to IFN therapy (Enomoto & Sato, 1995)

1.3.23 HCV replicon

Our understanding of the hepatitis C virus was greatly improved with the development of a selectable subgenomic replicon (Bartenschlager *et al*, 2003;Lohmann *et al*, 1999a). Replicons are subgenomic, self-replicating RNA molecules that contain all the nucleotide sequences required for RNA replication, transcription, and translation, but are not themselves infectious. The structural genes were deleted and replaced by a selectable marker (neomycin phosphotransferase, conferring G418-resistance) and a second IRES-element was introduced to allow translation of the HCV nonstructural region. This allowed the generation of human hepatoma cell lines expressing the self-replicating HCV RNAs and proteins.

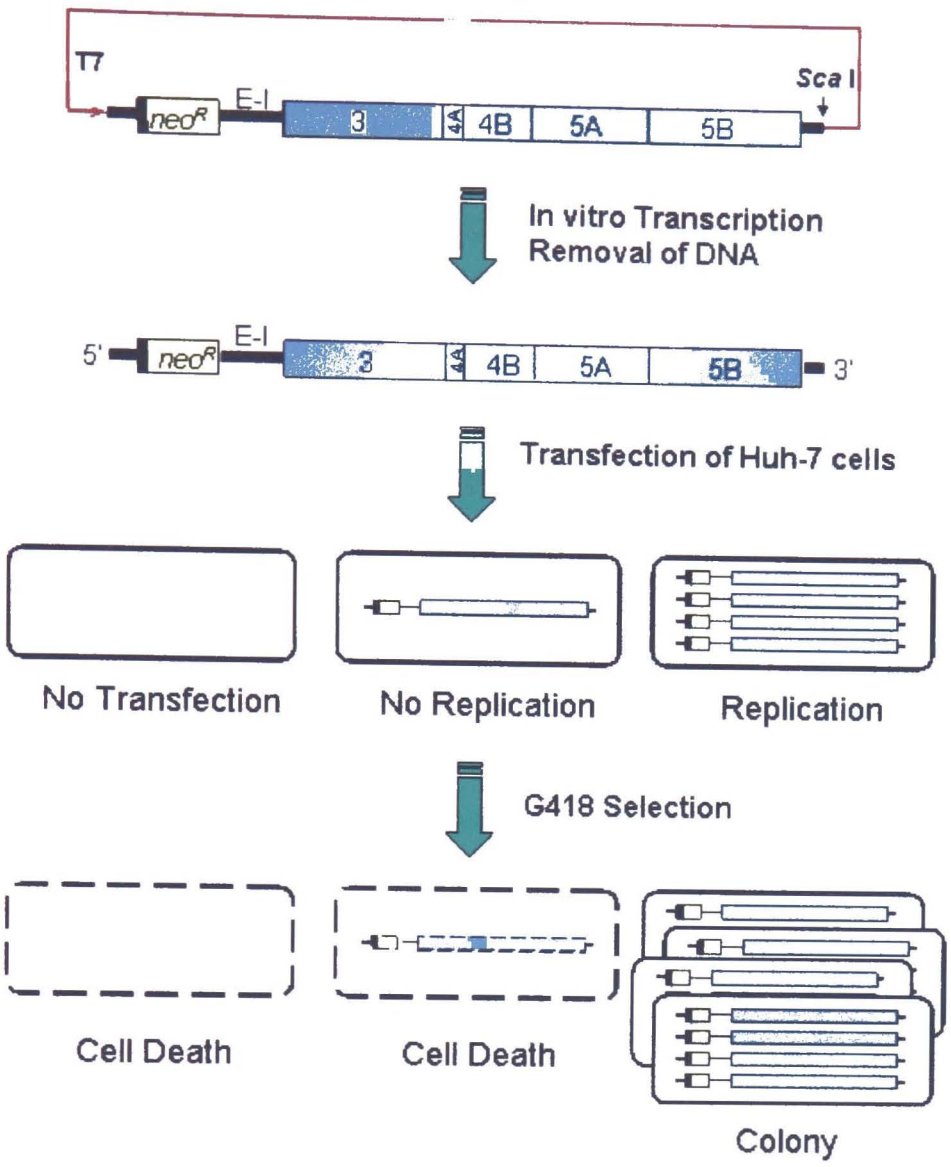


Fig 1.12 Schematic representation of the method used to establish HCV-replicon containing cell lines. The subgenomic RNA are transfected into Huh7 cells. The cells containing replicating HCV replicons develop resistance to G418 and will therefore form colonies. (Taken from http://www.med.uni-heidelberg.de/hyg/ hyg5/EN/ HCV.HTM)

The original HCV replicon could only replicate at a low level but replication was enhanced up to 10,000 fold through the acquiring of cell culture adaptive mutations (Krieger *et al*, 2001;Lohmann *et al*, 2003). Mutations tended to be clustered but not exclusive in the NS5A area, and although mutations in the NS4, NS5A and NS5B all enhanced replication of the replicon they were incompatible with each other. Whereas mutations in the NS3 region, only slightly enhanced replication on their own, in combination with other mutations they further enhanced the replication (Bartenschlager *et al*, 2004). Although the original replicon was derived from a 1b genotype others have since followed and there are now HCV replicons for genotypes 1a and 2a.

Although the replicon system advanced our knowledge of HCV, it still has it limitations. These were overcome recently by the development by three separate groups of a HCV infection system *in vitro* (Wakita *et al*, 2005); (Zhong *et al*, 2005;Lindenbach *et al*, 2005).

Using the JFH-1, a genotype 2a HCV isolate obtained from a patient with fulminant hepatitis, Wakita *et al.* showed that the JFH-1 isolate, replicated and secreted viral particles after transduction into Huh7 cells of transcribed RNA. When transfected into the Huh7.5 cell line, which are highly permissive for HCV RNA replication, the viral titres were increased. The secreted virus was infectious and this could be neutralized by CD81-specific antibodies and by immunoglobulins from chronically infected individuals. Lindenbach *et al.* used a full length genotype 2a subgenomic replicon, which was found to replicate efficiently within cell culture and more importantly it did so without adaptive mutations which are thought to be detrimental to the production of infectious particles in culture. By constructing a chimeric replicon they developed a system that allows for efficient replication of HCV. The virus was neutralised by an E2-specific human monoclonal antibody which showed that E2 was essential for viral entry. They also showed that the secreted virus could be blocked by blocking CD81.

1.3.24 Current Treatment

Treatment so far is based on the antiviral action of interferon alpha in combination with ribavirin, which clears the virus in around 30-40% of the patients. Ribavirin was one of the first antiviral drugs to be discovered. It is a synthetic guanosine analogue with broad spectrum activity against DNA and RNA viruses. It has been suggested that ribavarin monotherapy inhibits cellular DNA synthesis and promotes a CD4+ Th1 immune reponse (Hu *et al*, 2001), but recently Dixit *et al* suggested, based on mathematical modelling of viral kinetics in treated patients that ribavirin in the presence of pegylated IFNα, caused the produced virions to be less infectious,

thereby potentially reducing the number of hepatocytes that could be infected (Dixit *et al*, 2004). However other mathematical models have shown that ribavirin is a mutagenic agent that causes fatal errors in the replication of HCV producing non-viable virions (Crotty *et al*, 2000).

Another innovation in HCV therapy is the pegylation of the interferon protein. The pegylation of IFNα (a polyethelene glycol group is covalently attached to the IFNα molecule by a chemical process) increases the half life of the protein by several days (Glue *et al*, 2000) and increases the percentage of patients that clear the virus to approximately 55%. Moreover, in view of its slow clearance, it allows treatment to be reduced to weekly administration.

1.3.25 Clinical studies

Studies of hepatitis C viral kinetics have been used to evaluate different treatment regimes and are used to monitor response to therapy. On the basis of these kinetic studies HCV patient treatment-response categories have been defined. A sustained virological response (SVR) requires HCV RNA to be undetectable in serum 24 weeks after the end of therapy. McHutchinson et al demonstrated that 98% of patients with SVR were HCV RNA negative having completely cleared the infection (McHutchinson *et al*, 2002).

Combination therapy results in an overall 50-60% response rate in all patients, increasing to 80% response rate with genotypes 2 and 3. Two weeks into combination therapy the response rate is tested by quantitative PCR, patients who do not exhibiting a greater than 2 log drop in viral load are unlikely to clear the virus with continued therapy and are considered non-responders (NR), but there are also sub-categories of patients for whom treatment fail. Those who experience a significant decrease in HCV RNA during treatment can be defined as "partial

responders" and "responder-relapsers" become HCV RNA negative during therapy but relapse after the end of treatment (Fried *et al*, 2002).

Treatment outcome	Definition
Non-responder (NR)	No significant reduction in HCV RNA (>1 log) at any stage of treatment
Complete-responder (CR)	HCV RNA negative during treatment and have no HCV RNA in serum 6 months after the end of treatment.
Partial responder	Significant decrease of HCV RNA during treatment
Responder-relapsers	HCV RNA negative during treatment but relapse after the end of treatment

Table 1.6 Definitions of patient response to IFN treatment.

In a study by Zeuzem et al, sustained virological response after monotherapy with pegylated IFNα2a was 39% in comparison to 19% SVR with standard IFNα2a (Zeuzem *et al*, 2001). Clinical trials have shown that IFNα and ribavirin combination treatment significantly increases the rate of SVR both in untreated and previously treated but relapsed patients and the rate was increased further when ribavirin was used in combination with pegylated IFNα2a. SVR was achieved in 56% of patients treated with the combination pegylated IFNα2a and ribavirin therapy compared with ribavirin combined with standard IFN (45%) and 30% with pegIFNα2a alone (Fried *et al*, 2002). However, this treatment regime has a high relapse rate associated with genotypes 2 and 3 (24%) and 54% of patients with genotype 1. The current standard therapy uses a combination of pegylated IFN and ribavirin, but the duration and response to therapy are genotype specific: Genotype 1 patients require 48 weeks of

combination therapy for 50% successful viral elimination, while genotype 2 and 3 patients require 24 weeks of combination therapy for 80-90% viral elimination (Hayashi & Takehara, 2006).

1.3.23 Resistance to Treatment

Frequent viral load measurements and mathematical modelling determine viral kinetics in chronic HCV (Zeuzem *et al*, 2001). Studies of chronic HCV patients during IFNα treatment reveal a biphasic decline in HCV RNA levels after the first IFNα treatment. The initial phase of viral decline is rapid, approximately a 0.5 -2 log fall in HCV RNA levels within 48 hours which has been shown to be dose dependent i.e. the rate of viral decline is inversely proportional to the dose of IFNα, where the IFNα slows down virus production and/or viral release. If the IFNα treatment was 100% effective then viral replication would stop completely and HCV RNA would be undetectable within a matter of days, but this first phase of viral decline is followed by a slower second phase decline which is highly variable among patients. The second, slower phase appears to reflect the gradual clearance of the virally infected cells by the patient's immune system at the same time as the inhibition of HCV replication (Neumann *et al*, 1998;Zeuzem *et al*, 2001). During chronic HCV infection, HCV replication kinetics are at a steady state; virus production and *de novo* infection of the cells are counter-balanced by virion degradation and death of infected cells. The production-clearance rate of viral particles during this steady state is 10^{12}/day, where viral particles have a half life of approx. 3 hours (Neumann *et al*, 1998). The standard IFNα dosage regime of 3 million units 3 times a week was not sufficient to maintain such viral kinetics, but the addition of ribavirin and pegylated IFN helps maintain the viral clearance kinetic.

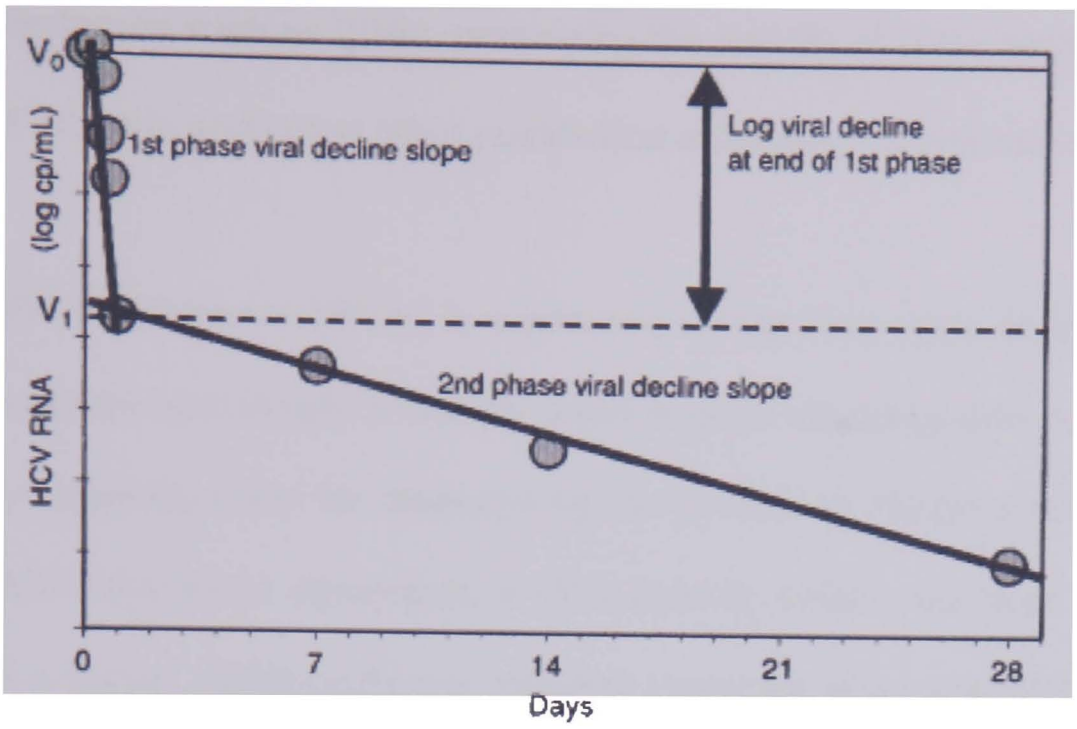


Fig 1.13 HCV viral kinetics after IFNα treatment.

Resistance to treatment is not only dependent on the type of therapy. Virological factors and the patient's immune response may also make a difference. Patient chartacteristics known to be associated with poor response to IFN treatment include older age, male gender and African or Hispanic ethnic background. A compounding factor was non-compliance due to the numerous side effects associated with IFN treatment such as depression, diarrhoea, nausea, fatigue and flu-like symptoms which can be severe.

It appears that the response to treatment may be a balancing act between viral replication and patient immune response. In the case of a strong replicating virus, the immune response may be irrelevant, but conversely in the case of a weal replicating virus a strong immune response may be sufficient to clear the disease.

1.3.23 Future Treatments

Due to the limitations of the current therapy, not only in response rates, but also cost and side effects, several new avenues such as siRNA, novel interferons and therapeutic vaccines are currently being researched, a few of which have reached first stage clinical trials. Improvements in the interferon area comes through novel

interferons such as IFNω, prolonging the half life of IFNα further with molecules such as albumin and using other nucleoside analogues to promote the induction of IFNα.

RNA interference (RNAi) is a process of silencing gene expression with small RNA duplexes specifically knocking down a gene message and subsequent protein level by targeting them for cleavage by ribonucleases. Several siRNA (silencer RNA) for HCV have been developed; a HCV-specific siRNA has been shown to inhibit levels of a fusion NS5B-luciferase reporter transcript when the siRNA and the target were co-transfected into mice (McCaffrey *et al*, 2002). A recent publication used RNAi to inhibit ongoing HCV replication in an HCV replicon system with siRNA targeting the NS3 and NS5 regions of the HCV genome being most effective (Kapadia *et al*, 2003).

Although earlier reports into development of an oral HCV serine protease inhibitor, BILN 2061 (Boehringer Ingelheim, Germany) were encouraging, patients infected with genotype 1, receiving a two-day course of treatment with BILN 2061, experienced a rapid drop in their HCV viral loads of 2 to 3 logs (100-1,000 fold decreases) (Hinrichsen *et al*, 2004), but patients infected with either genotype 2 or 3 did not experience the same drop in their viral load (Reiser *et al*, 2005) and recently due to adverse side-effects, plans to develop the drug have been put on hold.

Table 1.7 A sample of prospective HCV targeted therapeutics.

Compound Name	Company	Clinical Phase	Target	Mechanism of Action
BILN 2061	Boehringer-Ingelheim	II*	NS3-4A Pro	Serine protease inhibitor
VX-950	Vertex	Ib	NS3-4A Pro	Serine protease reversible covalent inhibitor
NM283	Novartis	II	NS5B pol	Nucleoside analogue
JTK-103	Japan Tobacco	II	NS5B pol	Non-nucleoside allosteric inhibitor
HCV-796	ViroPharma	Ia	NS5B pol	Non-nucleoside allosteric inhibitor

*Development halted due to cardiotoxicity in monkeys

1.4.1 HCV Inhibition of the Interferon Pathway.

Evading the antiviral response triggered by IFNα is crucial to the success of any virus. Several recent studies have clarified that HCV RNA is a potent trigger of IFN induction, leading to an antiviral state. Therefore HCV must have evolved several mechanisms to evade the IFN system and establish a persistant infection. Unravelling the mechanisms by which HCV evades the antiviral systems may lead to a greater understanding of the antiviral pathways and the development of better drugs to counteract ability of the virus to avoid elimination.

After HCV infection, the host response is initiated in the hepatocytes, leading to the expression of several ISGs, conferring an antiviral state within the cell. The dsRNA of the virus is detected extracellularly by TLR3 leading to the activation of the protein kinases TBK1 and IKKε and intracellularly by RIG-I, which binds to cardif, once again leading to the activation of TBK1 and IKKε. These kinases phosphorylate the transcription factor IRF3 which forms homodimers that translocate to the nucleus to

induce the expression of IFNβ. The activation of IKKε leads to the degradation of IκB releasing NF-κB, which translocates to the nucleus and functions with IRF3 again in inducing the expression of IFNβ. This is then released to activate the JAK/STAT pathway, resulting in the expression of ISGs. The amount of IFN produced and the expression of ISGs can predict the disease outcome. During the acute phase of disease, the infected hepatocytes continuously produce IFN and express ISGs, but by the chronic phase, the viral proteins by-pass the innate immune system and the antiviral actions of IFN thereby turning the tide in favour of viral replication.

1.4.2 Inhibition by the Core protein

HCV core protein has been shown to evade the IFN response by inducing the expression of SOCS3 (Suppressor of cytokine signalling 3), which is known to inhibit the JAK-STAT pathway (Bode *et al*, 2003). SOCS3 can inhibit the activity of Jak1 and Tyk2 as well as directly inhibiting the assembly of ISGF3 which is required for the expression of ISGs. However, mice expressing HCV proteins suggest that SOCS3 only plays a minor role in repressing the JAK STAT signalling. STAT1 phosphorylation still occurred in these mice, but the ability of STAT1 to bind DNA was impaired. A form of STAT1 (hypomethylated), which has been found in the liver of chronic HCV patients, has been shown to associate with the Protein inhibitor of activated STAT protein (PIAS) blocking the ability of the phosphorylated STAT1 to bind to the DNA sequence. The Protein phosphatase 2a (PP2A) in turn has been shown to produce hypomethylated STAT1 (Duong *et al*, 2004). Taken together the PP2A and PIAS proteins could contribute towards the inhibition of IFN signalling.

More recent studies, have shown that the first 23 amino acids of the core protein reduce the phosphorylation of STAT1 and inhibit the IFN signalling pathway. Conversely the C-terminal of STAT1, specifically the SH2 domain which is required

to form STAT1/2 homodimers, was required for the interaction between STAT1 and the core protein (Lin *et al*, 2006)

1.4.3 Inhibition by NS5A

HCV has developed several mechanisms to evade the antiviral response of interferon. The early focus of research was on the NS5a protein which contains the controversial Interferon sensitive determining region (ISDR) (discussed previously) and has also been shown to directly interact and inhibit PKR.

1.4.4 The Interferon sensitivity determining region.

The full-length sequence of NS5a from patients that responded and who failed to respond to treatment were compared (Enomoto N, 1995). Patients who responded to treatment were found to be infected with genotype 1b isolates with recurring mutations within a 40a.a. region in the carboxyl half of the NS5a protein. This region was subsequently termed the ISDR. Further research disputed these findings. Within the European and North American HCV isolates there is no correlation between the ISDR and response to treatment. (Paterson M, 1999). Several reasons have been postulated for the discrepancy between the European and Japanese ISDR studies. These include, IFN treatment regimes, treatment response rates, mutation frequencies, population genetics and the definition of treatment response. However, a recent meta-analysis and long-term follow up provided overall support for the association between the ISDR and treatment outcome (Pascu *et al*, 2004).

1.4.5 NS5A and PKR

As previously discussed, several viruses inhibit the antiviral response by targeting PKR. The NS5A protein of HCV has been shown to interact with the catalytic domain of PKR, disrupting PKR dimerisation and PKR-mediated eIF2α phosphorylation. The

ability of NS5a to bind PKR, is dependent upon the ISDR and a further 26 a.a downstream. By introducing specific mutations within the ISDR, identical to those in IFN-responsive HCV strains, the ability of NS5a to bind to PKR and inhibit its functions was abolished (Gale, Jr. *et al*, 1998).

However, the use of cell lines inducibly expressing NS5A to determine its mediated inhibition of PKR in HCV resistance has led to conflicting results. In support of the role of NS5A as an inhibitor of PKR, diminished phosphorylation of PKR and eIF2α in the presence of NS5A have been documented in human cells lines (Wang *et al*, 2003).

E2 has been shown to inhibit the kinase activity of PKR, blocking the downstream signalling. The E2 protein contains a sequence identical to the auto-phosphorylation site of PKR and the phosphorylation site of eIF-2α (Taylor *et al*, 1999).

A strong body of evidence is accumulating suggesting that the NS5A proteins, while capable of inhibiting PKR, may not influence the sustained response to IFN treatment (Paterson M, 1999) (Squadrito *et al*, 2002). Paterson *et al* cloned the NS5A gene from patients showing a clear non-response (NR) and complete response (CR) to treatment. These were expressed in HT1080 cells (human fibroblasts) under the control of an inducible promoter and using an antiviral assay their effect on IFNα mediated inhibition of EMCV measured. The NS5A-CR clone was found to be more inhibitory of the antiviral actions of IFNα than the NS5A-NR clone.

1.4.6 NS5A and IL-8

A further association between NS5A and the interferon response was discovered through a link with IL-8. In 2001, Polyak *et al* showed that NS5A could induce the expression of IL-8, (consistent with the finding that high levels of IL-8 are found in chronically infected HCV patients (Polyak *et al*, 2001a) and furthermore showed that the IL-8 inhibited the IFN induced antiviral response (Polyak *et al*, 2001b).

1.4.7 Inhibition of IFN induction by NS3/NS4A

Foy et al showed by using siRNA technology in Huh7 cells that silencing RIG-I, but not TRIF abolished signalling to the IFNβ promoter induced by Sendai virus. Further experiments showed that expression of RIG-I was suppressed with increasing amounts of NS3/4a, which inhibited the N-terminal region of RIG-I. Treatment of cells with an active site inhibitor of the NS3/4A protease relieved this suppression and restored intracellular antiviral defenses.

Four different groups then identified a CARD containing protein that links the viral sensors RIG-I and Mda5 via CARD-CARD interactions to the activation of IRF3 and NFκB. This protein was variously called IPS1, MAVS, VISA and Cardif.

IFNβ promoter stimulator 1 (IPS1) was identified by Kawai *et al* using cDNA libraries and they showed that over-expression of the protein activated IFNα, IFNβ and NFκB promoters to elicit an antiviral response that inhibited VSV replication. They also demonstrated that IPS1 associated with the CARD region of RIG-I and mda5 but could interact with FADD in a CARD independent manner (Kawai *et al*, 2005).

Using a bioinformatics search for proteins with CARD similar to RIG-I, Seth *et al* also identified the same protein and called it MAVS (Mitochondrial antiviral signalling) and

also showed that it activated NFκB and IRF3 and that the protein was localised to the mitrochondrial membrane (Seth & et al, 2005).

Further evidence of this proteins function as an adaptor was demonstrated by Xu *et al,* who named the protein VISA (virus-induced signalling adaptor) (Xu *et al*, 2005).

The adaptor protein was then shown to play an important role in the way that HCV disrupts RIG-I signalling to IRF3 and NFκB. Meylan et al showed that the HCV NS3/4a protease cleaves the Cardif protein during HCV infection *in vitro* at Cys-508, resulting in the dislocation of the N-terminal fraction of the protein from the mitochondria and disrupting the induction of IFNβ (Meylan & et al, 2005).

Table 1.8
Summary of the roles the HCV proteins play in inhibiting the IFN response (Qureshi, 2007)

HCV Protein	Role in IFN evasion
NS3/4a	Attenuate signalling from RIG-I and TRIF
Core	Upregulate SOCS3 expression, inhibits STAT1 activation
NS5a	Upregulate IL-8
NS5a and E2	Inhibit PKR activity
HCV proteins	Disruption of STAT1 and signalling via PP2A
HCV proteins	Removes ISG56 block

1.5 Summary

The inhibition of the interferon system by HCV is crucial to its success; this thesis investigates different systems that could potentially be used to study the interactions between various HCV derived proteins and the interferon system with the aim of targeting therapy more effectively. A full understanding of the interplay between HCV

and the IFN system will enable rational design of new antiviral drugs. Present *in vitro* systems rely on either a single genotype replicon or the newly developed JFH1 cells. Present animal models of HCV infection are flawed. Although the chimpanzes are infectable with HCV they do not go on to develop liver disease. An alternative method involves the use of the UPA-SCID mouse which exhibits abnormal liver development. These mice can be transplanted with human hepatocytes which are infectable with HCV. However, due to the differences between murine and human immune systems and specifically for this thesis, the IFN system, the UPA-SCID mouse is a poor model. Development of further models based on patient derived material will allow a broader spectrum of testing across genotypes.

Most viruses have now been discovered to encode specific IFN antagonists. By identifying the viral inhibitors, novel anti-viral drugs can be developed to block their activity. These novel drugs could prevent the viral antagonists from blocking the IFN signalling pathway and/or IFN response, thereby restoring the natural IFN response, enabling the patient to clear the infection or significantly improve the success of IFN therapy. The aim of this thesis is to investigate the potential of cell based replicon systems to act as a reporter assay and identify the individual proteins capable of inhibiting the IFN antiviral system.

As HCV is a member of the *Flaviviridae*, other members of the same family have been utilised to study the inhibitory effects of HCV. Although a dengue replicon was developed it was deemed inappropriate as a reporter system, but the effects of the dengue replicon on the interferon antiviral system have been studied in further detail to provide more insight into the mechanisms of dengue infection (chapter 4)

The other *Flavivirius* replicon system tested in this thesis was the Bovine viral Diarrhea virus (BVDV). This has previously been shown to be sensitive to type I IFN,

with which our studies concurred. The inhibitory effects of patient derived HCV proteins were tested in the BVDV reporter system (chapter 5) and further analysis carried out by reporter assays for both MxA and 6-16 (chapter 6). The advantages and disadvantages of each system will be further discussed in the relavent chapters.

In summary, the biological effects of patient derived HCV proteins were investigated as well as their impact on the antiviral effects of interferon.

2. METHODS

Background

To evaluate putative IFN inhibitors we developed different assay systems. Two approaches were taken to address this problem. One looked directly at interferon signalling in the cells using an interferon sensitive promoter construct coupled to a reporter gene. The other directly models interferon inhibition of virus by expression of a viral replicon containing a reporter gene. In these systems the effects of viral inhibitors that modify the effects of IFN can be assessed.

Cells treated with IFN will see a decrease in the RNA replication of the reporter whilst the IFNα/β antagonist will lessen/prevent the decrease in RNA replication of the reporter. The assay has been used to give a comprehensive analysis of HCV proteins from patients undergoing IFN +/- ribavirin treatment, showing a response or resistance to therapy as well as those who have relapsed and those who have gone on to develop HCC.

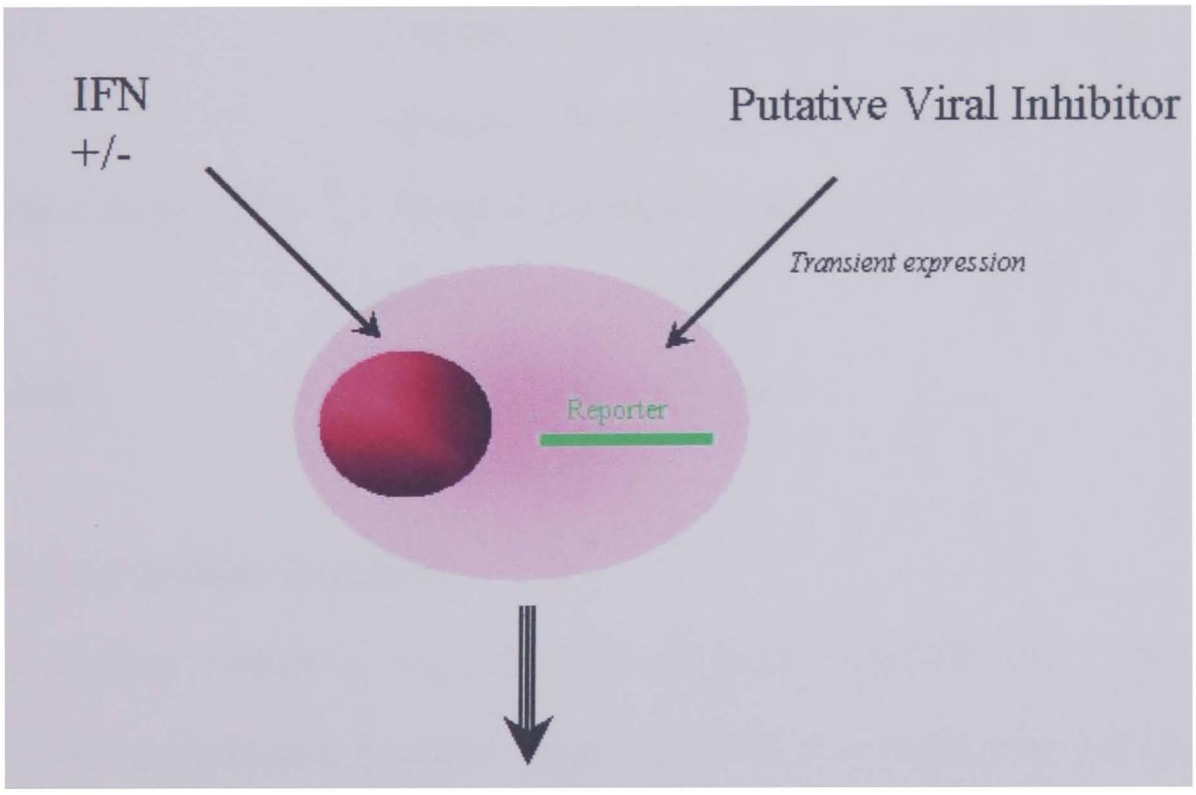


Fig 2.1 Schematic diagram showing how the reporter system would work. A putative IFN inhibitor is transiently transfected into cells expressing the reporter with and without interferon treatment and the reduction in the reporter assessed.

2.1 Materials

2.1.1 E. coli strains and Genotypes

Competent E. coli cells were purchased:

TOP10F' One Shot (Invitrogen)	F' The mcrA Δ(mrr-sdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG
JM109(DE3) (Promega)	endA1 recA1 gyrA96 thi hsdR17 (r_K^-, m_K^+) relA1 λ(DE3) supE44 Δ(lac-proAB) [F' traD36 proAB $laqI^q$ ZΔM15]
DH5α	F-Φ80lacZΔM15Δ(lacZYA-argF) U169 deoR recA1 EndA1 hsdR17(r_k-, m_k+) phoA supE44 λ- thi-1 gyrA96 relA1

2.1.2 E.coli growth media

SOC broth	2%(w/v) tryptone, 0.5%(w/v) yeast extract, 20mM glucose, 10mM NaCl, 2.5mM $MgCl_2$, 10mM $MgSO_4$
Luria Bertani Broth (LB)	1%(w/v) tryptone, 0.5%(w/v) yeast extract, 0.5%(w/v) NaCl
LB agar (LBA)	LB, 1.5%(w/v) agar-agar

2.1.3 Tissue Culture Reagents

All tissue culture reagents were purchased from Invitrogen/Life Technologies: DMEM (Dulbecco's Modified Eagle Medium), FBS (Foetal Bovine Serum), RPMI (Roswell Park Memorial Institute), PBS (Phospho Buffer Saline), 10XTE (Trypsin-EDTA), P/S (Penicillin Streptomycin), L-Glutamine

2.1.4 Molecular Weight Markers

100bp DNA ladder (Promega, Inc.): 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500bp fragments.

1Kb ladder (Promega, Inc.): 250, 253, 500, 750, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, 10000bp fragments

2.1.5 Buffers and Solutions

Molecular Biology Reagents

EDTA solution	0.5M EDTA [pH 8.0] (1L): add 186.1g EDTA•$2H_2O$ to 800ml distilled water. Add 20g NaOH pellets to approx. pH 8.0. Allow to dissolve and pH accurately to 8.0. Add water to 1L and autoclave to sterilize.
TAE Buffer	50X TAE stock (1L): add 242g Tris base (Trizma), 57.1ml glacial acetic acid and 100ml 0.5M EDTA [pH 8.0]. Make up to 1L with water.
TBE Buffer	5X TBE stock (1L): add 54g Tris base (trizma), 27.5ml boric acid and 20ml 0.5M EDTA [pH8.0]. Make up to 1L with water.
Sodium Acetate	3M NaAc [pH 5.2] (100ml): dissolve 40.8g NaAc•$3H_2O$ in 80ml distilled water. Adjust pH to 5.2 with glacial acetic acid. Make up to 100ml and autoclave to sterilize.
Agarose Loading Dye	0.25%(w/v) bromophenol blue, 0.25%(w/v) xylene cyanol FF in 40%(w/v) sucrose solution.
X-Gal	20mg/ml solution (10ml): dissolve 200mg X-Gal in 10ml dimethylformamide (DMF). Store in light proof container at –20°C.

10XMOPS Buffer	0.2M MOPS, 0.05M sodium acetate, 0.01M EDTA, [pH 5.5-7.0] (1L): add 41.86g MOPS, 4.102g sodium acetate, 3.72g EDTA in 800ml of DEPC-water and mix to dissolve. Make up to 1000ml with DEPC-water Adjust pH with NaOH and store at room temperature protected from light.
SSC Buffer	20X SSC (1L): add 175g NaCl and 95g Tri-sodium citrate in 700ml of water. Make up to 1L with water and stir until dissolved.
SSC Wash Buffer 1	2X SSC + 1%(w/v) SDS (1L): add 100ml of 20X SSC and 1g SDS in 900ml of water. Stir until dissolved.
SSC Wash Buffer 2	0.2X SSC + 1%(w/v) SDS (1L): add 10ml of 20X SSC and 1g of SDS in 990ml of water. Stir until dissolved.
SSC Wash Buffer 3	2X SSC (1L): add 100ml SSC in 900ml of water.
1M disodium hydrogen orthophosphate Anhydrous (1L)	Add 141.96g of disodium hydrogen orthophosphate anhydrous in 1L DEPC-water.
1M sodium dihydrogen orthophosphate 1-hydrate (1L)	Add 69g of sodium dihydrogen orthophosphate 1-hydrate in 1L DEPC-water.
1M Sodium phosphate buffer (1L)	Mix 666ml of disodium with 333ml of sodium dihydrogen and adjust pH to 7.2. Autoclave.
Northern Blot hybridization buffer	0.2M sodium phosphate buffer [pH 7.2], 1mM EDTA [pH 8.0], 1%(w/v) BSA, 7%(w/v) SDS, 45% (v/v) formamide (50ml): add 8ml from 1M sodium phosphate buffer [pH 7.2], 0.4ml from 0.1M EDTA [pH 8.0], 0.4g BSA in a double baked container and stir

	until dissolved. Then add 2.8g SDS and 18.2ml formamide.
PBS	137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4 \cdot 7H_2O$, 1.4mM KH_2PO_4. The final pH should be 7.1.
10 x TBS	12.11g TRIS [100mM], 87.66g NaCl [1500mM] make up to 1L with ddH_2O

2.1.6 Protein Analysis Reagents

Whole cell lysis buffer	2ml HEPES KOH [1M], 5mls NaCl [1M], 2mls NP40, 0.5g Na Deoxycholate, 0.2g SDS, 1ml Orthovanadate [100mM], 1ml EGTA [100mM], 10mls NaF [100mM], make up to 100mls with ddH2O
SDS-PAGE sample buffer	397µl ddH_2O, 63µl Tris-Cl (1M, pH6.8), 400µl SDS (10%), 100µl glycerol, 0.5µl BPB.
SDS-PAGE gel running buffer	3g Tris base, 14.5g Glycine, 1g SDS, make up to 1L with ddH_2O
Western Transfer buffer	2.9g Tris, 1.46g Glycine, 100ml Methanol, make up to 500ml with ddH_2O
Blocking Solution I	PBS with 5% milk
Blocking Solution II	TBS with 5% PVP and 0.5% FBS

2.1.7 Miscellaneous

DEPC-treated water	Add 1ml DEPC to 1L water. Leave overnight at 37ºC and autoclave to sterilize.
Methyl violet Stain	2%(v/v) formaldeyhyde and 0.25%(w/v) methyl violet.
IPTG (100mM stock)	Dissolve 0.238g IPTG in 10ml of distilled water and filter sterilize. Store at –20ºC.

Ampicillin (100mg/ml)	Dissolve 1g ampicillin in 10ml of distilled water and filter sterilize. Store at –20°C.
PBS-Tween	5% Tween added to 137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4 \cdot 7H_2O$, 1.4mM KH_2PO_4.
TBS tween	2% Tween added to 10mM TRIS and 150mM NaCl

2.1.8 Encephalomyocarditis Virus

A laboratory stock of EMCV (10^8 pfu/ml) was generously provided by Ms D.Watling (Imperial Cancer Research Fund, London, UK).

2.1.9 Interferons

The IFNα used in the majority of experiments was Roferon2a (9million units/ml) obtained from Roche. The IFNα subtypes were a kind gift from Norman Finter. The subtypes were HPLC purified from a mixture commercially licensed as Wellferon which was obtained from Sendai infected human lymphoblastoid cells. The mixture contains α-1, -2, -8, -10, -14, -17 and -21 with IFNα-4 and -7 as minor components.

2.1.10 Oligonucleotides

Oligonucleotides were synthesized by Sigma-Genosys. The primers were dissolved to 100μM in distilled water and stored at –20°C.

Table 2.1 Miscellaneous PCR primers

Primer	Sequence (5' to 3')
SP6	GATTTAGGTGACACTATAG
T7	TTAATACGACTCACTAT

Table 2.2 Primers for PCR amplification of constitutive genes

Primers	Sequence (5' to 3')
GAPDH (for)	ACAGTCCATGCCATCACTGCC
GAPDH (rev)	GCCTGCTTCACCACCTTCTTG
RPL13A (for)	CCTGGAGGAGAAGAGGAAAGAGA
RPL13A (rev)	TTGAGGACCTCTGTGTATTTGTCAA

Table 2.3 Primers for PCR amplification of Interferon stimulated genes

Primers	Sequence (5' to 3')
MxA (for)	AACAACCTGTGCAGCCAGTA
MxA (rev)	AAGGGCAACTCCTGAGAGTG
IRF7 (for)	GAGCCCTTACCTCCCCTGTTAT
IRF7 (rev)	CCACTGCAGCCCCTCATAG
25OAS (for)	ACAGGCAGAAGAGGACTGGA
25OAS (rev)	GCCAGGAGTCAGGAGACTTG
PKR (for)	TCTCTGGCGGTCTTCAGAAT
PKR (rev)	ACTCCCTGCTTCTGACGGTA

Table 2.4 Primers for PCR amplification of Dengue

Primers	Sequence (5' to 3')
Dengue NS1 (for)	CTGAAGTGTGGCAGTGGGATT
Dengue NS1 (rev)	CTTCAAAGCTAGCTTCAGCTATCCA
Dengue probe*	CACAGACAACGTGCACACATGGACAGA
RepT7 forward	GGCGGCCGCTAATACGACTCACTAT
Rep Rv	AGAACCTGTTGATTCAACAGCACCAT

*The Dengue probe is labelled with FAM and quenched with Tamra

Table 2.5 Primers for PCR amplification of the BVDV replicon

Primers	Sequence (5' to 3')
pdGN-BR.1	GCTGGAGCTCTTTGAGGA
pdGN-BR.2	GGCCATGCAACTTGTGACCCATAGAG
pdGN-BR.3	GTCACAAGTTGCATGGCCCAGTCCAAGC
pdGN-BR.4	GCATGTTTAAACCCACATTGATCCTAGCAGAAGC
pdGN-BR.4 HpaI	GCATGGTTAAACCCACATTGATCCTAGCAGAAGC
T7 9-BVDV	ATGAATTCGTTAATACGACTCACT
T7 15-BVDV	GTATCGATGAATTCGTTAATACGACTC ACT
3'UTR BVDV	GGGGGCTGTTAGAGGTCTTCCC

Table 2.6 Primers for PCR amplification of HCV 3b

Primers	Sequence (5' to 3')
3b 3'UTR	ACAGGGTTGGGGTGTTAACCTAC
3b 5'UTR	TCTTCACGCGGAAAGCGTCTAG
3b Core (for)	CTCGAGACCATGAGCACACTTC
3b Core (rev)	GAATTCATCTGGACGCGGGGCAAGTCAAGC
3b E1 (for)	CTCGAGACCATGCTGGAGTACAGGAATGCGTC
3b E1 (rev)	GAATTCACGCGTCCACTCCTGAAAACATGATTAG
3b E2/NS1 (for)	CTCGAGACCATGACAACACACACCACTGGTGGC
3b E2/NS1 (rev)	GAATTCATGCGTCCGCCAGAAGGAGG
3b NS2 (for)	CTCGAGACCATGCGCGTGTGTGTGGCCCTC
3b NS2 (rev)	GAATTCACAACCGCGCCGAAACGGGC
3b NS3 (for)	CTCGAGACCATGGGCCGCGAGTTGTTGCTGGG
3b NS3 (rev)	GAATTCACGTGGTGACTTCCAAGTCAGCTGACATG
3b NS4 (for)	CTCGAGACCATGAGCGCCTGGGTGCGGTTG
3b NS4 (rev)	GAATTCAGCAGGGACTAGGATAGTCCTCGTTG

3b NS5 (for)	CTCGAGACCATGAACGGTGACTGGTTACATG
3b NS5 (rev)	GAATTCATCACCGAGCAGGTAGGAGGAAAATGCC

Table 2.7 Primers for nested PCR amplification of NS5a*

Primers	Sequence (5' to 3')
1363	CAGTGGATGAACCGGCTGATA
1364	ATGTCCGGTTCCTGGCTAAGGGA
1365	ACTAAGACATTGAGCAGCA
1366	TGTGGTGACGTAGCAACGAGTTGCT

*These primers were also adapted for the PCR amplification of NS5a from different genotypes. All primers contained suitable restriction enzymes for cloning.

2.1.11 Vectors

The pGEM-T Easy vector system (Promega) was used to subclone PCR fragments. pGEM-T easy contains 3´-T overhangs at the insertion site providing a compatible overhang for PCR products generated by certain thermostable polymerases. These polymerases often add a single deoxyadenosine, in a template-independent fashion, to the 3´-ends of the amplified fragments.

The pIRES2DsRed2 vector (Clontech) was used to express the HCV Non-structural genes in mammalian cells. It is a bi-cistronic expression vector containing the cytomegalovirus (CMV) promoter and the IRES of the EMCV permitting both gene of interest and the Dsred2, a red fluorescent protein (RFP), derived from non-bioluminescent reef coral (class *Anthozoa*) to be translated from a single mRNA.

2.2 Methods

Most methods were conducted in accordance with standard procedures for Molecular Biology. For further references see:

1. Sambrook, J. Molecular Cloning-laboratory manuals E.F. Fritsch, and T. Maniatis-3rd edition; Cold Spring Harbor Laboratory Press
2. Current Protocols in Molecular Biology by Frederick M. Ausubel; John Wiley and Sons.

2.3 Agarose gel electrophoresis

Electrophoresis through agarose or polyacrylamide is a common method of separating and purifying fragments of DNA or RNA. The gels are stained with the fluorescent intercalating dye ethidium bromide and DNA bands in the gel can be visualized under ultraviolet light. This method can detect as little as 1 to 10ng of DNA.

The electrophoretic mobility of a DNA/RNA fragment is inversely proportional to the $\log_{10}$ of the number of base pairs in that fragment. By comparison to known molecular weight standards, the length of the DNA fragments can be ascertained. However, superhelical circular, nicked circular and linear forms of DNA of the same molecular weight migrate through agarose gels at different rates. Therefore any molecular weight standards used must be in the same form as the sample DNA.

To form a DNA agarose gel, 1g of agarose was dissolved in 100mls of 1xTAE buffer. When it had cooled to a temperature of around 55°C (termed 'hand hot'), 1µl of 10mg/ml Ethidium Bromide (Sigma) was added, the molten agarose was poured into the gel frame and allowed to set before adding enough 1xTAE buffer to cover the gel.

Prior to loading, 2μl of loading dye was added to the DNA sample and carefully pipetted into the wells. The gel was run at a 5V/cm until the bromophenol blue front had migrated at a sufficient distance. Gels were then visualized using a UV trans-illuminator and then photographed if required.

2.4 RNA extraction

2.4.1 Caution about RNases

When working with RNA samples it is necessary to take certain precautions to avoid RNAse contamination. RNAses are ubiquitous molecules in the laboratory environment and very insensitive to normal sterilization techniques, such as autoclaving.

A) Care should be taken to avoid contact with any dirty surfaces, pipettes, pipette tips, glassware. Gloves should be worn at all times and changed regularly. Sterile disposable plastic-ware should be preferably used and all solution should be treated with DEPC or purchased RNAse free.

B) The RNA gel tank and forming chamber should be washed thoroughly with detergent and water and left to dry after washing with ethanol. It should then be soaked in 3% hydrogen peroxide solution, which will inactivate any RNAses and washed with DEPC-water before pouring the formaldeyhyde gel solution.

C) All reagents should be kept on ice to reduce the activity of RNAses present. However, with the application of a little care, carefully autoclaved pipette tips, clean surfaces washed with 70% ethanol, RNAse contamination can be minimized.

2.4.2 RNA isolation from mammalian cells and biopsies

(The procedure was scaled according to the amount of RNA required for subsequent steps)

The cells were plated in 6-well plates and treated with the appropriate stimulus. The medium was removed and 1ml Trizol (Invitrogen) was added and left for 5 minutes to allow for complete dissolution of all cellular components. This system employs a formulation of guanidinium thiocyanate and urea in conjunction with a phenol solution. The Trizol solution was mixed and placed in a 1.5ml eppendorf. 200µl of chloroform were added and the mixture was shaken vigorously for 15sec and incubated at room temperature for 3 minutes. The organic and aqueous phases were separated by centrifugation at 12,000g for 15 minutes at 4ºC. The aqueous phase (top layer) was then transferred to a new microcentrifuge tube and the RNA was precipitated by the addition of an equal volume of isopropanol. The mixture was incubated at room temperature for 10 minutes and then centrifuged at 12,000g for 10 minutes at 4ºC. The RNA pellet was washed with 70% ethanol and resuspended in an appropriate amount of DEPC-treated water and stored at –80ºC until further use.

2.4.3 Extraction of HCV RNA from serum

HCV RNA was extracted from patient serum using the QIAamp Viral RNA mini kit (Qiagen). This system uses the selective binding properties of a silica gel based membrane. 140µl of the sample is lysed in buffer AVL with carrier RNA under highly denaturing conditions to inactivate RNases to ensure isolation of intact viral RNA before adding 560µl EtOH to provide optimum binding to the membrane. The membrane is then washed in two steps using firstly buffer AW1, then AW2 to remove contaminants. The RNA is eluted in 60µl of buffer AVE and stored at -80°C until further use.

2.4.4 Visualising the RNA

The integrity of the RNA preparation was assessed by gel electrophoresis. To form an RNA agarose gel, 1g of agarose, 75ml of DEPC-water and 10ml of 10XMOPS solution was poured into a 250ml conical flask and dissolved. At the same time, 17.5ml of formaldeyhyde was poured in a 50ml flask and heated to 50°C for 15 minutes. The agarose solution was then allowed to cool to around 50°C in a waterbath and the formaldeyhyde added slowly in a ventilated hood to protect the investigator from the intoxicated fumes of the formaldeyhyde solution. 2μl (stock 50μg/ml) of ethidium bromide was then also added. When all the components were dissolved, the solution was poured into a forming chamber, the comb added carefully and left to cool at 4°C. Prior to loading, 6μl of RNA-formaldeyhyde loading dye (Ambion, Inc.) was added to 20μl of RNA sample and then incubated at 65°C for 10 minutes. Immediately after the samples were immersed in ice for 5 minutes and loaded carefully into the wells.

2.5 Synthesis of cDNA from RNA

2.5.1 DNaseI treatment

DNaseI is an enzyme that degrades both double and single stranded DNA endonucleolytically, producing 3' –OH oligonucleotides without damaging the RNA prior to RT-PCRs, especially necessary in cases where the primers are not intron spanning. In the presence of Mg^{2+}, DNaseI attacks each strand of DNA independently and the sites of cleavage are distributed in a statistically random fashion. To 3μg of the total RNA, 3units of RQ1 RNase-free DNaseI (Promega) were added and 1μl of 10xbuffer (400mM Tris-HCl (pH8.0), 100mM MgSO4, 10mM CaCl2) and made up to 10ul with DEPC treated H_2O. The mixture was incubated at 37°C for 30 minutes, before stopping the reaction with 1μl RQ1 DNase Stop Solution [20mM EGTA (pH8.0)] and heat inactivating at 65°C for 10 minutes.

2.5.2 Reverse Transcriptase

The discovery and cloning of reverse transcriptases (RT; RNA-Dependent DNA polymerases) from retroviruses has altered the central dogma of molecular biology (DNA→RNA→protein). For the retroviruses, this enzyme catalyses the synthesis of proviral DNA from the RNA genome contained within the virion. For the molecular biologist, the enzyme is used to generate cDNA from RNA. The most commonly used are those from Avian Myeloblastosis (AMV) and Moloney Murine Leukaemia Virus (MMLV), MMLV is preferred for longer mRNA templates as its RNase H activity is weaker than the AMV RT enzyme. Like any other DNA polymerase, RT requires a primer for first strand synthesis, either oligo-dT (Promega) or random decamers (Promega) depending on the original mRNA template.

2μg of the DNaseI-treated RNA and 1μg of the first strand synthesis primer in a total volume of 10μl H_2O were incubated at 70°C for 5 minutes to melt secondary structure within the template. The tube was then immediately cooled on ice to prevent the secondary structure from reforming. The following components were added in order.

Table 2.8 Reverse Transcription reaction components (Promega)

Components	Amount
MMLV 5x Reaction Buffer	5μl
dNTPs (10mM each)	5μl
RNasin Ribonuclease Inhibitor	25 units
MMLV RT	200 units
Nuclease-Free H2O to final volume	25μl

The reaction was mixed and incubated for 60 minutes at 37°C. Once completed, the mixture was heated to 72°C for 10 minutes and returned to ice or stored at -20°C for future use.

2.6 Polymerase chain reaction (PCR)

The invention of PCR and its subsequent evolution and refinement as a research tool has had a profound effect upon the way molecular biology has progressed in the last decade. This technique relies upon the specificity of two primers for their substrate. Following an initial denaturation step, the temperature is lowered to allow for the primers to anneal to the target sequence, followed by an amplification/elongation step. This is repeated for many cycles (25-30 rounds). It is therefore theoretically possible to significantly amplify a single molecule within a mixture.

The major advance in PCR technology came with the introduction of thermostable polymerases, purified from thermophilic bacteria such as *Thermus aquaticus*, that remain stable at high temperatures. More recent advances include the introduction of polymerase with 3'-5' proofreading activity. Additionally, thermal cyclers with short ramping times have minimized any manual interference in the amplification process and have increased performance. More recent advances in PCR have been the development of the real-time, quantitative PCR, which allows the researcher to follow the experiment in real-time and accurately quantify the gene of interest.

2.6.1 Primer design

There are certain simple rules that should be followed when designing primers:

A) The primer sequences should not be complementary to each other. The formation of primer dimers will adversely affect the quantity of the desired product.

B) The primers should roughly have similar annealing temperatures, which depend on the length and G/C content of the primers. There are different ways of calculating the melting temperature of the primers but an accurate determination is generally not necessary.

C) The primer can usually be designed with a short stretch of unrelated bases at the 5'-end, which allows for the insertion of a short sequence, restriction site or altered nucleotide into the template sequence.

D) In general, a length of 15-20 bases is suitable for a primer. However, if mutations are to be introduced then longer primers might be designed, which will tolerate the mismatches better and allow for stabilization of the annealing process. The mismatch region should be flanked by regions of good primer-template match, unless they are located at the 5'-end of the primer. Usually, in the case of mutational PCR, the mismatches are designed in the middle of a lengthy primer.

E) As a general rule, the 3'-end base(s) (especially the last one) should have a high G/C content, which will stabilize the amplification process.

To aid with the primer design several web based programs were used

e.g. Primer3 http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi,

Exon Primer http://ihg.gsf.de/ihg/ExonPrimer.html,

2.6.2 Standard PCR reaction conditions

The PCR reaction is usually performed in 25, 50 or 100μl volumes. The reaction buffer is provided as an optimised 10X solution. The reaction mixture is

supplemented with an excess of reaction substrates: dNTPs (an equimolar mixture of dATP, dCTP, dGTP and dTTP to an overall concentration of 200μM), template DNA, primers (0.2μg/50μl) and 1.5mM $MgCl_2$.

Reaction tubes are then placed in the thermal cycler and the DNA denatured at 95°C for 5 minutes. The DNA is then amplified by 30 cycles of 50secs at 58°C (annealing temperature), followed by 50secs at 72°C (polymerisation) and 50secs at 94°C for denaturation. At the end of the amplification cycles, an additional polymerisation step is added for 7 minutes to "tidy-up" the incomplete ends of the product. Usually, 5μl of the PCR product is run on an agarose gel to determine product formation. The rest can be stored at -20°C.

2.6.3 PCR contamination

PCR contamination is a very common phenomenon in areas where researchers are working with DNA high-copy number tasks, such as PCR. To ensure that such contamination is avoided, separate areas were designated for setting up the PCR reaction, which could be UV irradiated, along with separate micro-pipettes, reagents, equipment and laboratory coats and sterilized pipette tips.

To prevent accidental contamination, negative PCR controls were typically included. Should these give positive results, then the experiment and current batch of reagent aliquots were discarded.

2.6.4 Hi-Fidelity PCR

One of the accepted trade-offs of thermostable DNA polymerases, is the high error rate. The Phusion high fidelity DNA polymerase has good proof reading ability and the ability to amplify long fragment lengths. Phusion's error rate is 50-fold lower than

that of *Thermus aquaticus* (Taq), and 6-fold lower than that of *Pyrococcus furiosus* (Pfu). The Phusion polymerase possesses processivity 10-fold greater than Pfu and Taq.

2.6.5 Amplification of HCV genes from patient serum

When a PCR is running under optimum conditions, the concentration of DNA product approximately doubles at every cycle. As the concentration of HCV RNA in serum isolated from infected patients is very low an additional step using nested primers was employed.

50μl PCR reactions were carried out using Phusion HF PCR buffer (Finnzyme), 100pmoles of primers (see table 2.5 and 2.6), 1.5mM $MgCl_2$ and 1 unit of Phusion high fidelity DNA polymerase (Finnzyme). 200μl thin-walled reaction tubes were used in a PTC 100 thermocycler (MJ Instruments). The reactions were heated to 94°C for 2 min before 40 cycles of: 94°C for 30secs (denaturation), 53°C(dependent on primer) for 30 secs (primer-template annealing) and 72°C for 1 min 30 secs (polymerisation). This was followed by 72°C for 2 min and then held at 4°C until analysed on a 1% agarose gel.

2.6.6 Site-Directed mutagenesis and gene assembly by PCR

Site-directed mutagenesis allows the introduction of specific mutations in specific genes, in specific locations within the gene, something not possible by chemical or enzymatic means.

PCR primers were design that contained mismatches to the target sequences. During each round of amplification, the modified nucleotide became incorporated into the target sequence and after sufficient rounds it became the dominant species.

The generated PCR products were smaller than the full size template, which allowed for gel purification of the amplified mutant sequences. It is of course necessary at the end of the gene assembly process to sequence the spliced product to check whether the correct mutation has been introduced.

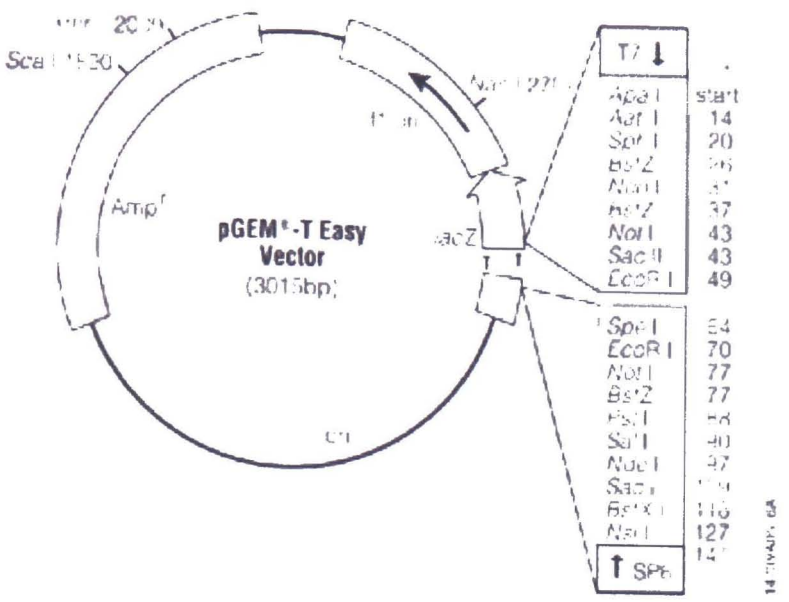
2.7 Making DNA

For maintenance, storage, manipulation and replication of nucleotide sequences, genes of interest are frequently transferred to bacterial plasmids. These double-stranded closed circular DNA molecules behave as accessory genetic units that replicate independently of the bacterial chromosome. By transferring a nucleotide sequence to a plasmid (cloning), transforming a laboratory strain of E. coli with the recombinant plasmid, the replicating bacteria can be used to reproduce multiple copies of the genes of interest.

2.7.1 Transfer vectors (pGEM-T Easy)

The commercially available pGEM-T Easy Vector System (Promega) was used during this study. The vectors are commercially prepared by cutting vector pGEM-5Zf(+) with EcoRV and adding a 3' terminal thymidine to both ends. These 3' terminal overhangs at the insertion site improve the ligation efficiency of a PCR product into the plasmid not only by preventing the vector from re-circularising during the ligation but also by taking advantage of the template independent activity of some thermo-stable polymerases to add a single deoxyadenosine to the 3' ends of the amplified fragments.

Fig 2.2 Transfer plasmid pGEM-T Easy, Obtained from Promega.



2.7.2 Extraction and purification of plasmid DNA

Plasmids are generally prepared from bacterial cultures grown is the presence of a selective agent such as an antibiotic. Isolation of plasmid DNA involved three steps:

A) Growth of the bacterial culture

B) Harvesting and lysis of the bacteria

C) Purification of the plasmid DNA

2.7.3 Growth of the bacterial culture

The plasmids were purified from cultures grown in liquid medium, containing the appropriate antibiotics that have been inoculated from a single bacterial colony picked from a freshly streaked selective agar plate. Antibiotic selection was applied at all stages of growth to maintain selective pressure at all times. Overnight cultures were grown for no more than 12-16 hours to prevent ampicillin depletion (hydrolysis) in the culture by the β-lactamase, encoded by the plasmid-linked bla gene.

2.7.4 Alkaline lysis (qiaprep miniprep system)

This method uses alkaline conditions to lyse the bacterial cells, followed by binding of plasmid DNA to an anion-exchange column under appropriate low salt and pH conditions. RNA, proteins and other contaminants are removed by a medium-salt wash. The plasmid DNA was eluted in a high-salt buffer.

The bacterial pellet was resuspended in 250μl of cold Buffer P1 and vortexed vigorously until no cell clumps were visible. 250μl of Buffer P2 was added and the tube gently inverted 4-6 times until the solution became clear. Chromosomal DNA was precipitated by the addition of 350μl of Buffer N3, which also neutralised the lysate and adjusted it to high-salt binding conditions necessary for the adsorption of the DNA to the QIAprep column. The mixture was incubated on ice for 10 minutes before centrifugation at top speed for 10min at 4°C. The supernatant was applied to the column and centrifuged for 30 sec at room temperature. The column was washed with 0.5ml of Buffer PB which inactivates any endonucleases present (essential when working with endA$^+$ strains such as the JM series, HB101 and its derivatives) and ensures that the plasmid DNA is not degraded, followed by a wash with 0.75ml of Buffer PE. The flowthrough was discarded and the column was centrifuged again for an additional 1min to remove residual wash buffer before eluting with 50μl PE. The eluted plasmid DNA was stored at -20°C.

2.7.5 Large scale DNA preparation

The Qiagen Plasmid Midi kit was used for large-scale DNA preparations, using 100ml overnight *E. coli* cultures. The method is otherwise a scaled up version of the MiniPrep protocol with an additional endotoxin removal step.

2.8 Purifying DNA

2.8.1 Recovery and purification of DNA

Several times during the course of experiments it became necessary to purify the DNA fragments obtained by certain applications, such as PCR or restriction digestion, from other contaminants present (primers, dNTPs, undigested plasmid DNA, and enzymes).

The QIAquick system (Qiagen) was chosen to perform that task because of certain advantages it presented, namely, convenience and reliability. The QIAquick system combines the ease of spin-column technology with the selective binding properties of silica-gel membrane. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is finally eluted with Tris buffer or water.

2.8.2 Ethanol/Isopropanol precipitation of DNA

Precipitation of DNA (or RNA) during a molecular biological procedure is often required to concentrate or purify the sample. This can be accomplished within the limits of a microfuge tube. Due to the restricted volume of the eppendorf tube, the maximum volume that can be practically precipitated using this procedure is 500μl. 1/10 volumes of 3M sodium acetate (pH 4.8) is added, followed by 2.5 volumes of cold ethanol or 1 volume of cold isopropanol, The solution was vortexed thoroughly and placed in the –80°C freezer for 30 minutes. The tube is then spun at 12,000g for 15 minutes at 4°C. The supernatant is aspirated and the pellet dried in a vacuum dessicator. A co-precipitant was often added to the nucleic acid solution to improve visualization of the pellet, usually GlycoBlue (Ambion).

2.9 Cloning

2.9.1 Restriction endonuclease digestion of DNA

Type II restriction endonucleases cleave unprotected DNA at well-characterized, pre-determined recognition sequences. The typical recognition sequence is an exact palindrome of 4, 5, 6, 7 or 8 base pairs with an axis of rotational symmetry. Cleavage of d$DNA by a restriction enzyme can generate a number of different ends. Usually, these ends have 5'-phosphate and 3'-hydroxyl ends although exceptions are known. Such a cut may yield either 'blunt ends' (both ends having the same length) or a single stranded 3' or 5' overhang, dependent on the enzyme. Usually, a DNA concentration of 5μg/10μl was digested in a volume of 50μl. To this, 5μl of 10X reaction buffer was added, plus enzyme to a concentration of 2 units/μg DNA (typically 1-2μl). One unit of enzyme is generally sufficient to digest 1μg of DNA in an hour. The reagents were mixed and incubated at 37°C for an hour, or longer if complete digestion was required.

2.9.2 Dephosphorylation of linearized DNA fragments

During a ligation reaction the 5' end phospho-group and the 3' hydroxyl-group become the substrate for the T4 DNA ligase resulting in a phosphodiester bond. By removing the 5' end phosphate from the DNA vector using the calf intestinal alkaline phosphotase (CIAP), it is unable to self-ligate.

Table 2.9 Components of a Dephosphorylation reaction

Time	**0.5h (5' overhangs)** **1h (blunt ends)**
10X Alkaline Phosphatase Reaction Buffer	4 µl
DNA	1pmol ends
Enzyme	1 Unit
H_20	Up to 40 µl
Temperature	37°C (5' overhangs) 50°C (blunt ends)

2.9.3 Ligation of DNA fragments

The reaction that allows for the insertion of the desired gene into a vector is catalyzed by T4 DNA ligase and involves the ATP-dependent formation of a phosphodiester bond between the 3' hydroxyl end of a double-stranded DNA fragment and the 5' phosphate end of the same or another DNA fragment. The ligation reaction can take place between fragments that possess complementary cohesive ends or blunt ends to produce circular recombinant molecules.

The following reaction conditions are for ligation of DNA fragments with complementary cohesive ends. During the capture cloning of PCR products where pGEM-T Easy transfer vectors were used a molar ratio of 3:1 (insert:vector) was recommended.

Table 2.10 Components of a Ligation Reaction

Time (h)	1/24
5X Ligase Reaction Buffer (μl)	4
Insert:Vector Ratio (molar)	3:1
Insert Ends (fmol)	3-30
Vector Ends (fmol)	9-90
Total DNA	0.01-0.1
Ligase (Units)	0.1
Temperature (°C)	23-26/14
Final Reaction volume (μl)	20

All the components were added into an autoclaved 0.5-ml eppendorf tube and centrifuged briefly to bring all the contents to the bottom of the tube.

2.9.4 Transformation of competent E. coli cells with recombinant DNA

The competent cells were thawed and kept on ice until needed. The cells were gently mixed and 1-50ng of ligated DNA or 0.1ng of supercoiled plasmid DNA was added to the cells and the tube swirled gently. The cells were incubated on ice for 30min. Each transformation reaction was heat-pulsed at 42°C for 20-60 seconds depending on the type of cells (30 seconds for TOP10F', 45 seconds for other cell types). Immediately after the heat pulse the reactions were incubated on ice for 2 minutes. 950μl of preheated SOC solution was added to the cells and the reactions incubated at 37°C for 1 hour with vigorous shaking (225rpm). An appropriate amount of the transformation reaction (100-200μl) was plated onto an LB agar plate supplemented with the appropriate antibiotic.

2.9.5 Identification of colonies that contain recombinant plasmids

2.9.5.i α-Complementation (Blue/White screening)

The pGEM-T Easy vector, along with many of the vectors in current use, carries a short segment of DNA that contains the coding information for the first 146 amino acids of the β-galactosidase gene (lacZ). Situated within this coding region is a multiple cloning site that does not disrupt the reading frame of the α-peptide coding region of β-galactosidase but results in the harmless addition of a few aminoacids in the amino fragment of the enzyme. The plasmid is inserted into a host cell that encodes the carboxy fragments of enzyme but not the amino fragment that is plasmid encoded. Neither fragment is active by itself, but in cells that have acquired the plasmid vector the two fragments can associate to form enzymatically active protein. This type of association is called α-complementation. The fully functional α-peptide can catalyze the cleavage of X-gal (5-bromo-4-chloro-indonyl-b-D-galactoside) substrate and produce a characteristic blue colour. In contrast, insertion of a DNA fragment into the multiple cloning site almost always disrupts the coding reading frame and results in the production of an amino fragment unable for α-complementation. Therefore, bacterial clones that contain an insert produce white-coloured colonies thus allowing recombinant clones to be directly identified by colour screening on indicator Luria-Bertani agar plates containing IPTG and X-gal. The agar plates also contain the antibiotic ampicillin to select against clones that do not contain any plasmid.

The procedure for testing bacterial clones for α-complementation was as follows:

To a pre-made LB agar plate supplemented with 100μg/ml of ampicillin, 40μl of a 20mg/ml stock of X-gal and 6μl of a 100mM stock of IPTG (isopropylthio-b-D-galactoside) was added. The solution was spread over the entire surface of the plate using sterile technique before incubatating at 37°C for 1hour in order for the solution

to be absorbed. The plate is inoculated with the transformed bacteria, dried quickly and then placed inverted overnight in the 37°C incubator. The next day the colonies have fully developed. Colonies that do not contain the insert are blue, whilst those that do are coloured white. Sometimes, colonies that contain the plasmid are coloured blue because the insert has been cloned in-frame with the β-galactosidase amino fragment resulting in the production of a protein able for α -complementation.

The JM series of cells (eg. JM109) carry the amino fragment of β-galactosidase on an F' episome. Strains containing an F' episome should always be maintained on minimal medium plates (M9) supplemented with thiamine-HCl. This selects for the presence of the F' episome which carries a nutritional requirement for growth (proline biosynthesis) and decreases the number of false positives.

2.9.5.ii Antibiotic selection

Several of the plasmid vectors used in this study carried the bla reading frame that encoded for β-lactamase, an enzyme that degrades the antibiotic ampicillin. By using ampicillin sensitive host strains (competent cells for the transformations reactions) we can select for transformants that have successfully acquired the plasmid that confers ampicillin resistance. This type of selection is only able to detect clones that have incorporated the antibiotic resistance conferring plasmid and cannot discriminate between insert and non-insert carrying clones. However, this does not diminish in the least the importance of this selection method. In fact, while α-complementation selection can be used with pGEM-T Easy vectors (contains the amino fragment of β-galactosidase).

2.9.5.iii PCR screening

E. coli colonies expected to contain insert (white) were screened by PCR amplification across the pGEM-T MCS using the SP6 and T7 primers (table 2.1). A small amount of each colony was added to 25μl standard PCR reaction (see 2.6.2). PCR products were examined by electrophopresis in 1% agarose TAE gel and visualised by UV irradiation.

2.9.6 DNA sequencing

An ABI Prism 377 Dye Terminator Cycle Sequencing Kit was used with AmpliTaq DNA Polymerase FS Ready Reaction mix (Applied Biosystems, CA, USA). The AmpliTAq DNA Polymerase FS enzyme was designed specifically for use with fluorescent labelled dye terminators and has no 5'-3' nuclease activity. The 'Ready Reaction mix' contains all the reagents necessary for DNA sequencing (except DNA and primers). This system uses dITP instead of dGTP to minimize band compressions. 20μl reactions were setup, containing 6μl of Ready Reaction mix, approximately 0.5μg of DNA and 3-5 pmoles of primer. Once thermocycling was complete, the reaction products were ethanol precipitated and resuspended in 5μl of formamide dextran blue dye. 2μl sequencing reactions were loaded per lane of the gel. (In later experiments the DNA sequencing was done courtesy of the Genome centre, Bart's and the London, Charter House Square, London). DNA sequence data was analysed using BioEdit and the sequence checked using the BLAST program http://www.ncbi.nlm.nih.gov/BLAST/.

2.10 Real-Time PCR

Real-time PCR is an extraordinarily powerful technique, which relies on two important discoveries: the finding that the Taq polymerase possesses 5'→3'–exonuclease activity and the construction of dual-labelled oligonucleotide probes,

which emit a fluorescence signal only on cleavage, based on the fluorescent resonance energy transfer (FRET). Unless otherwise mentioned, all real-time PCR reactions were carried out on the Rotor-Gene, Corbett Research (Mortlake, Sydney, Australia).

2.10.1 SYBR green

SYBR green is a DNA-binding dye that incorporates into dsDNA, it is undetectable in its free form but once bound to dsDNA it starts to emit fluorescence. The excitation and emission maxima of SYBR green I are 494nm and 521nm respectively and the fluorescent signal increases proportionally with increasing amount of amplicon generated with each cycle and therefore can be used in the quantification of DNA. However, as SYBR green binds to any dsDNA, detecting not only the specific target, but also non-specific PCR products and primer-dimers, a method of eliminating unspecific products is needed:

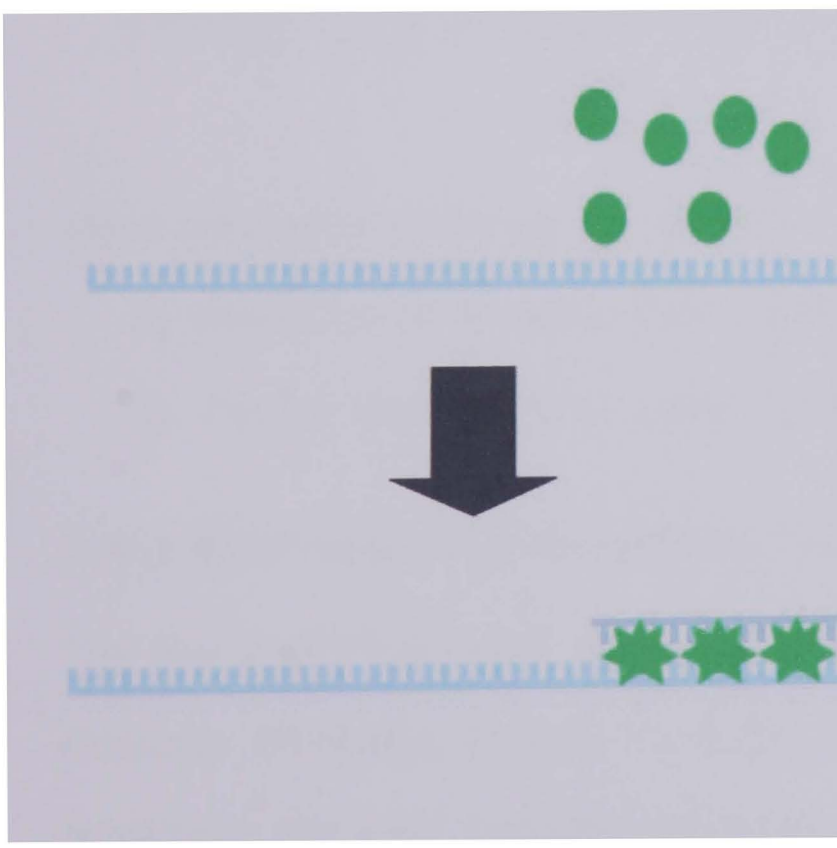


Fig 2.3 Mechanism of SYBR green action. Unbound SYBR green does not emit fluorescence. As soon as the SYBR green binds to dsDNA target fluorescence occurs.

2.10.2 Melting curve analysis

The melting temperature (Tm) of a DNA double helix depends on its base composition (%GC, and its length, especially if it is very short). All PCR products for a particular primer pair should have the same melting temperature unless there is

contamination, mis-priming or primer-dimer artifact; therefore by checking the Tm we can confirm the specificity of the PCR reaction as SYBR green does not distinguish between one DNA species and another. After the cycling has been completed, the temperature is raised by 1°C/sec and the change in fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence will rapidly decrease. The software plots the rate of change of the fluorescence with temperature against the temperature resulting in the Tm of the product.

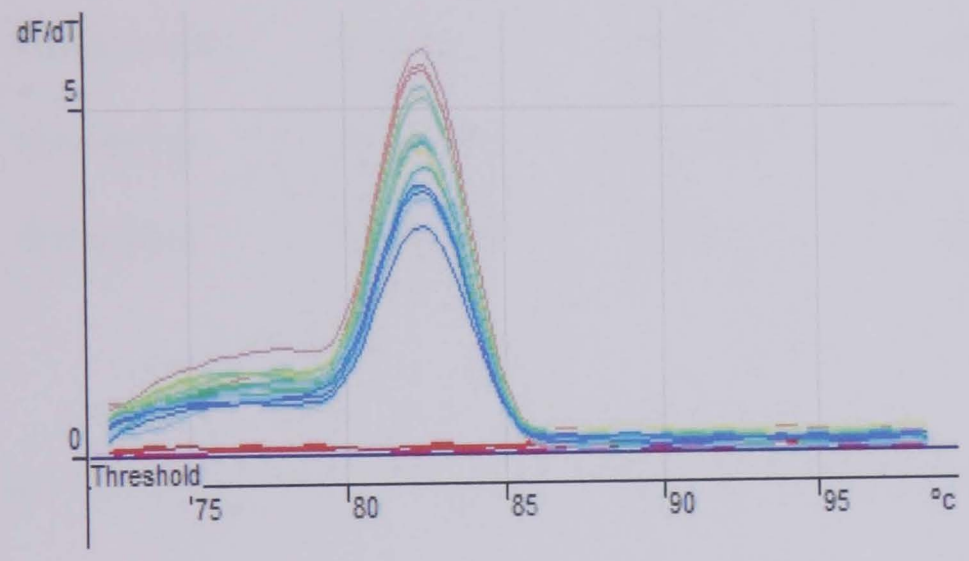


Fig 2.4 Melting curve analysis. The change in fluorescence with temperature indicates whether the PCR product is identical. Pure homogenous PCR products produce a single, sharply defined melting curve with a narrow peak. In contrast, primer-dimers melt at a relatively low temperatures.

Other considerations for using SYBR green

A) Ideally, the length of the PCR product is 100-150bp

B) The Tm of the primers should be between 50-60°C

2.10.3 SYBR green Real time PCR set up

The real time PCRs were set up using a CAS 1200 Robotics system, Corbett Research (Mortlake, Sydney, Australia) using QuantiTect SYBR green 2x Mix containing, Hot start Taq DNA polymerase, SYBR green I, ROX, dNTPs, buffer (Tris.Cl, KCl, $(NH_4)_2SO_4$, 5mM $MgCl_2$) (Qiagen) and 1μM final concentration of each primer (Sigma-Genosys), made up to 25μl with nuclease-free H_2O.

2.10.4 Real time SYBR green PCR conditions

The PCR using Qiagen SYBR green I must start with an initial incubation of 15 minutes at 95°C to activate the HotStart Taq DNA polymerase.

Table 2.11 Cycling conditions for SYBR green

Step	Time	Temperature	Ramp	Additional comments
Initial activation step	15 min	95°C	20°C/sec	
Denaturation	15 sec	94°C	20°C/sec	
Annealing	20-30 sec	50-60°C	20°C/sec	
Extension	10-30 sec	72°C	20°C/sec	Aquire data to appropriate channel

2.10.5 Taqman probes

In Taqman assays the Taq polymerase enzyme cleaves an internal labelled nonextendable probe during the extension phase of the PCR. Three oligonucleotides are used; a forward and reverse primer and a probe. The probe is dual-labelled, with a reporter dye e.g. FAM (6-carboxyfluorescein), covalently attached at the 5' end and a quencher dye e.g TAMRA (6-carboxytetramethylrhodamine), bound to the 3' end by a linker. As long as the probe is intact (during annealing), the fluorescence energy is quenched by the proximity between the fluprophore and quencher. During the extension phase of the PCR the probe is cleaved by the 5'→3' enzymatic activity of the Taq polymerase, thereby the

quencher and the fluorophore are separated allowing fluorescence emission from the reporter dye.

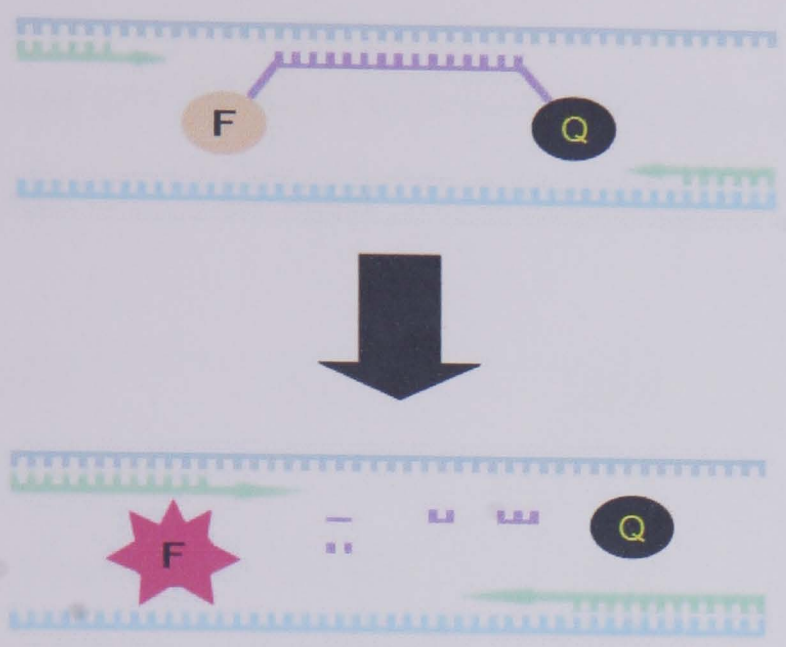


Fig 2.5 Schematic diagram showing how taqman probes detect PCR products. After denaturation, the primers and probe anneal to the target. Fluorescence doesn't occur because of the proximity between flurophore (F) and quencher (Q). The probe is cleaved during extension by the 5' to 3' enzymatic activity of Taq, separating the fluorophore and quencher allowing fluorescence from the reporter dye.

2.10.6 Dengue real-time PCR

The quantitative dengue taqman PCR was carried out with Qiagen hot start taq and set up using the CAS 1200 robotics system (Corbett Research). The PCR reaction was set up to be 25µl containing:

Table 2.12 Dengue taqman PCR components

Components	Amount
Water	Up to 25µl
10xBuffer (containing 15mM Mg^{2+})	2.5µl
Mg^{2+} (25mM)	0.75µl
Forward Primer (10mM)	0.75µl
Reverse Primer (10mM)	0.75µl
Probe (5mM)	1µl
dNTPs (10µM)	2µl
Taq	0.2µl

The PCR reaction was carried out on the Rotorgene (Corbett Research) using the conditions below.

Table 2.13 Cycling conditions for dengue taqman PCR

Step	Time	Temperature	Ramp	Additional comments
Initial activation step	15 min	95°C	20°C/sec	
Denaturation	15 sec	94°C	20°C/sec	
Annealing	20-30 sec	60°C	20°C/sec	
Extension	10-30 sec	72°C	20°C/sec	Aquire data to FAM channel

2.10.7 Generation of Standard curves

Standard curves can be used for both absolute and relative quantification. In order to generate a standard curve, at least 5 different concentrations of the standard should be measured, and the unknown target should fall within the range tested. For absolute quantification of DNA, the copy number or concentration of the nucleic acids used as standards must be known.

Table 2.14 Molar conversions for nucleic acid templates

Nucleic Acid/Genomic DNA	Size	pmol/ug	Molecules/ug
1Kb DNA	1000bp	1.52	9.1×10^{11}
Homo sapiens	3.3×10^{9}bp in haploid gene	4.7×10^{7}	2.8×10^{5} (for single copy gene)

Using genomic DNA, a standard curve was generated from 30ng down to 3pg in a ten fold dilution set up using the CAS-1200 from Rotorgene. After completing the cycling reactions, the data was displayed as change in fluorescence against cycles.

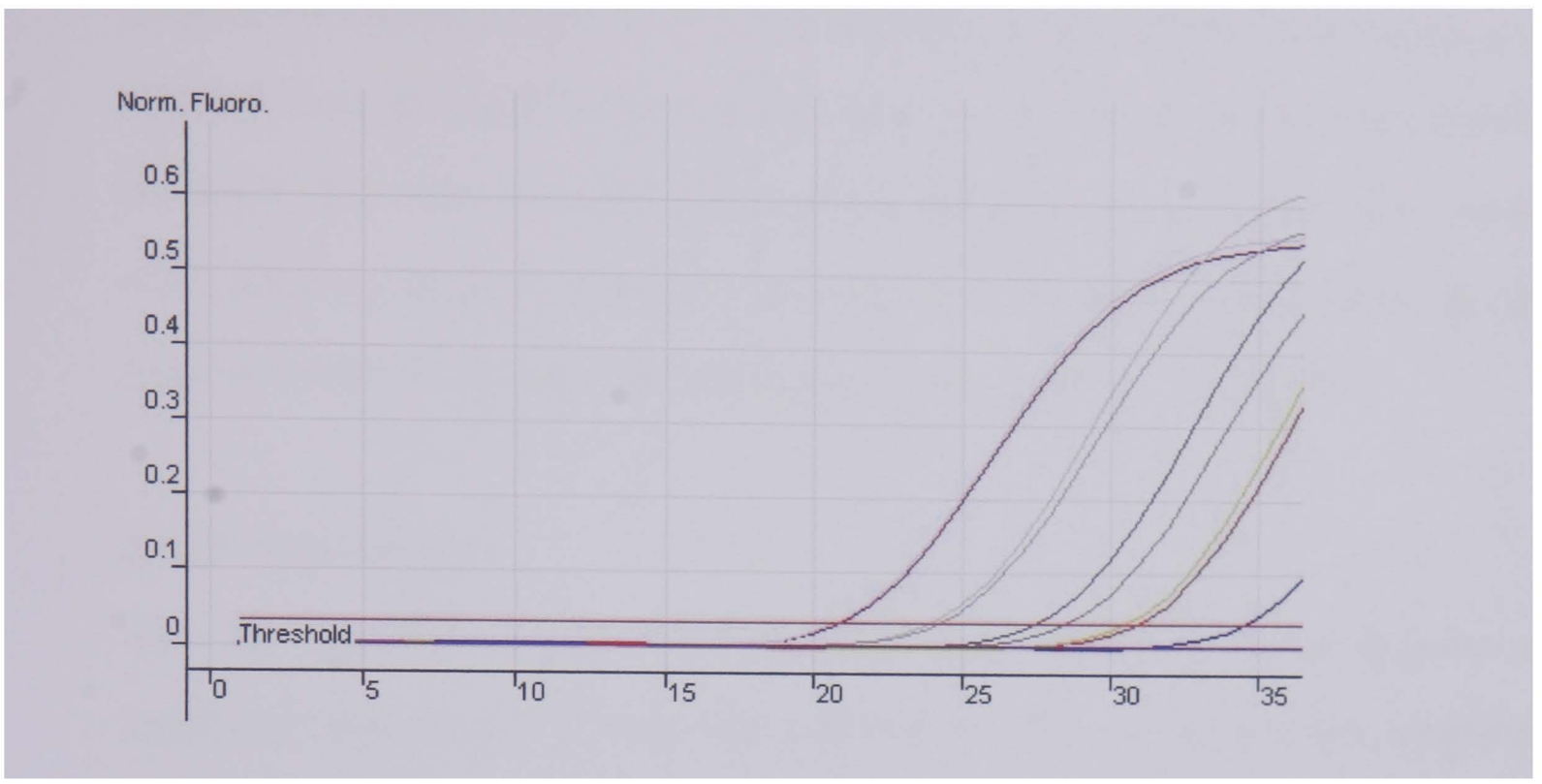


Fig 2.6 Example of a PCR run used to set up a standard curve. A ten-fold dilution of human genomic DNA was used as a template.

From Fig 2.6, a standard curve was extrapolated by using a best fit line from the log-linear region of each curve. A standard curve of the C_T (cycle threshold) against the concentrations was plotted.

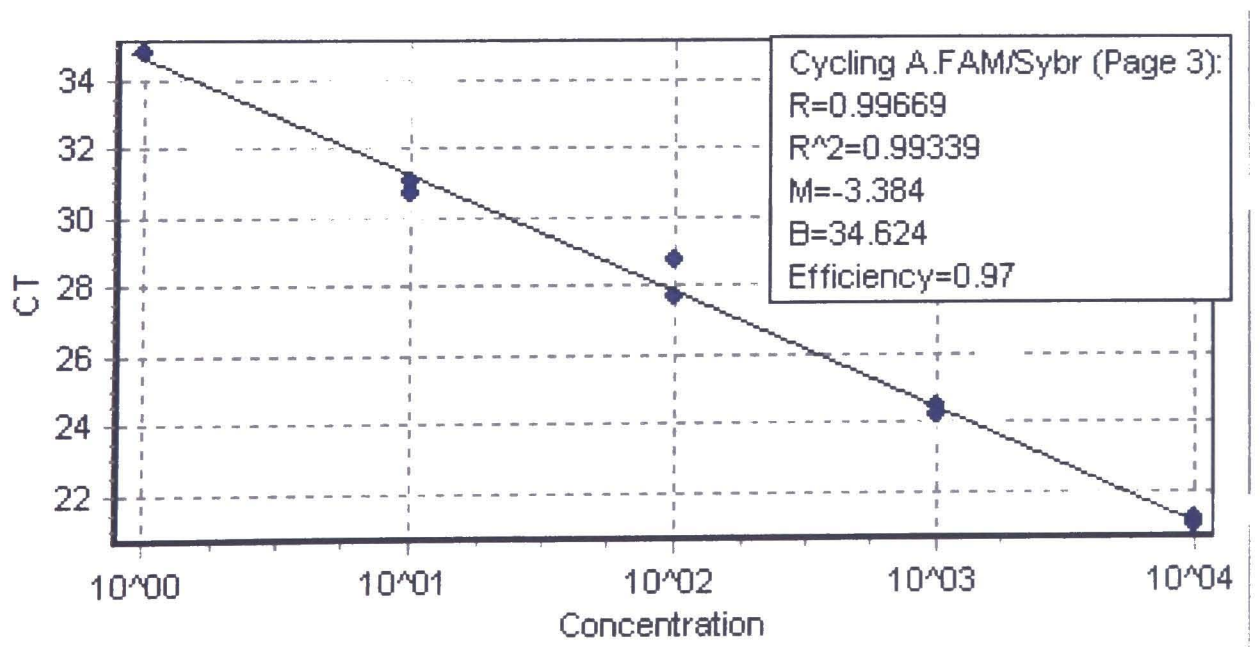


Fig 2.7 Standard curve plot data obtained from fig 2.6.

2.10.8 Constitutively expressed genes

To correct for experimental variation in individual RTs and PCRs the experiment can be normalised to a constitutively expressed gene. Normalisation to housekeeping genes is not always straight forward. It is important to find one that is expressed at a constant level among different tissues and is not influenced by the experimental treatment. The most common housekeeping genes in use at the moment are β-Actin, which encodes a cytoskeleton protein and is expressed among almost all cell types and GAPDH, an abundant glycolytic enzyme, present in most cells.

2.10.9 Data Analysis

After the run was completed, the threshold cycle (C_T) value (cycle at which a significant increase with an exponential growth of PCR product was first detected) was manually set. The Ct value is inversely proportional to the amount of product present. The threshold should be placed above any baseline activity and within the exponential increase phase (which looks linear in the log transformation).

The Ct of the gene of interest was normalised to the housekeeping gene, either using the ratio or the algorithm;

$$\text{Relative expression} = 2^{(\text{Ct GAPDH} - \text{Ct Gene of interest})}$$

The experiment was discarded, if

1. The negative (no template) controls were positive
2. The no RT enzyme controls were positive, indicating the presence of genomic DNA contamination.

3. There was a difference of 3 or more cycles in the housekeeping gene, which indicates that the expression of the normaliser is either up or down-regulated and therefore cannot be used.

2.11 Macro-arrays

Radiolabelled cDNA was generated from 20 μg total RNA by reverse transcription with Superscript II (Gibco) in the presence of ^{32}P-dCTP. Residual RNA was hydrolysed by alkaline treatment at 70°C for 20 min and the cDNA was purified using G-50 columns (Amersham Pharmacia). Before hybridisation to the macroarrays the labelled cDNA was mixed with 50 μg COT-DNA (Gibco) and 10 μg Poly-A DNA (Sigma, St. Louis, USA), denatured at 95°C for 5 minutes and hybridised for 1h to minimise non-specific binding. Preparation of the macroarrays (representing 150 known ISGs), hybridisation of the radioactive cDNAs and scanning and analysis of the macroarrays were carried out as described previously (Schlaak et al, JBC, 2002).

(This work was carried out at the Department of Gastroenterology, University of Essen, Germany by Dr. Schlaak).

2.12 *In vitro* transcription

This was carried out using Ambion T7 MegaScript, which is designed for the large scale production of RNA. For in vitro transcriptions DNA with a promoter site is required as a template. This can be either linearised plasmid DNA or PCR products that contain an RNA polymerase promoter. A PCR primer was designed to incorporate the T7 promoter included in the BVDV plasmid to match with the 3' UTR at the opposite end to use as a template. To obtain enough template, 25 PCR reactions were performed and pooled together before being purified using a Qiagen

PCR purification kit. The following reaction components are for the in vitro transcription, which are assembled on ice under RNA working conditions.

Table 2.15 Components for an *in vitro* transcription

Components	Amount
Nuclease-free water	To 50µl
ATP solution	5µl
CTP solution	5µl
GTP solution	5µl
UTP solution	5µl
10x Reaction Buffer	5µl
2.5µg linear template DNA	2.5µg
2µl Enzyme mix	5µl

The reaction was mixed thoroughly and incubated at 37°C for 15 minutes and then at 37°C overnight. The DNA template was removed by DNaseI treatment and the RNA recovered by Lithium chloride (LiCl) precipitation (ppt). 1µl of DNaseI was added to the reaction mixture and incubated at 37°C for 15 min, the reaction was stopped and the RNA ppt by adding 30µl Nuclease-free water and 30µl LiCl ppt solution. The mixture was left at -20°C for 30 min before centrifugation at 4°C to recover the RNA pellet which was washed with 70% Ethanol and re-suspended in DEPC treated water and measured by UV spectrophotometer.

For dengue replicon RNA, the template was again prepared from a pooled PCR reaction using RepT7 and Rep Rv primers (see table 2.4). This reaction also involved the addition of a cap analog (5µl) and dropping the amount of GTP to 1µl

for the first 15minutes before adding a further 1μl of GTP and incubating the reaction at 37°C for a further 2 hours.

2.13 ELISA

Dengue NS1 ELISA

A 96 well plate was coated overnight at 4°C with 50μl of coating buffer containing Rabbit αNS1 (1:1500) before being washed with PBS Tween 20 (all washes are carried out 3-4 times). 150μl of 1% gelatin in PBS with Azide was placed in the wells and left for 1 hour at room temperature to block. The contents of the plate were then emptied and washed again with PBS Tween 20. The samples were serially diluted in PBS Tween 20 + 0.25% gelatin, and together with a standard curve of Dengue NS1 (100-5ng/ml), left to incubate for 1 hour at 37°C before being washed with PBS-Tween 20. 100μl of 1H7.4 diluted to 1/1000 in PBS Tween gelatin was added to the plate and incubated for 1 hour at 37°C before being washed with PBS Tween 20. 100μl of 1/1000 dilution of goat anti mouse IgG/Peroxidase conjugate was added to each well and again incubated for 1 hour at 37°C. The plate was then washed three times with final wash solution and 100μl of TMB substrate solution were added for 15 minutes at room temperature in the dark. The incubation was stopped with 100μl of 1M H_2SO_4 and the plate mixed with swirling for 15 seconds. The absorbance at 450nm was measured in a microplate reader within 5 minutes after the addition of the stop solution.

2.14.i SDS-Polyacrylamide gel electrophoresis

The BIO-RAD minigel format was used for routine acrylamide gel electrophoresis. The plates were cleaned with water and ethanol before use and assembled. The use of SDS in the gel and in the loading buffer produces protein denaturation and coats the proteins with a negative charge. Thus the proteins will migrate through the

gel on the basis of size rather than charge or shape when subjected to electrophoresis. Prior to electrophoresis the sample (bacterial pellet or precipitated protein) were re-suspended in 100μl of loading dye and boiled for 10 minutes to denature the proteins. The sample was then centrifuged briefly and loaded on the gel.

2.14.ii Sample preparation for STAT2 Westerns

Due to difficulties in achieving consistent results for STAT2 immunoblotting an alternative sample preparation method was devised.

The cell pellet was centrifuged and washed twice in PBS before adding 100μl of whole cell lysis buffer (WCL) containing 10μl Sigma protease inhibitor cocktail and 10μl PMSF [100mM] and left to incubate on ice for 10 min. The lysate was centrifuged at 14,000rpm at 4°C for 15 min and the nucleic acid pellet removed. The lysate can be stored at -20°C at this stage. To 20μl of the lysate, 2.5μl DTT [1M] and 7.5μl gel loading buffer (Invitrogen) was added before heating to 70°C for 10 min. The sample was then centrifuged briefly and loaded onto the gel.

2.15 Western blotting

The protein samples were separated by SDS-PAGE and the stacking gel removed. The running gel was soaked in protein transfer buffer for 10 minutes for equilibration to prevent any distortions within the gel during the transfer process. A sheet of Hybond PVDF membrane (Amersham) was cut to size and activated in methanol and then washed briefly in transfer buffer. The electroblotting cassette was assembled and the transfer process was carried out at 25V for an hour. Following transfer, the membrane was removed and rinsed briefly in PBS. Non-specific binding sites were blocked by immersing the membrane in 5% (w/v) blocking

reagent in PBS overnight. The membrane was rinsed five times with washing buffer for 10 minutes each time, then incubated with an appropriate dilution of the primary antibody in blocking solution for an hour at 4°C. The washing steps were then repeated as earlier, then incubated for 1 hour with an appropriate dilution of secondary antibody in blocking solution and washed again. The ECL reagents supplied with the Amersham Hybond ECL western blotting kit were mixed and the membrane immersed in them. After 5 minutes incubation the membrane was wrapped in clingfilm and exposed to a sheet of autoradiography film for 30secs-5 minutes before being developed.

2.16 Cell maintenance

The following cell lines were used in this thesis. Unless otherwise stated culture mediums were supplemented with 10% fetal bovine serum.

A549 cells	DMEM
HT1080 cells	DMEM
2FTGH cells	DMEM + 200mg/ml hygromycin B
Huh7	DMEM
K562	RPMI
K562ΔCPAC2A	RPMI + 3ng/ml Puromycin
K562ΔCPAC2A cured	RPMI
ThP1	RPMI
ThP1ΔCPAC2A	RPMI + 3ng/ml Puromycin
Huh7/LN-BR	DMEM + 250µg/ml G418
HL116	DMEM

Cell lines were maintained using standard tissue culture techniques. Frozen stocks of cells were stored in liquid nitrogen. Cells were trypsinised, pelleted and resuspended in 90%FCS, 10% DMSO at a final concentration of 1-10X10^6 cells/ml. Then they were dispensed as 1ml aliquots into cryovials (Costar). The cells were initially frozen at –80°C for 24 hours and then transferred to liquid nitrogen. Cell recovered from liquid nitrogen were initially thawed by agitation in a 37°C waterbath. The cell aliquot was then washed by centrifugation, resuspended in fresh medium and placed in a T25 flask. No drug selection was added in the first 48 hours after thawing, to permit the cells to recover.

2.17 Transfection

A variety of strategies are available for the delivery of genes into eukaryotic cells. They fall into three categories;

1. Biochemical
2. Physical
3. Viral-mediated.

The experimental conditions necessary to transfect cultured cell lines with DNA vary from one cell line to the next. Whether the cells are being transfected for transient or stable expression, the fraction of cells transfected is always low and therefore it is desirable to optimise the transfection conditions for each individual cell line using a positive control. An ideal method to transfer genes into the cells of interest depends upon.

1. High efficiency of transfer
2. Low toxicity
3. Reproducibility
4. Suitability for *in vivo* and *in vitro* applications.

2.17.1 Liposome mediated (DNA)

Cells were transfected with Fugene6 transfection reagent (Roche) using a scaled down version of the protocol supplied. The cells (generally Huh7) were grown overnight in 12 well plates in supplemented DMEM up to 80% confluency. The Fugene 6 was added to DMEM to make up to 100µl and left at room temperature for 5 minutes before adding 0.5µg of DNA. This was incubated for a minimum of 15 minutes again at room temperature. The mixture was then added drop-wise to the cells and left for 24-48 hours, depending upon the experiment.

2.17.2 TransMessenger (RNA)

Cells were grown on coverslips in 24 well plates overnight in supplemented DMEM up to 30% confluent. On the day of the transfection 4µl Enhancer R was added to Buffer EC-R and mixed gently before adding 2µg of RNA to a final volume of 100ul and mixed by gentle vortexing for 10 seconds before incubating at room temperature for 5 minutes. 8µl of TransMessenger Transfection reagent was added to the mix and gently vortexed for 10 seconds before allowing the transfection-complex to be formed at room temperature for 10 minutes. In the meantime the cells were washed twice with sterile PBS. 100µl of DMEM without serum was added to the Transfection mix and added dropwise onto the cells on the coverslip and left for 48 hours before visualising using the fluorescent microscope.

2.17.3 Electroporation

Electroporation is the use of a trans-membrane electric field to induce pores in the cell membrane thereby allowing molecules to pass from one side to the other. Electroporation is affected by three major factors.

1. Cell to cell variability causes some cells to be more sensitive to electroporation than others.

2. For pores to be formed the pulse amplitude and duration had to be above the lower limit threshold.

3. The number of pores and the effective pore diameter increases with the product of amplitude and diameter.

Cells that were approximately 90% confluent were trypsinised and washed twice in electroporation buffer before adding $1x10^6$ cells/100ul to 1mm cuvettes. The cells were then pulsed using an Advance Pulse Agile Electroporation system (model PA4000) (CytoPulse Sciences, Inc.) at an electric field of 1600V/cm, pulse width of 0.2milli-seconds, twice with 0.55second intervals between pulses. The cells were then pulsed again at 400V/cm with a pulse width of 0.8milli-seconds four times with a 0.125second interval between pulses. The cells were then transferred to a 6 well plate containing coverslips and left for 48 hours in DMEM containing 10% serum before visualising using a fluorescent microscope.

2.17.4 Curing of the dengue replicon cells

K562 cells that were stably expressing ΔCprME-PAC2A were removed from puromycin selection and passaged continuously in RPMI containing 10% FBS and 500μg/ml glycirrhizic acid. At intervals, cells were checked for replicon expression by indirect immunofluorescence for dengue NS1. Once the cell line appeared to be cured of the replicon, it was subsequently grown in RPMI containing 10% FBS. The continued absence of replicon in this cell line was checked by indirect immunofluorescence for dengue NS1 and RT-PCR for dengue RNA.

2.18 X-GAL staining

2.18.1 Adherent Cells in Monolayer Tissue Culture

This technique was used to visualise the β–galactosidase gene product from the lacZ gene of pSFV3-LacZ (Invitrogen) when expressed in mammalian cells following transfection.

The culture medium was removed from the 6-well plates and the cells were fixed by the addition of 5% paraformaldehyde (PFA) for 10 minutes in the dark. The PFA was then removed and the cells were washed 4 times with PBS. The cells were then covered with freshly prepared X-Gal staining solution (1mg/ml X-Gal, 4mM potassium ferricyanide, 4mM potassium ferrocyanide and 2mM magnesium chloride). These were incubated at 37°C until satisfactory staining had been achieved (usually between 2hours-18hours). Stained cells were stored at 4°C after replacing the X-Gal staining solution with cold PBS prior to photography.

2.18.2 Adherent Cells, 96 well format.

As a transient transfection control, a β-Gal plasmid was transfected at 20 times less the concentration of the reporter plasmid. The cells were lysed in 100µl of the passive lysis buffer (PLB) supplied with the luciferase kit (Promega, UK) and left shaking at room temperature for 15 minutes before transferring 30µl to a separate plate. 20µl Reporter Buffer and 50µl Assay buffer were then added before incubating at 37°C until a faint yellow colour developed (usually between 3-6hours). The reaction was stopped with sodium carbonate and the plate read at 415nm.

2.19 Luciferase Assays

The appropriate cells were seeded at $1x10^3$/well in a 96-well Microlite plate (Thermolife Sciences, UK) in 100µl of growth medium containing 10% FCS and

stimulated and/or transfected as necessary. After a further 24 hours, the cells were washed twice with PBS before being lysed in 100µl passive lysis buffer (PLB) supplied with the luciferase assay kit (Promega). 25µl of luciferase assay reagent was added to 20µl of the lysed cells and the luciferase expression was read using a microplate luminometer (EG & G Berthold).

2.20 FACS

For each test, 0.25 - 0.5 x 10^6 cells were pelleted and resuspended in 50µl of a previously determined concentration of antibody diluted in RPMI holding medium containing 0.01% NaN_3 and incubated on ice for 30 minutes. Where necessary, the cells were washed and a second layer of FITC or PE conjugated goat anti-mouse IgG2 $(Fab')_2$ was added for 30 minutes on ice. The cells were then washed and resuspended to 1-2 x 10^6 cells/ml for analysis on a FACScan flow cytometer using Cell Quest software (Becton Dickinson).

2.21 Fluorescence microscopy

Cells were grown overnight on sterilised coverslips in their appropriate medium before washing twice with PBS. These were then briefly washed in ddH_20 to get rid of any PBS crystals and then fixed in 50:50 acetone/methanol at -20°C for 1min or fixed in 4% paraformaldehyde in PBS at room temperature for 30 minutes. The cells were then washed twice in PBS, 10 minutes each time before being allowed to air dry. The cells containing the red or green fluorescent proteins were then mounted cell-side down on a slide containing mounting solution, the excess being carefully removed before sealing the coverslip and being visualised under the fluorescent microscope.

2.21.1 Dengue IF

For the NS1 immunofluorescence the cells were grown as above on sterile coverslips and fixed in 50:50 acetone/methanol for 2 minutes. The cells were then washed briefly in ddH2O before being transferred to an empty 24-well plate and incubated with the primary antibody diluted 1:200 in PBS/0.1% BSA at room teperature for 45 minutes. The coverslips were washed 3 times with PBS/0.1%BSA before incubating them with goat anti-mouse F(ab')2-FITC conjugate (Jackson Immunochemicals) diluted 1:100 in PBS/0.1%BSA at room temperature for 45 minutes. The coverslips were washed 3 times with PBS, drained and inverted onto a drop of fluorescent mounting medium on a glass microscope slide. The edges of the coverslip were sealed with nail varnish and the cells viewed immediately using a fluorescent microscope. The Hoescht normalisation antibody was diluted 1:250 in PBS/0.1% BSA for 5 min at room temperature.

2.22 Growing EMCV

EMCV is a member of the cardiovirus genus of the family *Picornaviridae*. It is a positive-sense RNA virus and causes diseases such as type I diabetes, encephalitis and myocarditis in mice. EMCV has been widely used for studies on the molecular mechanisms of virus replication and for quantifying the antiviral activity of type I interferons.

To grow a stock of the virus a permissible cell line such as A549 was used. The cells were 70% confluent before washing with PBS and replacing with DMEM containing 2% serum and 1μl of EMCV stock (M.O.I 5) for 1 hour. The medium was then replaced with supplemented DMEM and the cells left in the incubator until there were no visible signs of cell growth. The cell debris was then removed by centrifugation at 200g for 7 minutes and the supernatant containing the EMCV was aliquoted into cryovials and stored at -80°C until required.

2.22.1 Plaque Assay – to quantify the amount of EMCV

$1x10^6$ A549 cells were plated out in a 6 well plate and left overnight in a 37°C incubator. When confluent (usually after 18 hours) the medium was replaced with a 10-fold dilution of EMCV in duplicate and left to adsorb for 1 hour in the 37°C incubator. After an hour the virus is replaced with 2.5mls of CarboxyMethylCellulose (CMC)/DMEM and left overnight. The CMC limits the spread of the viruses thereby making the plaques easier to count. The following day the CMC/DMEM is removed and the wells stained with methyl violet for 20 minutes before washing with PBS and counting the plaques

If the dilution of 0.01μl virus per ml produces 100 plaques, then this corresponds to 1pfu/μl of diluted virus. For an antiviral assay 0.5pfu/cell is required. If 100 cells are plated, we assume that they double overnight to 200 cells, therefore 100 pfu of EMCV will be required.

2.22.2 Antiviral assay

The standard antiviral assay used here is based on two facts. First, EMCV virus is sensitive to the antiviral effects of interferon and second, that the cell lines used allow EMCV replication.

The assay is carried out in a 96-well format. Cells were plated at a density of $1X10^4$ cells per well in a flat-bottomed 96-well plate. After 24 hours the medium is removed and a titration of interferon is added to the cells. After another 24 hours, the medium is removed and the cells are incubated with an EMC virus solution of 0.5pfu/cell (in DMEM containing 2% serum). After 1 hour the medium is removed and replaced with DMEM containing 10% serum. Approximately 16-24 hours later, cell viability is determined by staining with methyl violet and the absorbance is measured at 570nm

in a plate reader. Assays were usually performed in duplicate and data were normalized, plotted and a dose-response curve fitted using GraphPad Prism (GraphPad Software, Inc., CA, USA). The EC_{50} (concentration that interferon is 50% effective) was calculated using this software.

2.22.3 Trans-rescue Assay

This assay was modified from the above antiviral assay to accommodate the fact that the K562 cells are non-adherent. The same principle applies; $2x10^6$ non-adherent cells are treated overnight with varying doses of IFNα2a. The next day $1x10^6$ cells are counted and 0.5pfu/cell EMCV added in DMEM containing 2% serum for 1 hour at 37°C before being centrifuged and grown in 6well plates in DMEM containing 10% serum. After 18 hours the cells are again centrifuged at 6000rpm for 2 minutes and the supernatants collected. The supernatants are then added as a two-fold dilution onto an A549 monolayer in a 96 well format for 1 hour before being replaced with DMEM containing 10% serum and again left overnight. The following day, cell viability is determined by staining with methyl violet and reading the absorbance at 570nm in a plate reader. The OD is then plotted against the dilution of the EMCV.

2.23 Bioinformatics

Bioedit, WinMDI, Autoassembler, VectorNTI, pDRAW32, GraphPad Prism.

3. Results: Amplifying, Cloning and Expressing HCV fragments

Although the main emphasis of the thesis is the actual construction and testing of a reporter system for an interferon inhibitor, various HCV proteins were obtained from patients to test and compare their inhibitory effect on the interferon system with treatment outcome.

In order to determine the inhibitory effects of the HCV proteins on the cellular response to IFNα, the HCV genes were cloned for expression. HCV samples were obtained from both liver and serum samples from patients with different genotypes. Their treatment outcome was retrospectively used to compare to the results obtained with the reporter system.

Several studies have concentrated on various genes in HCV e.g. NS5a and core as being instrumental in inhibiting the interferon response. These genes were amplified and were then transfected into the reporter assay and their affect on the IFN system determined.

3.1 Experimental Outline

The genes encoding different HCV proteins were cloned for expression. Two NS5a constructs was generated and a pair of previously cloned NS5a constructs was obtained. The NS5a ORFs were subcloned into an expression vector for transient expression *in vitro*.

3.1.1 Cloning and expressing the HCV genes

The HCV genes were originally cloned into the pGEM-T vector system prior to sub-cloning into the pIRES2DsRed2 expression system.

3.1.2 Patient Details

Serum samples and liver biopsies were obtained from (with informed consent, ethics approval No. P1-04-039) HCV infected patients attending the hepatology clinic at the Royal London Hospital, Whitechapel, London, UK. The patients selected were newly diagnosed and being assessed for Pegylated Interferon alpha-2a (Pegasys) and Ribavirin (Copegus) treatment. All patients were diagnosed by detecting IgG antibodies to HCV and all were viraemic (HCV RNA detected using the Roche Amplicor assay, detection levels of HCV RNA as low as 50IU/ml), performed by the Virology Department, Bart's and the Royal London). Patients were excluded if they had evidence of co-infections with HIV or HBV.

Table 3.1 Details of patient samples used for the amplification of the HCV genes.

Patient ID	Serum or Blood	HCV Genotype
#001	Serum	3b
#002	Liver	1
#003	Liver	1
#004	Liver	3b
#005	Liver	3a
#006	Liver	1a
#007	Liver	3
#008	Liver	3a
#009	Serum	3b
#010	Serum	3b
#011	Serum	3b
#012	Serum	3b
#013	Serum	3b
#014	Liver	1

#015	Liver	3
#016	Liver	1
#017	Liver	1

3.1.3 Designing PCR primers for the amplification of individual HCV genes

Using pDRAW32, primer3 and BLAST, initial PCR primers were designed to amplify the various structural and non-structural genes of HCV containing overhanging sequence for the appropriate restriction site (XhoI and EcoRI), Kozac sequence and a translation initiation codon - ATG (see list of primers, table 2.3). Most eukaryotic mRNAs contain a short recognition sequence that facilitates the initial binding of mRNA to the small subunit of the ribosome. The consensus sequence for initiation of translation in vertebrates; the minimum Kozak sequence is: ACCATGG.

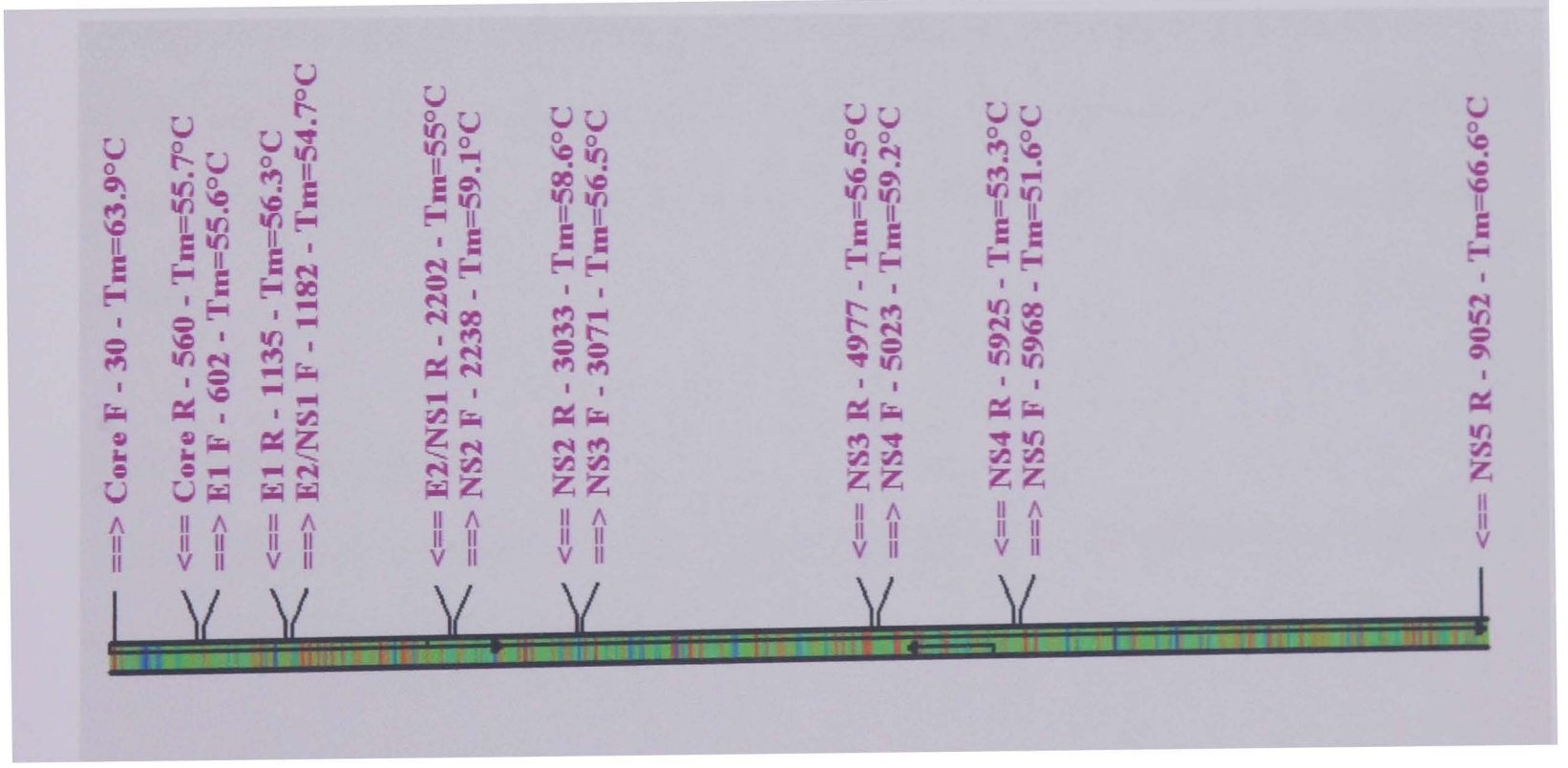


Fig 3.1 HCV genotype 3b, showing primers designed using the pDRAW32 program. The various colours show the percentage GC in 22bp blocks in the genome, red being 31% to blue 90%.

The majority of patients included in the study were infected with either genotype 1 or 3b and therefore primer design was based on these sequences.

3.1.4 Optimisation of cDNA Reaction Conditions

The first strand synthesis is an important step for the amplification of a gene and should be optimised to obtain the best possible results. First strand synthesis can be primed using a variety of primers, e.g. random hexamers, oligo dTs or sequence specific primers. Random primers and oliogo dTs are used to create a pool of cDNAs but sequence specific primers have been shown to generate the highest yield of the specific product. In this case, random hexamers and sequence specific primers were used before deciding on a 50:50 mixture, due to the lack of a polyA tail in the HCV genome. The choice of reverse transcriptase enzyme is now vast. For this purpose the RT enzyme must be sensitive enough to use on low RNA yields, produce a long length accurate transcript (up to ~5Kb), cope with GC rich regions and be able to work at temperature up to 60°C (higher temperatures produce more faithful cDNA especially if the template is GC rich). Although several RT enzymes were tested, the Phusion AMV RT enzyme was selected as it fulfilled all the above criteria.

3.1.5 PCR problems

Due to the low level of HCV in some patient serum/liver samples additional methods were used to amplify the required genes.

3.1.6 NS5a Nested PCR

Although several attempts were made to amplify the full length NS5 gene with different first strand synthesis methods, different PCR enzymes and conditions, none were successful; it was decided to concentrate on the NS5a region which has been extensively studied in relation to the ISDR. Primers 1364-1366 (previously

designed by U. Kumar at St Mary's Hospital Medical School, London, UK) were used in a nested PCR reaction to obtain the NS5a PCR product.

3.2 PCR Amplification

3.2.1 PCR Amplification of the NS5a gene

Although nested primers improved the PCR efficiency, different PCR primers were designed, depending upon the genotype and various combinations were found to be successful for each patient. For genotype 1 samples, the original nested primers worked well, but in other cases, the primers had to be re-designed to incorporate the changes. Using the HCV alignment website (http://hcv.lanl.gov/content/hcv-db/GET_ALIGNMENTS/alignments.html) and BioEdit, primers were re-designed to be specific for the 3b genotype.

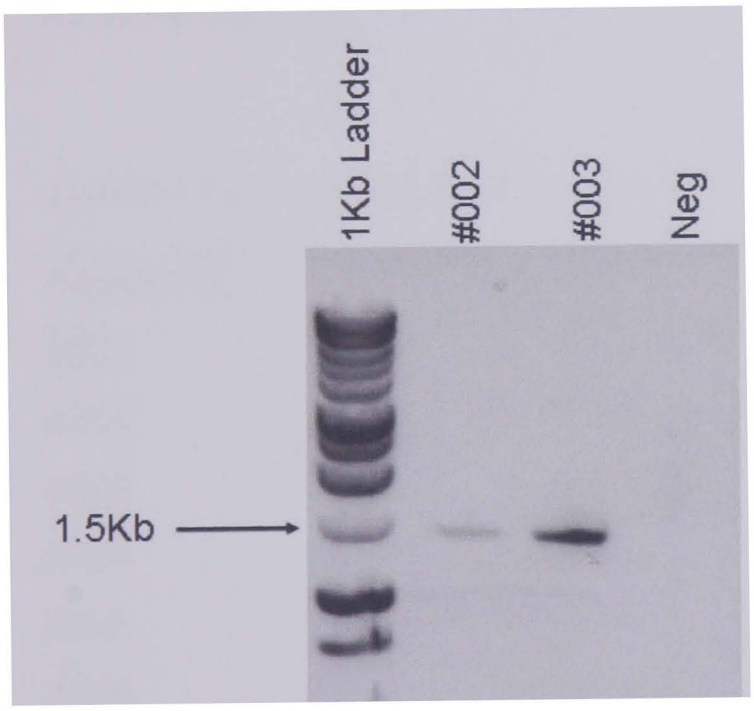


Fig 3.2 PCR amplification of full length NS5a from patient samples. Nested primers specifically designed for genotype 1 incorporating the sticky ends, kozac sequence and the ATG. 1% agarose TAE gel. Predicted PCR product = 1426bp. Marker lane = 1Kb ladder (Promega).

PCR amplification was attempted from all samples, but using the original nested PCR primers, the best products were obtained with samples #002 and #003 (Fig. 3.2). Samples #006, #007 and #008 were also successfully amplified using these primers although at a much lower yield. Amplification of other samples, known to be genotype 3b was attempted using the genotype specific primers.

Fig 3.3 PCR amplification of NS5a genotype 3b from patient samples. Nested PCR using genotype specific primers, incorporating the sticky ends, kozac sequence and ATG. 1% agarose TAE gel. Predicted PCR product = 1426bp. Marker lane = 1Kb ladder (Promega).

PCR products were obtained from samples #001 and #009. Although the first strand synthesis step was carried out with a mixture of random hexamers and the NS5 reverse primer, sample #001 was also primed with the specific primer alone and gave a greater yield than the mix; this did not correspond to any increase in product for other samples.

Table 3.2 Success of amplification of the NS5a HCV gene by nested PCR

Sample ID	Primer set
#001	3b 1364-1366
#002	1364-1366
#003	1364-1366
#004	Failed
#005	Failed
#006	1364-1366
#007	1364-1366
#008	1364-1366
#009	3B 1364-1366
#010	Failed
#011	Failed
#012	Failed
#013	Failed

3.2.2 PCR amplification of NS3

Attempts to amplify the full length NS3 gene from serum and liver biopsies were more successful than the full length NS5 gene and it wasn't necessary to use nested PCR. Amplification was attempted from all the samples but the success rate was limited to samples #001 and #009.

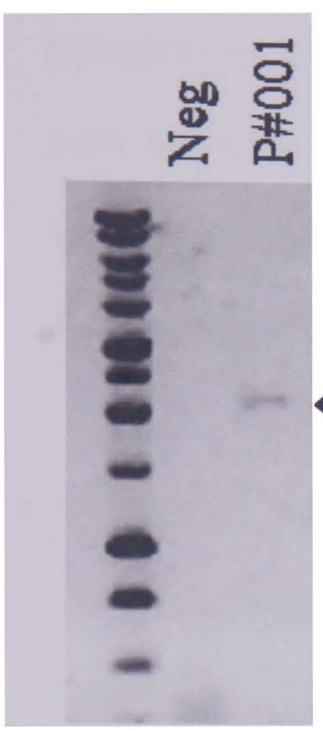


Fig 3.4 PCR amplification of the NS3 gene. Sample #001 was amplified using primers incorporating sticky ends, the kozac sequence and ATG. 1% agarose TAE gel. Predicted PCR product = 1972bp. Marker lane = 1Kb ladder (Promega).

As the amplification of NS5a and NS3 were successful with sample #001, it was decided to concentrate on amplifying the rest of the genes from this patient. So far PCR products have also been obtained for the NS4 gene from this sample.

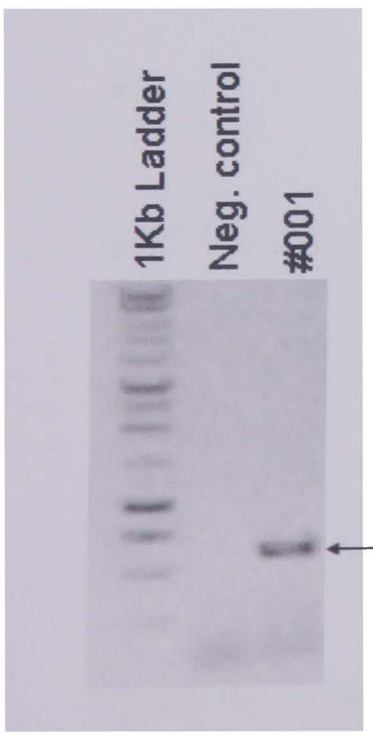


Fig 3.5 PCR amplification of the NS4 gene. Sample #001 was amplified using primers incorporating sticky ends, the kozac sequence and ATG. 1% agarose TAE gel. Predicted PCR product = 964bp. Marker lane = 1Kb ladder (Promega).

The PCR products obtained were used in subsequent cloning steps.

3.3 Cloning the HCV genes

All HCV primers used in this thesis were designed with XhoI and EcoRI sites for insertion into pIRES2-DsRed2 vector, but were sub-cloned into pGEM-T for ease of transfer between different expression systems and also as a fail-safe mechanism. The pIRES2-DsRed2 vector is a bi-cistronic vector containing an EMCV IRES between the multiple cloning site and the red fluorescent protein thereby allowing both the gene of interest and the DSRed2 gene to be translated from a single bicistronic RNA. The RFP allows the transfected cells to be detected by either fluorescent microscopy or FACS which will be convenient in later assays.

As the pfu enzymes do not allow the addition of an A-tail for cloning into a pGEM-T transfer vector, an A-tail was added to all PCR products using Qiagen taq and dATP [1mM] at 70°C for 15min before they were purified using Qiagen PCR purification kit. The modified PCR products were then ligating into the pGEM-T vector. This additional step overcame some problems with PCR yield and allowed the PCR fragment to be sequenced using SP6 and T7 primers (see table 2.1)

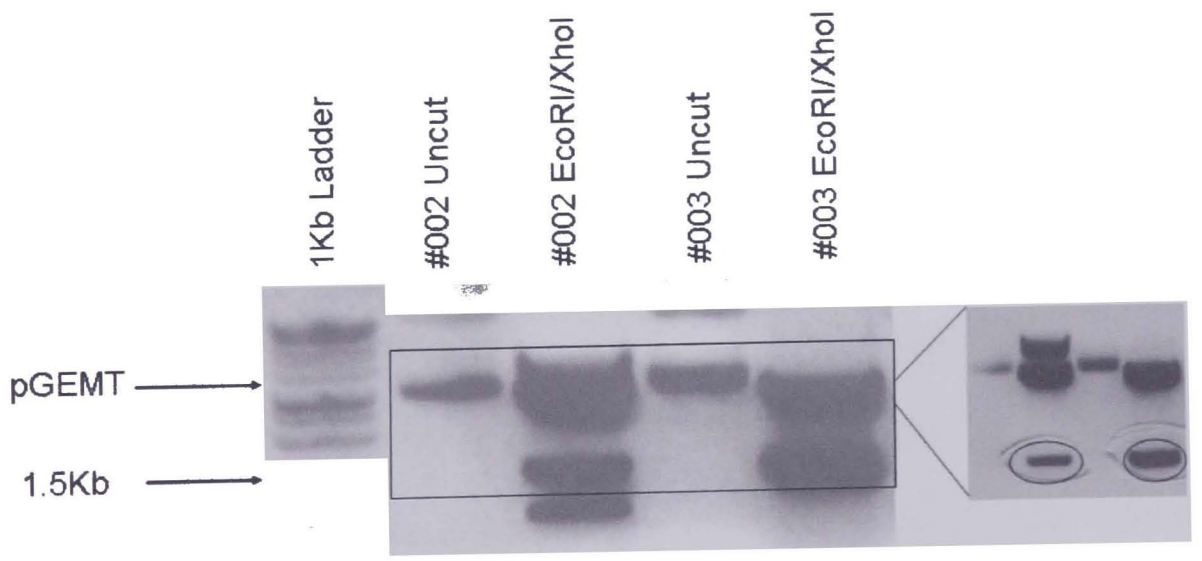


Fig 3.6 Gel electrophoresis of double digested pGEM-T samples containing the NS5a PCR products. Purified plasmid DNA was digested with XhoI and EcoRI to obtain the NS5a PCR product with sticky ends (1.5Kb). The inset gel shows a further separation of the bands to ensure that there was no cross contamination whilst cleaning up the NS5a band, circled bands were excised, cleaned and used for subsequent cloning steps.

After the digested fragment was obtained and cleaned from the pGEM-T vector it was ligated into the desired pIRES2-DsRed2 vector overnight and grown up in DH5α cells. The resulting colonies were tested for the presence of the NS5a gene again by double digestion using XhoI and EcoRI. Positive colonies were grown up further and the plasmid DNA isolated using a Qiagen endo free midi-kit to ensure high quality DNA was obtained for transfections and further experiments.

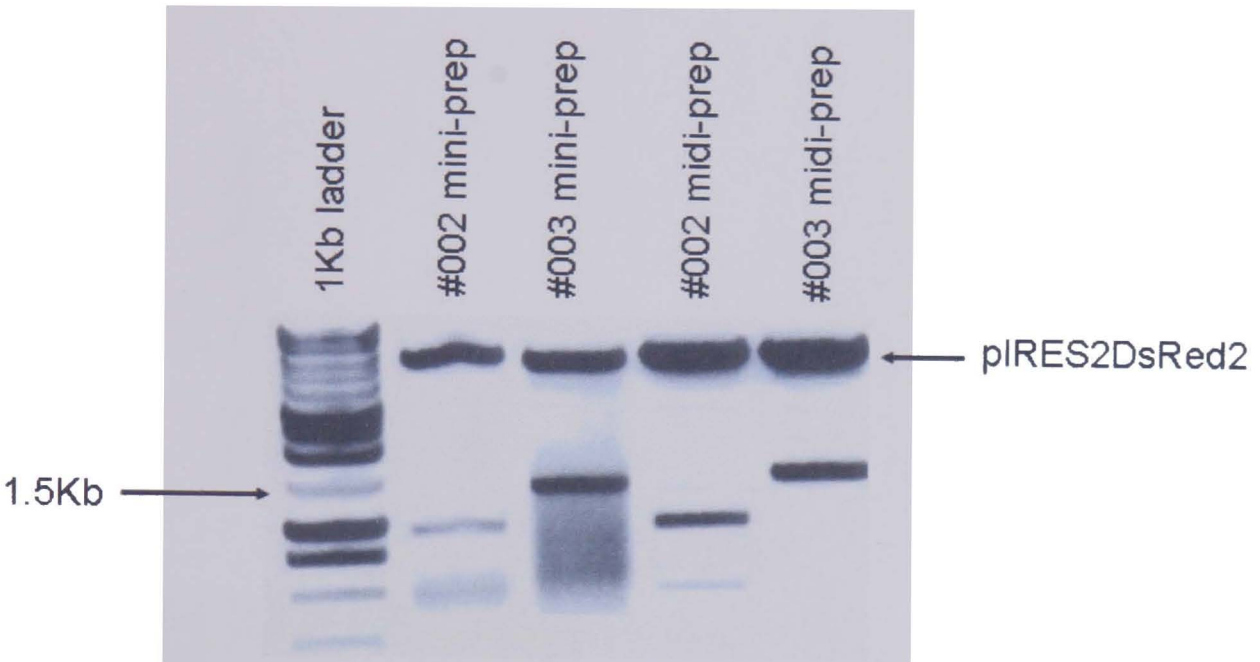


Fig 3.7 Gel electrophoresis of the NS5a gene double digested from the expression vector pIRES2-DsRed2. Colonies from samples #002 and #003 were screened for the presence of the HCV gene by a "quick, crude" mini-prep. Positive colonies were then grown up further and purified using and endo-free kit. The above double digestion shows the presence of the NS5a gene and the difference in the quality of the DNA.

Several different genotypes and quasispecies of the HCV genes exist. Although samples #002 and #003 were reported to be derived from patients infected with HCV genotype 1, a difference in their NS5a gene was seen by restriction digest with #002 having either an extra EcoRI or XhoI restriction site. Although the junction across the plasmid and PCR product was sequenced to ensure that the NS5a PCR products were in frame and therefore would express their proteins, full-length sequencing of the NS5a genes was not completed.

3.4 Expression of the NS5a *in vitro*

Expression of the NS5a from the pIRES2-DsRed2 constructs was tested using Western blotting and antibodies specific for the NS5a protein. The individual constructs were transfected into Huh7 cells using Fugene6 and left for 24-48hours before detection. The protein of the expected size for phosphorylated NS5a (56-58KDa) (Kaneko *et al*, 1994a) was detected using an anti-NS5a rabbit serum.

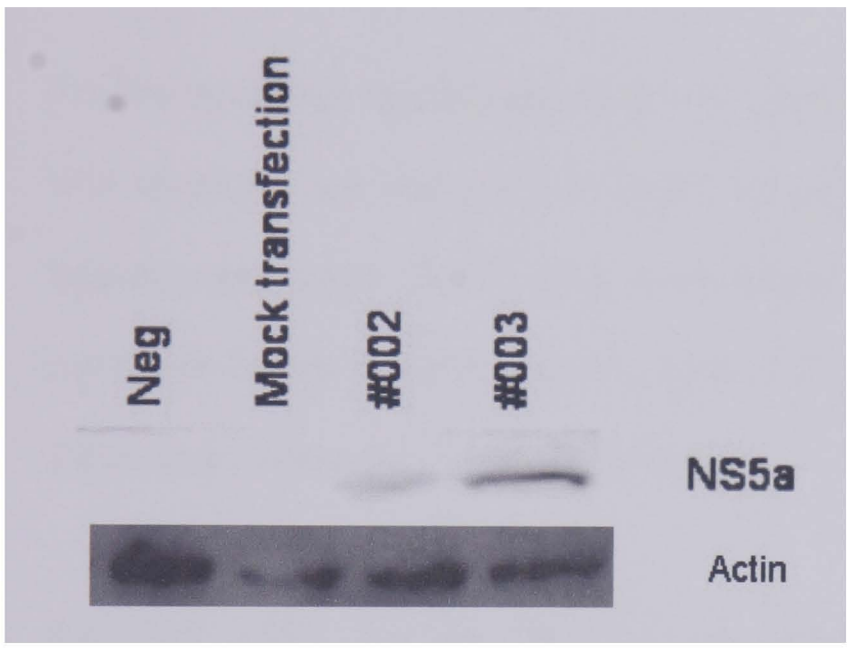


Fig 3.8 Detection and expression of NS5A protein derived from patient samples. Huh7 cells were transfected using Fugene6 with pIRES2-DsRed2-NS5A or mock transfected for 24 hours. Cells were lysed and loaded onto a Tris-Glycine 10% polyacrylamide pre-cast gel. Prestained protein standards were used. Pooled rabbit anti-NS5A serum was used in a Western Blot to detect NS5A protein.

3.5 Expressing and detecting the RFP

To confirm that the pIRES2-DsRed2 plasmid would function as part of a reporter assay in a FACS based system, BHK cells were transfected with 2μg of the vector using Fugene6 at a ratio of 3:2 and analysed by flow cytometry at 24hrs.

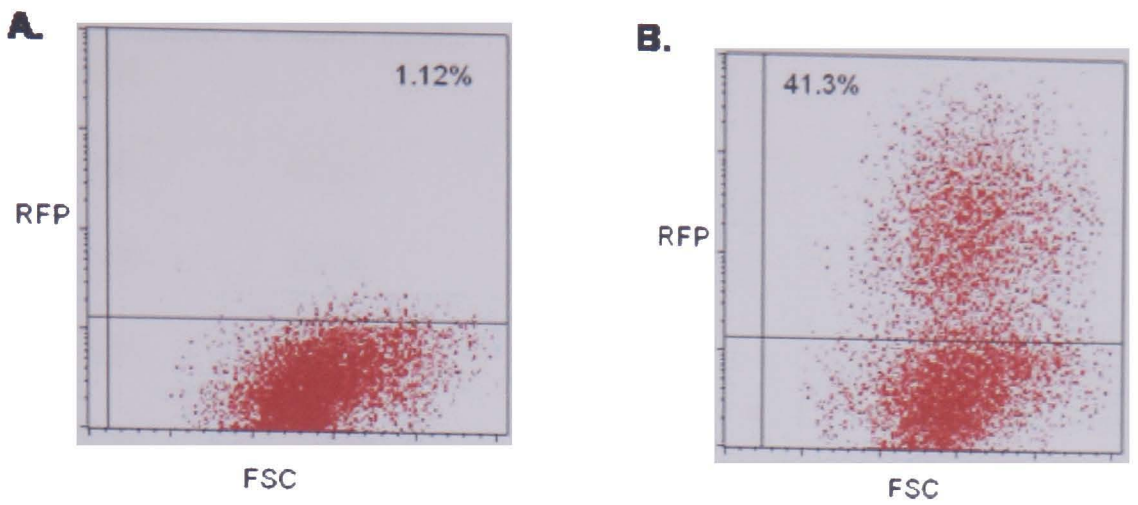


Fig 3.9 BHK cells transfected with pIRES2DsRed2. BHK cells were plated and left overnight to achieve ~70-80% confluency before being transfected with pIRES2-DsRed2 using Fugene6 and analysed by flow cytometry A. Mock Transfection and B. pIRES2-DsRed2.

For the designed reporter assay the experiment needs to be conducted in a suitable, IFN sensitive cell line such as Huh7 or HepG2, both liver cell lines as HCV is a hepatotropic virus. Huh7 cells were plated out and left to grow until ~70-80% confluent before transfecting at a ratio of 3:2 with Fugene6. The cells were left for 24hrs then analysed by flow cytometry.

Although ~45% transfection rate was seen with BHK cells, this could not be achieved using Huh7 cells where the transfection efficiency was normally 5%. The protocol was modified to optimise the transfection, varying the ratios, increasing the time between transfection and FACS analysis up to 72hrs and changing to another liver cell line but to no avail. The highest transfection efficiency achieved with the liver cell lines was only 8%.

Transfection Reagent	Cell Type	Reagent:DNA	Amount DNA (μg)	Length of Transfection (hrs)
Fugene6	BHK	3:1	2	48
Fugene6	BHK	3:2	2	48
Fugene6	BHK	6:1	2	48
Fugene6	HeLa S3	3:1	2	24
Fugene6	HeLa S3	3:1	2	24
Fugene6	Huh7	3:2	2	24
Fugene6	Huh7	3:2	2	24
Fugene6	Huh7	3:2	4	24
Fugene6	Huh7	3:2	4	48
Fugene6	Huh7	3:2	2	48
Fugene6	Huh7	3:2	2	72
Fugene6	HepG2	3:2	4	24
Fugene6	HepG2	3:2	4	48
Fugene6	HepG2	3:2	2	48

Table 3.3 Methods, conditions and cells tested to optimise the pIRES2-DsRed2 transfection.

To ensure that the problem did not lie with the FACS analysis, i.e. that the cells were transfected but in the liver cell lines the red fluorescence was too weak to differentiate from background, Huh7 cells were co-transfected at a ration of 1:1 with a GFP containing plasmid.

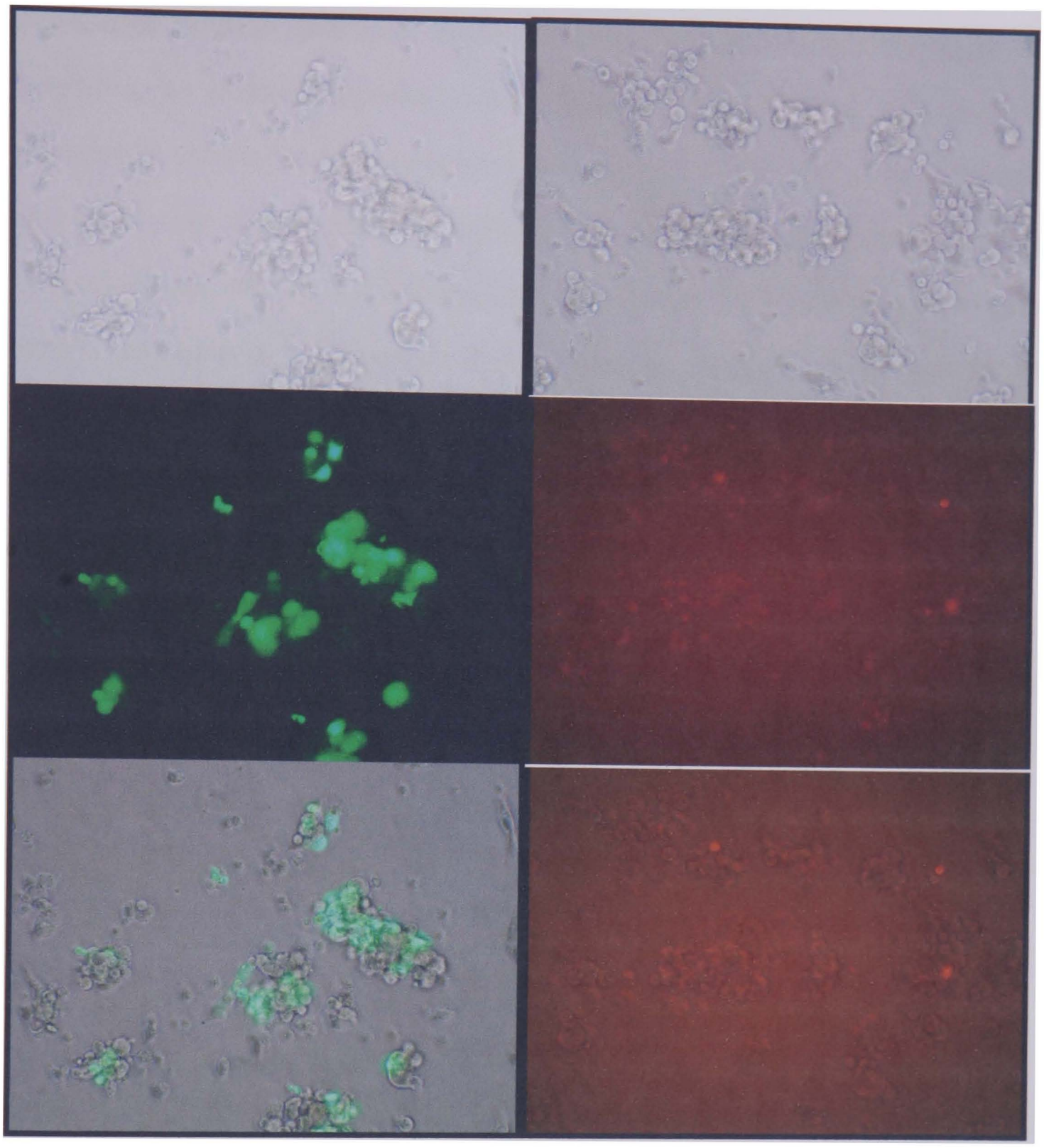


Fig 3.10: Fluorescence microscopy to demonstrate the different transfection efficiencies using GFP and RFP plasmids. HepG2 cells were transfected using Fugene6 and left overnight before being visualised under the fluorescent microscope. Left hand panels show the cells under light microscopy, the GFP transfected cells and the overlay between them. The right hand panels show the cells under light microscopy, the RFP transfected cells and the overlay between them.

Whilst transfection of approximately 5% of cells was observed in cells transfected with the RFP-expressing vector, a 10-fold greater percentage was demonstrated in the cells transfected with GFP-expressing plasmid. This experiment clearly demonstrated that it was not the transfection method which was at fault, but a problem inherent with the pIRES vector. Furthermore, having achieved high

transfection levels in BHK cells, as evidenced by RFP expression detected by flow cytometry, the problem appeared specific to the human cell lines being used. It is important to note that when using fluorescence microscopy, a significant proportion of the cells transfected with the RFP-expressing vector appeared to fluoresce at very low levels. Whilst it was impossible to conclusively determine whether this was merely background fluorescence, if it was not, a plausible explanation for the observed phenomena could be that RFP expression was very low and consequently not picked up when analysing the flow cytometry data.

Although the vector chosen did not appear to be suitable for FACS analysis there are several other RFP vectors available as well as other colours which could be detected in a dual assay with our green fluorescent reporter. Work is ongoing in the lab to address these transfection and FACS issues. To address whether the reporter system would be effective, two previously characterised NS5a clones have been used.

3.6 Previous Patient-Derived Clones (NS5a-CR and NS5a-NR)

The native NS5a clones, termed NS5a-CR (complete responder) and NS5a-NR (non-responder) were derived from serum of patients whose IFN-treatment outcome was known. NS5a-CR was cloned from pre-treatment serum from a patient who showed a complete and sustained response to IFN-treatment. NS5a-NR was generated from serum taking during treatment from a patient who showed no response to IFN-treatment (Paterson M, 1999). These NS5a PCR products were previously cloned into the pHook expression vector from Invitrogen and in this thesis were used as an initial test of the reporter system as they represent an extreme in the IFN-response phenotypes and therefore would give maximal differences in the reporter assay.

4. RESULTS

Developing and testing a Dengue replicon to use as the reporter system.

Dengue was chosen as a prospective reporter system due to it's phylogenetic relationship to HCV and the effect of type I interferon on early dengue viral replication. We aimed to generate an IFN sensitive dengue replicon and assess the effects of different HCV proteins on its sensitivity to IFN.

4.1.1 INTRODUCTION

Dengue virus is a mosquito-borne flavivirus that most commonly causes a mild acute febrile illness (dengue fever) and less frequently causes a severe life-threatening illness characterised by disordered haemostasis and increased vascular permeability (dengue hemorrhagic fever/dengue shock syndrome; DHF/DSS).

Dengue's name comes from the Swahili *Ki denga pepo*, which refers to the sudden onset of cramps and illness caused by evil spirits. The name was used later by English speakers to describe an epidemic in the Spanish West Indies in 1827. Dengue has since been known by several pseudonyms including; breakbone fever, dandy fever, denguero, bouquet fever, giraffe fever, polka fever, or the 5-day or 7-day fever.

The clinical symptoms of dengue are mentioned as early as 992 AD in a Chinese encyclopaedia (Gubler, 1998) but the first detailed description was the 1780 outbreak in Philadelphia by Benjamin Rush (Rush, 1789). Although Rush observed that the outbreak was ongoing in an unusually warm summer with an uncommonly large population of mosquitoes, he failed to link the disease with the insect. It wasn't until the early 20th century after the mosquito was identified as the vector for yellow fever and malaria that it was established that dengue was also a mosquito-borne disease.

The primary vector of the dengue virus is the *Aedes aegypti* mosquito; a small highly domesticated black and white tropical insect that prefers to feed on humans and typically lays it's eggs in artificial containers, such as tyres containing water. As a consequence, dengue is an urban-dwelling acquired disease.

Fig 4.1 Aedes aegypti taking a blood meal from a human (taken from http://www.cbwinfo.com/Biological/Vectors/Aedes.html)

Dengue infection does not have a direct pathogenic effect on the mosquito. After ingestion of a blood meal, there is infection of the epithelial cells lining the mid-gut. The virus then moves into the haemocle and infects the salivary gland, where it can be secreted again to cause infection in humans. Although several attempts have been made to eradicate or control the World's mosquito population, especially in the 1950s, it has proven difficult to use insecticide on a large scale and mosquito levels have surged back to pre-elimination attempts. This is one of the factors implicated as being responsible for the increased incidence of dengue fever in the world, the others being the introduction of new viral strains and the increase airplane travel.

In recent years, the geographic range of dengue in tropical and subtropical regions of the world has extended and DHF/DSS is occurring in new areas of the world and with increased incidence (Jacobs, 2000;Guzman & Kouri, 2003). Currently, there are an estimated 50 million cases of dengue each year and 500,000 cases of DHF requiring hospitalisation (2002).

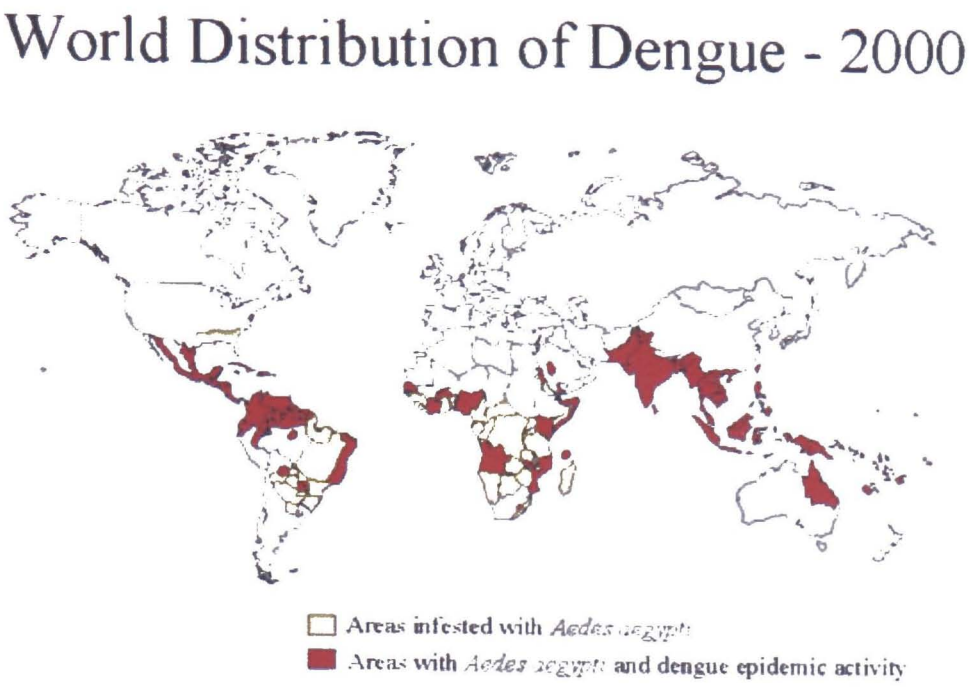


Fig 4.2 World distribution of dengue virus and their mosquito vector, *Aedes aegypti*, in 2000 (taken from http://www.cdc.gov/ncidod/dvbid/dengue/map-distribution-2000.htm)

4.1.2 STRUCTURE AND GENOME

The dengue virus comprises a 10Kb single stranded positive sense RNA, wrapped in an icosahedral nucleocapsid, within a lipid envelope. The genome consists of a 5' cap (m^7G5ppp'A), three structural proteins (capsid (C), prM, the precursor to the membrane (M) protein and the envelope (E) protein and seven non-structural proteins but lacking a 3' polyadenylation tail. The genome is translated as a large polyprotein that is cleaved co- and post-translationally by cellular proteases and a virally encoded serine protease.

4.1.3 STRUCTURAL PROTEINS

The Capsid (C) protein

The C protein is a highly basic ~11Kd protein (25% lysine and argentine residues) that forms a ribonucleoprotein complex with packaged genomic RNA. The nascent C protein contains a hydrophobic domain that acts as a signal sequence for the translocation of prM into the lumen of the ER. Mature protein is generated by a viral serine protease upstream of this hydrophobic domain.(Stadler *et al*, 1997)

The prM protein

The prM protein is cleaved by the host protease, furin (Stadler *et al*, 1997), located in the golgi to form the structural protein M (8Kd) and the N terminal Pr segment which is secreted into the extracellular medium where antibodies against it are thought to provide immunity. Although the cleavage of prM does not impair viral release it is required to provide a highly infectious virus.

Envelope protein

The envelope (E) protein consists of 494 amino acids in three antigenic domains. Domain I is the central structural domain, containing the N-terminus. This is flanked by domain II which contains a fusion peptide at its distal end and domain III, an immunoglobulin-like domain that is thought to contain the putative receptor binding site. The E protein is important in viral entry into host cells (Chen *et al*, 1996) as well as being the major target of the humoral immune response. It contains two glycosylation sites at Asn-63 and Asn-153 located in domains I and II respectively and is anchored to the ER membrane. E protein homodimers disassociate at low pH and each monomer re-associates with two adjacent E proteins to form a trimer that extends outward from the virion surface exposing the hydrophobic fusogenic domains. Initial attachment is thought to be due to basic glycoaminoglycan (GAG)-binding motifs on the C-terminus of E protein, that bind the heparin sulphates of the GAG family. The dengue virus structure has recently been determined and its surface is unusually smooth with the E protein organising itself to form a protective shell around the virus.

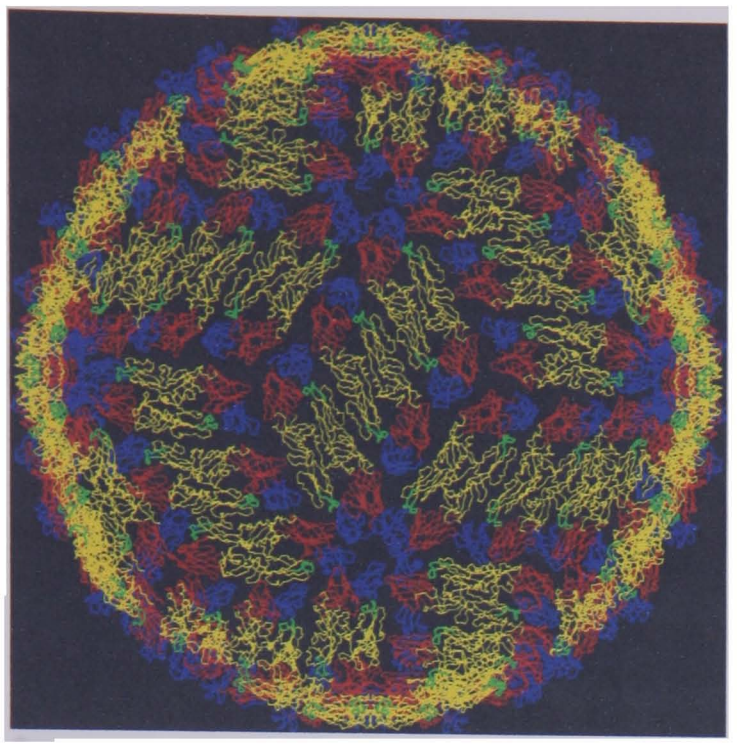


Fig 4.3 The dengue virus – the first flavivirus structure to be determined. The virus surface is unusually smooth and its membrane is completely enclosed by a protein shell. This computer illustration shows how the major protein, called "E" for envelope protein, organizes itself to form a protective shell around the virus. The protein is colour-coded blue, green and yellow to show the three specific domains of the protein. (Purdue University computer illustration)(Zhang *et al*, 2003)

4.1.4 NON-STRUCTURAL PROTEINS

NS1 protein

The NS1 glycoprotein, which is approx 46Kd exists as a cell-associated, cell-surface or a secreted form. NS1 is translocated into the ER lumen and released from the C-terminus of the E protein and is cleaved from NS2A nearly immediately after synthesis by an unidentified host protease. NS1 is slowly secreted from mammalian cells, where three NS1 dimers come together into a soluble hexameric form, but is not secreted from mosquito cells. Dengue NS1 is expressed on the surface of infected cells and the secreted form of NS1 elicits strong humoral immune responses, and immunisation with purified or recombinant NS1 can be protective.

NS2A and NS2B proteins

NS2A and NS2B are relatively small proteins (22 and 14Kd respectively). They are cleaved from each other by a serine protease. NS2a is required for the correct proteolytic cleavage of the C terminus of NS1 and NS2B is a membrane associated protein that forms a complex with NS3, it acts therefore as a cofactor for the serine protease function of NS3.

NS3 protein

NS3 is a large (70Kd) cytoplasmic protein that associates with the membrane through it's interaction with NS2B. It contains several enzymatic activities that implicate this protein in polyprotein processing and RNA replication. The N-terminal residues along with NS2b form the serine protease function which mediates cleavages at the NS2a/NS2b, NS2b/NS3, NS3/NS4a and NS4b/NS5 junctions as well as cleavages that generate the C-termini of the mature core protein. The C-terminal residues of NS3 share significant homology with other RNA helicases containing the motif Asp-Glu-Ala, unwinding the RNA in preparation for RNA replication.

NS4A and NS4B proteins

Both the NS4 proteins are small hydrophobic proteins that are membrane associated. Although no definitive role has been given to these proteins, NS4a and NS4b appear to be co-localised in the site of viral RNA replication.

NS5 protein

NS5 is a large, highly conserved protein which functions as the RNA dependent RNA polymerase (RdRP) of the virus. It includes the motif Gly-Asp-Asp (GDD) common to all RdRPs.

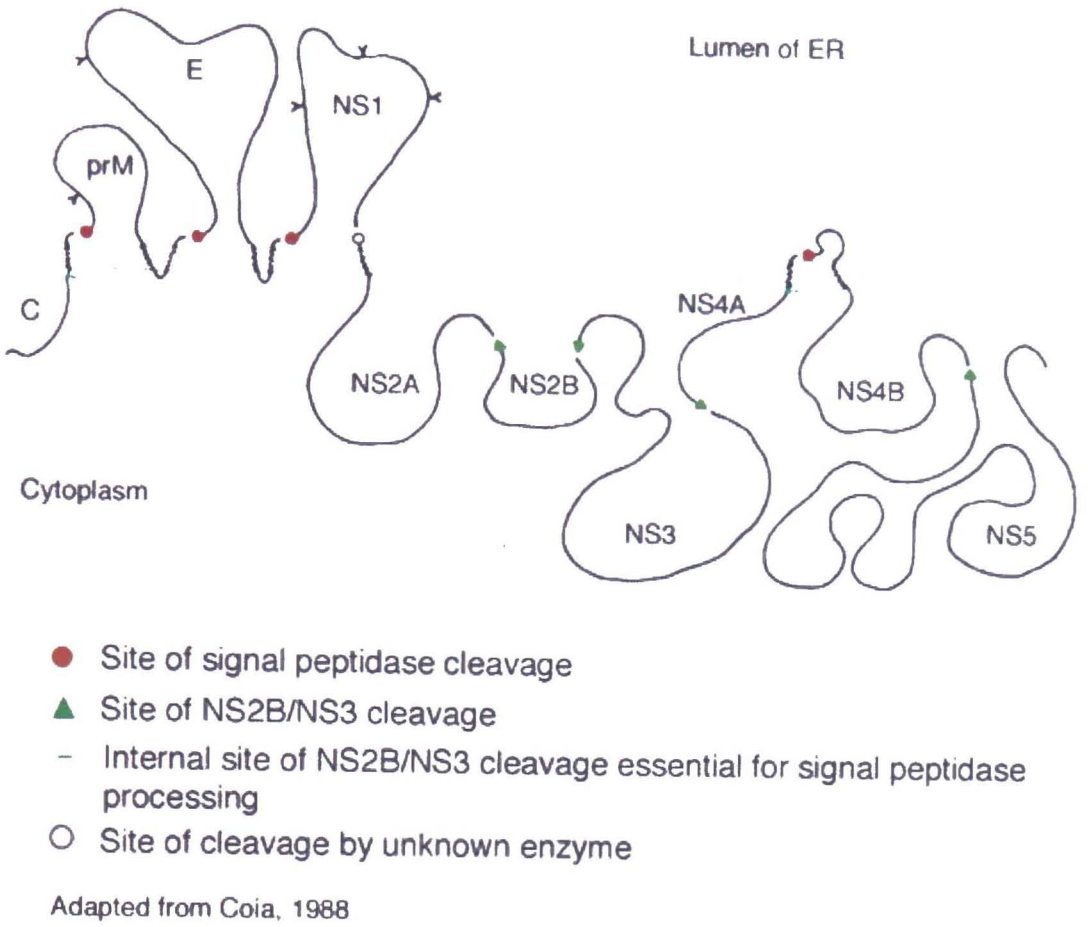


Fig 4.4. Organisation of the dengue virus proteins. Schematic diagram showing how the proteins of the dengue virus are organised within an infected cells. Adapted from Coia 1998

4.1.5 Replication cycle of the dengue virus

The dengue virus can replicate in a variety of cells *in vitro*, including cells of myeloid, lymphoid, epithelial, endothelial and fibroblastic lineages, but monocytes are the cells that are thought to support dengue replication *in vivo* (Diamond *et al*, 2000a). The receptor for the dengue virus is yet to be found, but several different molecules from different cells have been postulated including a highly sulphated type of heparan sulfate present on Vero and BHK cells (Hung *et al*, 1999), two glycoproteins of 40 and 45Kd identified on C6/36 cells (*Aedes albopictus* larvae cell line)(Salas-Benito & del Angel, 1997) and a lectin called DC-SIGN in dendritic cells (Tassaneetrithep *et al*, 2003). The 45Kd protein from the C6/36 cells has recently been shown to bind to the dengue type 4 (Reyes-del Valle & del Angel, 2004). After binding the virions are taken up by receptor mediated endocytosis and the viral nucleocapsid is released into the cytosol. A 3,391 a.a polyprotein is translated from the viral RNA at the surface of the endoplasmic reticulum (ER). Translation begins

at the first AUG codon of the RNA genome and while the ribosome moves forward, the polyprotein is processed into individual proteins. Proteases are encoded by both virus and host. Some proteolytic cleavages (c/prM, prM/E and NS4a/NS4b) occur in the ER and are mediated by host enzyme signal peptidases. The main protease activity is due to the serine protease domain of the NS3 in association with NS2b, which cleaves the viral protein between NS2a/NS2b, NS2b/NS3, NS3/NS4a and NS4a/NS5.

During viral RNA replication, catalysed by the non-strucutral proteins, a negative strand RNA intermediate is generated which becomes the template for the synthesis of several positive sense RNA that serve as both mRNAs for large scale virion production and as virion RNA for assembly into progeny virions. Progeny virions assemble by budding through intracellular membranes into cytoplasmic vesicles. These vesicles follow the host secretory pathway, fuse with the plasma membrane and release mature virions into the extracellular compartment.

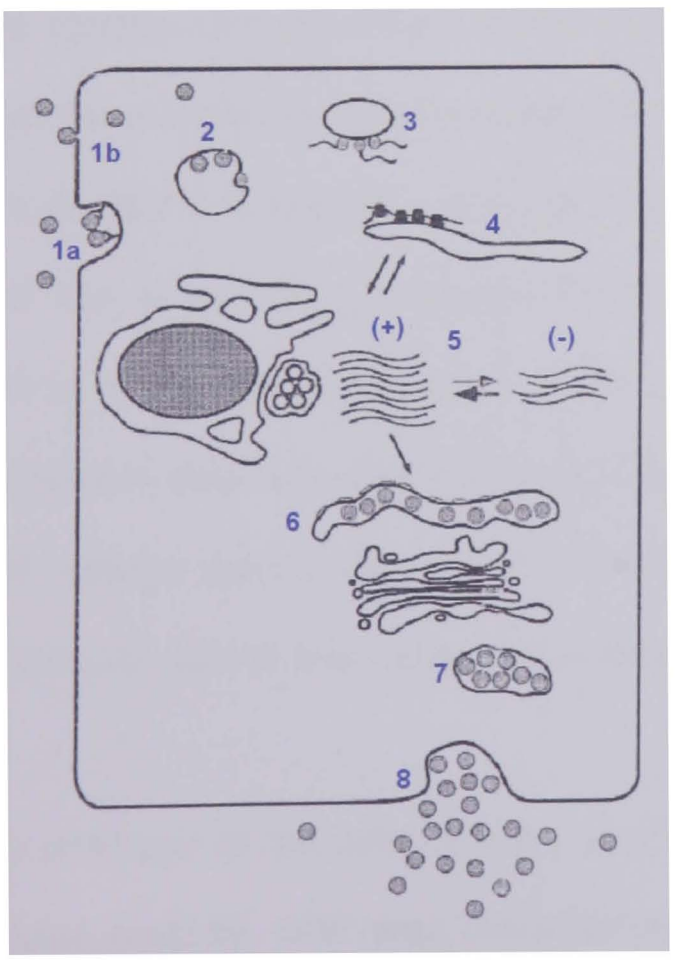


Fig 4.5 Life cycle of the dengue virus (Adapted from Fields).
1a. Attachment and binding
1b. Receptor mediated endocytosis
2. Low pH dependent membrane fusion
3. Uncoating
4. Translation and polyprotein processing
5. Membrane associated RNA replication
6. Virion morphogenesis in intracellular vesicles
7. Virion transport and glycoprotein maturation
8. Vesicle fusion at the plasma membrane: virion release.

4.1.6 Determinants of disease severity

Although the most common outcome of dengue is an acute febrile illness (DF) similar to influenza, a minority of cases lead to spontaneous haemorrhaging (DHF) and most seriously to dengue shock syndrome (DSS), characterised by circulatory failure. Several factors are thought to contribute towards the pathogenesis of DHF, including virus type and host immune status. Peak viral titre, early in the course of dengue infection correlates with disease severity in humans (Vaughn *et al*, 2000) and therefore anything that adds to or subtracts from the virus' ability to replicate can itself become a determinant of disease. Dengue occurs in four serotypes (Den 1-4) and immunity from one serotype does not protect against infection from the others. Several studies have noted that sequential infection with the different serotypes are more likely to produce DHF(Chungue *et al*, 1994;Morens, 1994) due to pre-circulating anti-dengue antibodies (known as antibody-dependent enhancement). These non-neutralising antibodies when bound by cells expressing the Fcγ, increase viral replication in these cells and increase cytokine production and complement activation. Endothelial cells cross-reacted with anti Dengue NS1 have been shown to have activated NF-κB leading to an increase in the expression of IL-6, and IL-8; elevated levels of which are also seen in the serum of cases with DHF (Lin *et al*, 2005). Expression of ICAM-1 (adhesion molecule) induced by anti-NS1 is partly regulated by NF-κB and also by MCP-1 which is also increased in endothelial cells activated by the anti-NS1(Lin *et al*, 2005). This is also elevated in DHF patient serum, which could contribute to the adherence of immune cells to endothelial cells in the inflammatory response associated with DHF.

The genotype of the dengue virus also seems to be associated with a difference in disease severity. DHF was unknown in the Americas until an outbreak in Cuba in 1981, which introduced Den2, a genotype previously associated with DHF in South East Asia to a new continent and with 116,000 hospitalised patients, 34,000 cases

of haemorrhagic fever and 158 deaths. Although the four serotypes of dengue are capable of causing DHF cases, dengue 2 and 3 are most often associated with severe disease. Serotype 2 has been recently shown to have a selective advantage over its more benign counterparts by being able to replicate to a higher titre in human dendritic cells and infecting a greater proportion of the vector mosquito (Cologna *et al*, 2005).

4.1.7 Dengue and Interferon

The immune factors that control Dengue virus infection or contribute to the severe disease are neither well understood nor easy to examine in humans. The *A. aegypti* is not a very efficient vector of the dengue virus. It requires a titre of 10^5-10^7 virus particles per ml of human blood, and thereby allowing only the highly virulent strain of the virus to be transmitted. Experimental evidence suggests that the IFN system may play an important role in limiting dengue virus replication, since knockout mice that lack type 1 IFN receptors develop severe infection after challenge with dengue virus (Johnson & Roehrig, 1999) and pre-treatment of cultured cells with IFN-α/β dramatically reduces dengue virus replication (Diamond & Harris, 2001;Diamond *et al*, 2000b). In this critical early phase of dengue infection, prior to full recruitment of antigen-specific defences, innate cellular antiviral mechanisms may play an important role in suppressing viral replication.

However, IFN-α/β has little effect on dengue replication if cells are treated with IFN after rather than before viral replication has been established (Diamond & Harris, 2001;Diamond *et al*, 2000b). Major IFN-regulated antiviral pathways mediated by PKR, 2' -5' OA$ and MxA appear to exert little effect on dengue virus replication in cell culture.

In view of the data showing that early Dengue replication can be inhibited by type I IFNs and as Dengue is known to give rise to a self limiting infection we speculated that a Dengue replicon would be sensitive to IFN and, in view of the similarities between Dengue and HCV, would prove to be a useful model for examining the IFN inhibitory properties of HCV proteins.

Therefore human cell lines that continuously expressed self-replicating subgenomic dengue RNA (replicons) were established (cells kindly prepared in conjunction with Dr M. Jacobs). The cells containing the dengue replicons were then analyzed in regards to their response to IFNα/β and their suitability as a reporter tested.

4.2 RESULTS

4.2.1 Cell lines stably expressing dengue replicons.

K562 (human leukoerythroblastic) and THP-1 (human monocytic) cell lines stably expressing the dengue replicon ΔCprME-PAC2A were used (designated K562ΔCprME-PAC2A and THP-1ΔCprME-PAC2A respectively). ΔCprME-PAC2A corresponds to the genome of the New Guinea C strain of dengue virus type 2 but contains a large in-frame deletion in the structural region, retaining only the first 27 codons of the C gene and the last 24 codons of the E gene. This replicon also contains an antibiotic selection cassette encoding puromycin N-acetyl transferase (PAC) followed by an artificial protein "cleavage" site (foot-and-mouth disease virus protein 2A) in place of the deleted structural genes. Replicon-containing cells were maintained in RPMI containing 10% FBS and 3μg/ml puromycin (Sigma) and checked for replicon expression before use by indirect immunofluorescence for dengue NS1. K562 and THP-1 cells without replicons were maintained in the same medium without puromycin. (This work was carried out in Dr M. Jacob's lab at the Royal Free Hospital).

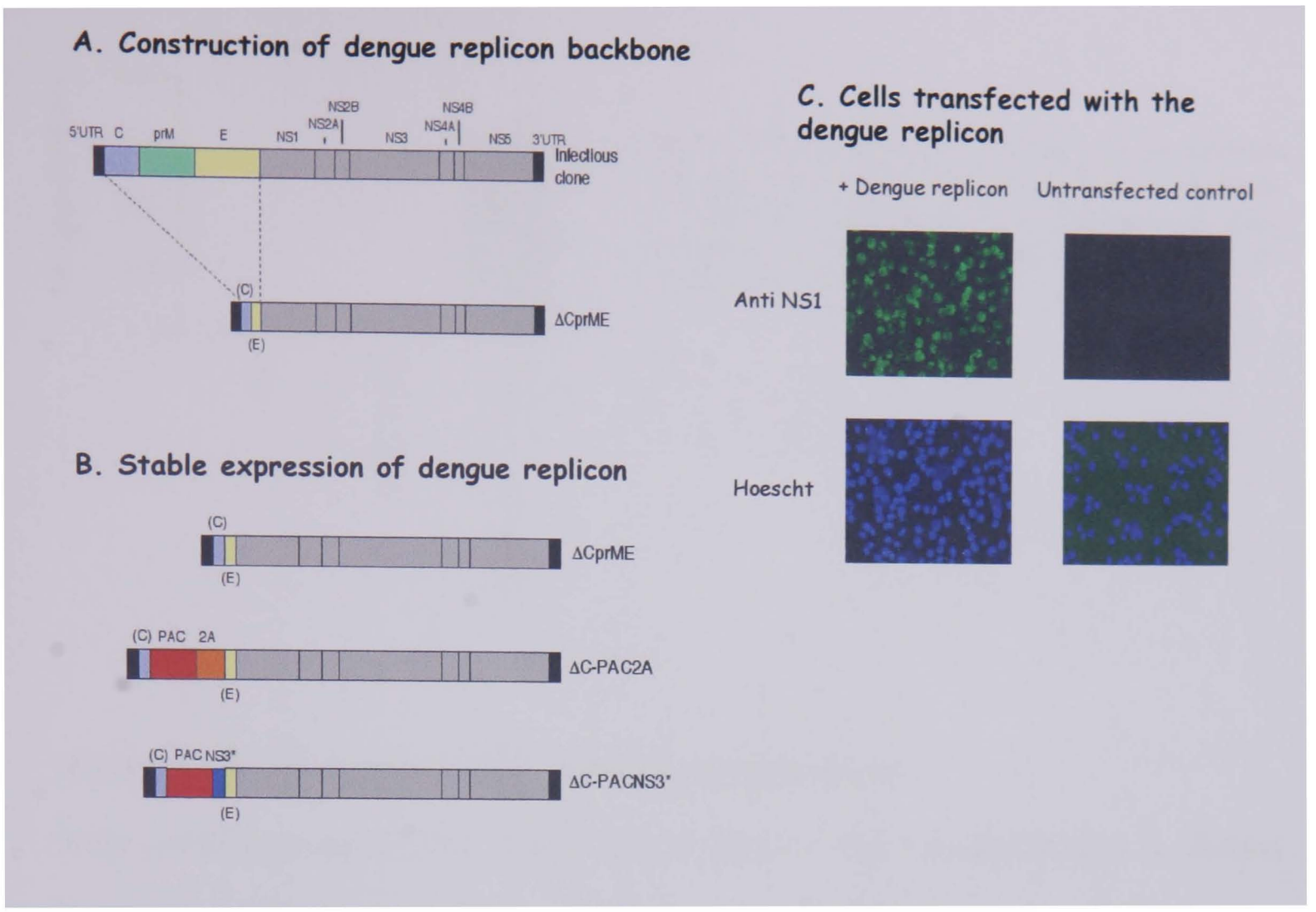


Fig 4.6 Construction and expression of the Dengue replicon. **A.** The dengue replicon was predominately obtained from the 7 non-structural genes and the flanking UTR (untranslated regions) of the dengue virus. **B.** Construction of a Dengue replicon that contains an antibiotic selection cassette encoding puromycin N-acetyl transferase (PAC) and foot and mouth disease virus protein 2A instead of the dengue structural genes. **C.** Expression of dengue NS1 protein detected by immunofluorescence with a fluoroscein labelled antibody in transfected cells, K562ΔCprME-PAC2a, (upper left) and non-transfected cells, K562 (upper right). The lower panels show the stained live cells.

4.2.2 Cured K562 cell line.

K562 cells stably expressing ΔCprME-PAC2A were removed from puromycin selection and passaged continuously in RPMI containing 10% FBS and 500μg/ml glycirrhizic acid, a known antiviral compound. At intervals, cells were checked for replicon expression by indirect immunofluorescence for dengue NS1. Once the cell line appeared to be cured of the replicon, it was subsequently grown in RPMI containing 10% FBS. The continued absence of replicon in this cell line was checked by RT-PCR for dengue RNA.

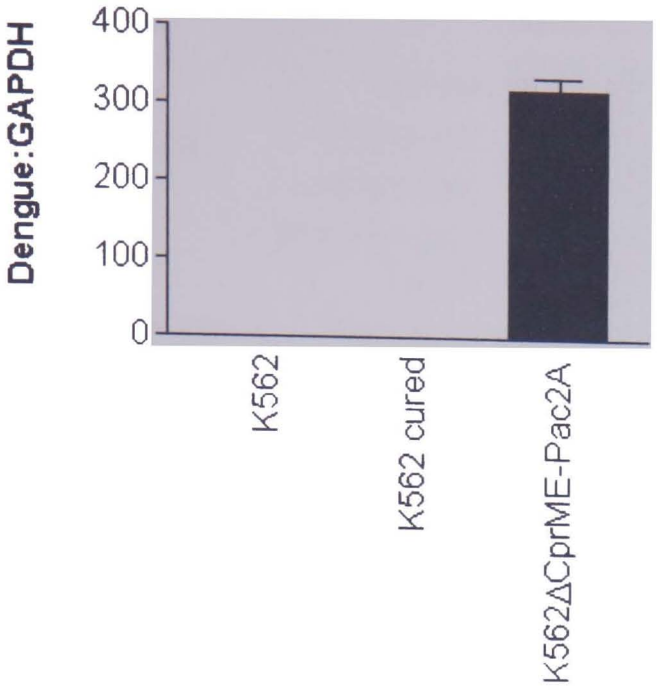


Fig 4.7 Cells continuously treated with glyccerhizic acid are "cured" from the dengue replicon. ΔCprME-PAC2A RNA levels were measured using quantitative RT-PCR and normalised against the housekeeping gene GAPDH

4.2.3 Interferon response (EMCV trans rescue assays)

After continuous expression of the dengue replicon was established and confirmed by IF (Fig 4.6C) the sensitivity of the replicon to the antiviral effects of IFNα2a was tested. The basis of this technique is that inhibition of the antiviral action of IFN by dengue replicons results in rescue of EMCV replication, which is detected in a modified plaque assay on A549 cells. K562 cells with and without the dengue replicons were grown in RPMI containing 10% FBS with 10 and 100 IU/ml IFN-α2a (Roferon-A, Roche) for 24 hours. 1 x 10^6 cells (with or without the dengue replicon) were then infected with 5 x 10^5 pfu encephalomyocarditis virus (EMCV) in RPMI + 2% FBS for 1 hour. Cells were washed and cultured for a further 24 hours in RPMI + 10% FBS. Culture supernatants were then harvested, and doubling dilutions were plated on to confluent A549 cells in a 96-well plate for 1 hour before replacing with RPMI + 10% FBS. After a further 24 hours, the A549 cells were fixed and stained with methyl violet and the optical density in each well was read at 570nm in an automated plate reader.

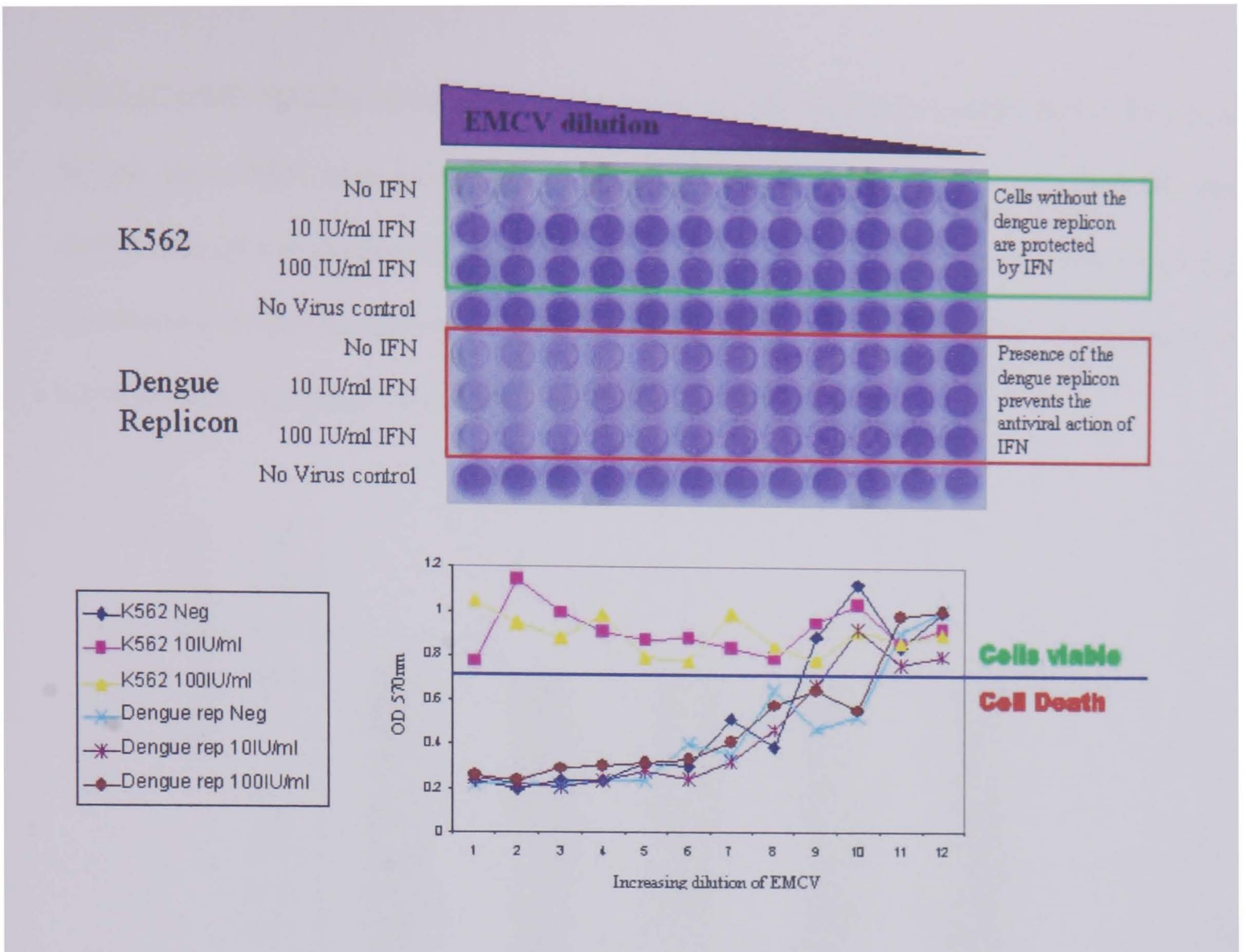


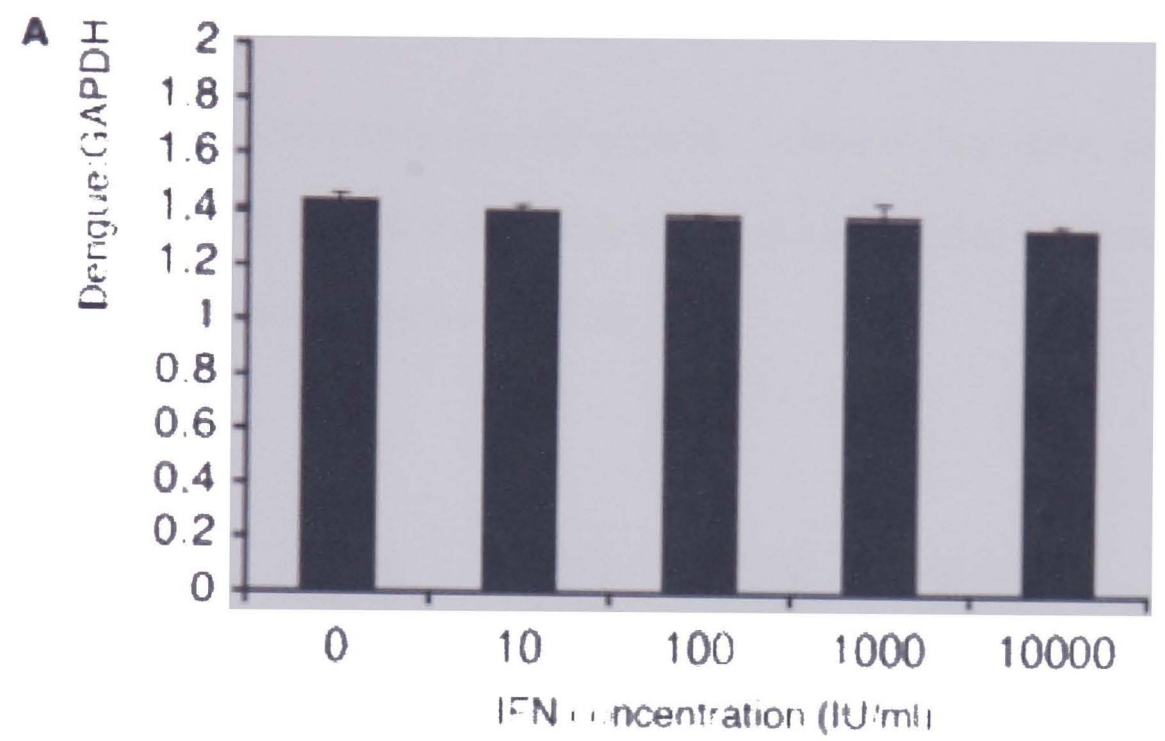
Fig 4.8 EMCV replication in the replicon containing cells is not affected in the presence of IFNα2, suggesting that the dengue replicon inhibits the IFN antiviral pathway. Cells with and without the dengue replicon were treated with IFNa for 24hours before adding 0.5pfu EMCV for 1hour, the cells were then cultured for another 24hours before harvesting the supernatants. The supernatants were plated in a serial dilution onto a A549 monolayer for 1hour before replacing with fresh medium and grown overnight. The cells were stained for viability the next day and the OD measured at 570nm

As expected, pre-treatment of K562 cells with either 10 or 100 IU/ml IFN-α2a dramatically inhibited replication of EMCV. In contrast, pre-treatment of K562ΔCprME-PAC2A cells with the same concentrations of IFN-α2a had no effect on EMCV replication. The data implies that the antiviral action of IFN is inhibited in dengue replicon-containing cells and suggests that the dengue virus has evolved countermeasures to subvert the IFN response.

4.2.4 Response to Interferon

As shown above, IFNα2a has no antiviral effect in the dengue replicon cells. This may also suggest that the dengue replicon is not sensitive to IFNα2a. To further test this hypothesis, the dengue replicon RNA and protein levels were measured.

K562ΔCprME-PAC2a cells were treated with up to 10000IU/ml IFNα2a for 24 hours before total RNA was extracted and reverse transcribed. The PCR reaction was performed and analysed on the Rotorgene (Corbett Research) using primers and a fluorescent probe designed to the NS1 region of the dengue virus. The amount of replicon was quantified and normalised against GAPDH.



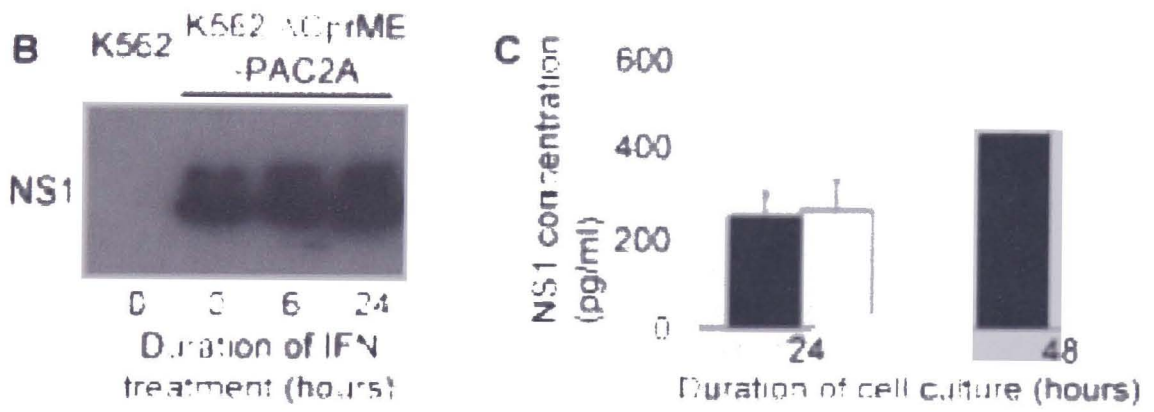


Fig 4.9 Dengue replicon RNA replication is resistant to IFNα. A. K562ΔCprME-PAC2A cells were grown in the presence of up to 10000IU/ml IFNα2a for 24 hours. Dengue replicon RNA levels were measured using quantitative PCR and normalised to GAPDH **B**. K562ΔCprME-PAC2a cells were grown in the presence of 100IU/ml IFNα2a for 0, 6 and 24 hours. Cell lysates ($2x10^5$ cells/reaction) were separated by SDS-PAGE and dengue NS1 protein were analysed by immunoblotting. K562 cells were included as a negative control. **C**. K562ΔCprME-PAC2A cells were grown in the absence (black bars) or presence (white bars) of 100IU/ml IFNα2a for 24 and 48 hours. The cumulative concentration of NS1 in the culture supernatants at each time point were measured by ELISA.

Interferon treatment up to 10,000IU/ml did not have any effect on the dengue replicon RNA levels (fig 4.9A). To confirm that this observation holds true for protein levels, the intracellular amounts of NS1 were measured with a Western blot (fig 4.9B) and secreted NS1 measured using a specific ELISA (fig 4.9C). The protein levels confirmed that interferon alpha did not inhibit the expression of the dengue replicon.

To ensure that the inhibition was not specific to Roferon, the same experiment was repeated using the natural interferons obtained from Wellcome (kind gift form N. Finter see materials and methods 2.1.9).

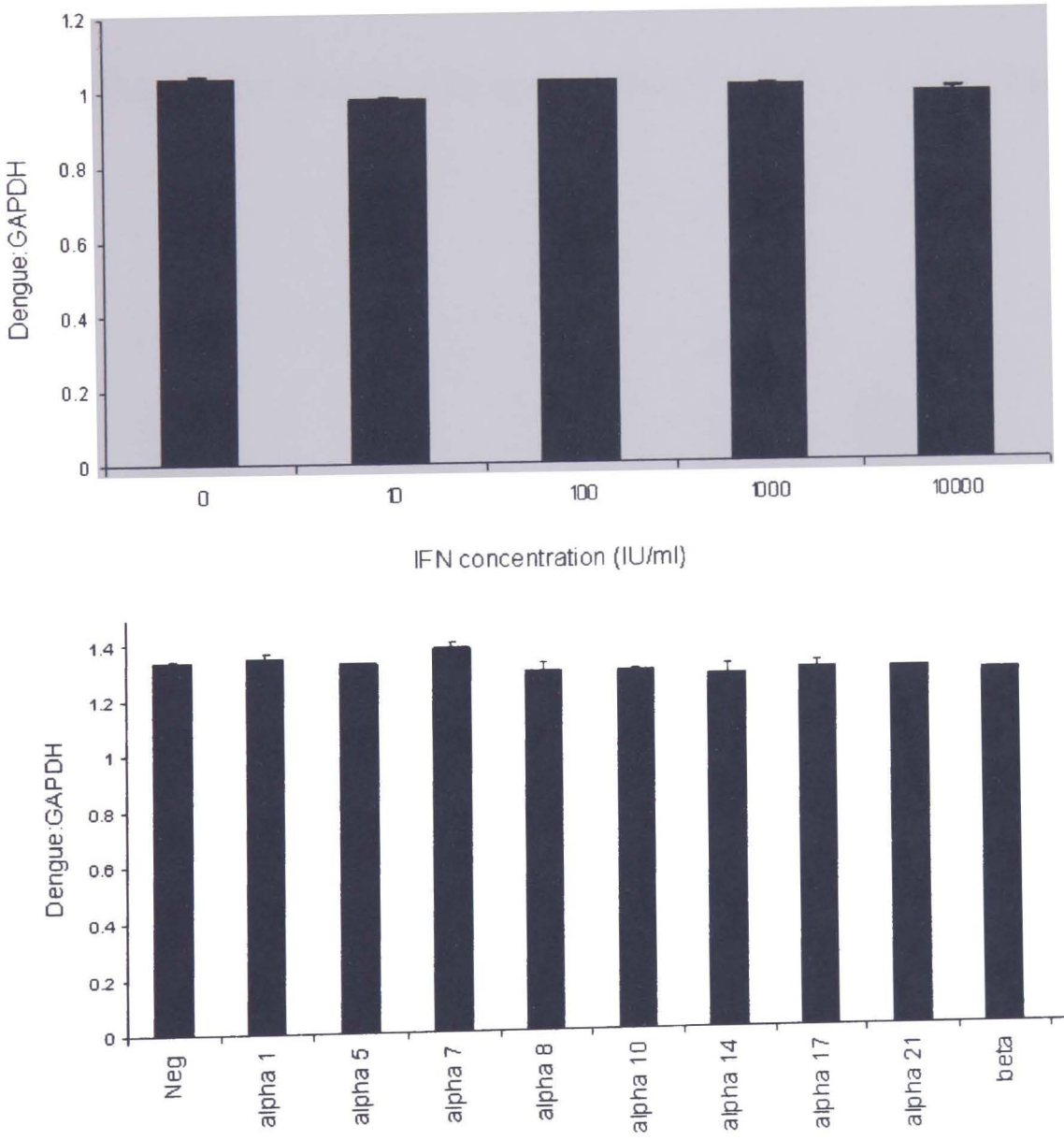


Fig 4.10 Dengue replicon RNA replication is resistant to natural IFNα2, other IFNα subtypes and IFNβ. A. K562ΔCprME-PAC2A cells were grown in the presence of up to 10,000IU/ml IFNα2 for 6 hours. **B.** K562ΔCprME-PAC2A cells grown in the presence of 100IU/ml of the type I Interferon subtypes for 6 hours. Dengue replicon RNA levels were measured using quantitative PCR and normalised to GAPDH.

These experiments demonstrate that dengue inhibits the cellular response to type I IFN. This clearly prohibits the use of a dengue replicon as a reporter for IFN inhibitors but provides an ideal system to analyse the effects of a flavivirus on the cellular response to IFN.

4.2.5 ISG gene induction is inhibited in replicon-containing cells.

Viruses have evolved a diverse range of mechanisms to counter the IFN response. In order to test whether the observed inhibition of the antiviral effect of IFN in replicon-containing cells was due at least in part to inhibition of IFN-induced signal transduction, we measured induction of MxA and PKR gene transcription. K562 cells with and without the replicon were stimulated with IFN-α2a for 6 and 24 hours and gene transcription analysed by quantitative RT-PCR and normalised to GAPDH.

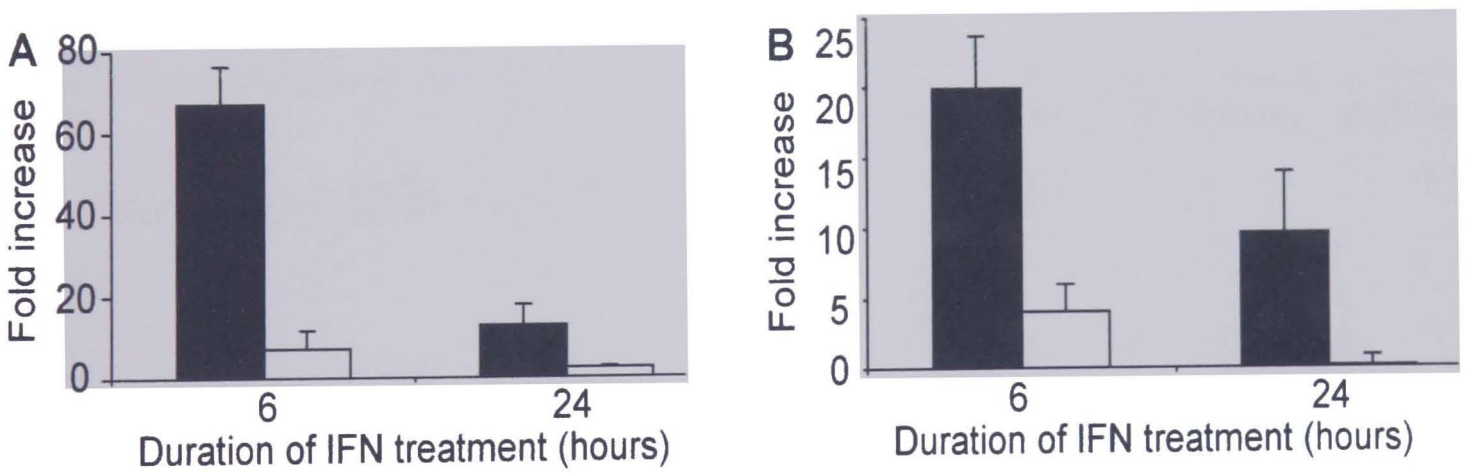


Fig 4.11 Real time PCR to measure the effects of the dengue replicon on Interferon inducible genes. K562 and K562ΔCprME-PAC2A cells were treated with 100IU/ml IFNα2a for 6 and 24 hours before measuring the interferon inducible genes by real time PCR. **A.** Induction of MxA, K562 cells (black), K562ΔCprME.PAC2A (white), B. PKR gene transcription.

As shown (fig 4.11) IFN induced MxA and PKR gene transcription in K562 cells but the dengue replicon markedly decreased induction of both MxA and PKR genes suggesting that IFN signal transduction is not fully functional in cells containing the dengue replicon.

4.2.6 The effect of the Dengue replicon on ISGs, studied using macro-array

Although the two ISGs tested showed a down-regulation in gene expression in the presence of the dengue replicon, to obtain a broader spectrum of the signalling pathway we used a macro-array specifically designed for the type I IFNs.

Preparation of the macroarrays (representing 150 known ISGs), hybridisation of the radioactive cDNAs, scanning and analysis of the macroarrays were carried out in Dr J. Schlaak's lab in Essen, Germany. (Schlaak et al, JBC, 2002). K562 cells with and without the dengue replicon were treated with 100IU/ml IFNα2a for 6 and 24 hours before extracting total RNA (RNA extraction was performed by myself in London, the macroarray was performed in Germany and results were analysed back in London). Results shown are for 24hours but similar data was obtained at 6hours.

Table 4.1 Table of macro-array data for ISGs. K562 cells with and without the dengue replicon where treated with 100IU/ml IFNα2a for 24 hours before extracting total RNA which was then analysed using a type I interferon specific macroaaray. All genes with a two-fold or greater induction in K562 cells without the dengue replicon are shown.

ISGs	24h no replicon	24h with replicon
IFN-alpha induced protein 27(IFI27)	55.8	11.5
VCAM-1	30.6	2.6
MxA	17.9	1.8
IFN-alpha induced protein (clone IFI-616)	12.9	1.1
met proto-oncogene (hepatocyte growth factor)	7.9	1.8
PSMB9	7.9	0.4
IFN-induced protein 17	7.5	1.3
Vipirin(cig5)	7.2	1.3
IL-15	6.5	1.1
9-27mrna	6.4	1.2
STAT1	6.2	1.1
STAT4	6.2	1.5
IFIT1	6.1	1.3
KIAA0284	5.6	1.5

STAT1 (91kDa)	4.9	1.8
IFN induced transmemberane protein 3(1-8U)(IFITM3)	4.9	1.4
INDO	4.8	0.6
IL6	4.8	0.6
IFN induced transmemberane protein 2(1-8D)(IFITM2)	4.5	1.1
MAP2K4	4.5	1.0
IFI35	4.4	1.1
Homo sapiens STAT	4.1	1.6
Raf(c-raf-1)	3.6	2.0
FK506 binding protein 6	3.6	1.9
DEAD-box protein p72	3.5	1.4
IFP 35	3.5	1.3
IFN induced leucine zipper protein (IFIT4)	3.4	1.7
c-myc	3.3	0.7
MIP-1b/CCL4	3.2	1.5
2-5OAS	3.1	0.9
c-myc	3.1	0.7
Gadd45	3.1	0.7
Reticulocalbin	3.1	2.3
ISGF3G	3.1	1.2
IRF1	3.1	1.6
IL-1 alpha	3.0	0.8
Pi3-kinase	2.9	1.9
NF-IL-6	2.9	0.8
TIMP-4	2.9	1.4
GBP2	2.9	1.5
TGF-bR3	2.8	1.2
HLA-E	2.8	1.5
DDX17	2.7	0.5
TAP1	2.7	1.2
Interleukin 2	2.7	1.2
IFI44	2.7	1.1
CBFA (core binding factor A1)	2.7	1.4
Interleukin 13 receptor, alpha 1	2.7	2.1

TIMP-3	2.7	2.9
FKHRL1	2.6	2.0
p57Kip2	2.6	1.2
Catechol o-methyltransferase	2.6	2.4
MAPK6	2.6	0.7
Phospholipid scramblase 1	2.5	1.3
IFI16	2.5	1.1
IFN induced protein with tetratricopeptide repeats 1	2.5	1.6
translocation T(4:11)of ALL-1 gene to chromosome 4	2.4	1.0
TGIF (TG interacting factor)	2.4	1.6
CBP (CREB binding protein)	2.4	2.2
bcl-2	2.4	1.3
SnoN	2.4	1.3
PDGF-alpha	2.4	1.7
IFN induced protein 44(IFI44)	2.4	2.0
ADAM-10	2.4	1.1
Trk oncogene	2.4	1.2
CCR5	2.4	1.2
DEAD box binding protein 1	2.4	0.7
n-myc	2.3	1.7
APOL3	2.3	1.1
(cpp32)	2.3	1.7
IFN regulatory factor 1	2.3	1.6
collagen alpha 1 (I)	2.3	1.6
IFN-GR1	2.3	1.2
MAPK13	2.3	1.6
Mad4	2.3	1.3
ATF-2(creb-2)	2.3	1.4
MAPKAPK2	2.3	1.0
Transcription factor 3 (TFE3)	2.2	2.0
Integrin-beta-6	2.2	1.5
MAPK9	2.2	1.5
Similar to small inducible cytikine subfamily B(Cys-X-Cys),member 11	2.2	1.3
MAP3K1	2.2	1.2

Alpha-1-antiproteinase	2.2	2.1
MCP-1/CCL2	2.1	0.8
Integrin beta 7	2.1	1.8
BST2	2.1	1.3
L-selectin	2.1	2.3
Placental calcium-binding protein	2.1	2.3
Interleukin 2 receptor, gamma	2.1	1.4
TIMP-2	2.1	1.5
PDK1	2.1	1.9
MAP2K2	2.1	1.7
MAPK8	2.1	1.0
MAPK8IP2	2.1	1.7
STAT5B	2.1	2.4
ubiquitin-conjugating enzyme E2L 6(UBE2L6)	2.1	1.8
MAP3K14	2.1	1.8
RGS2	2.0	1.4
RHO	2.0	1.3
Alpha-crystallin	2.0	1.9
MAPKAPK3	2.0	1.6
Cyclin D1	2.0	1.7
Interferon regulatory factor 5	2.0	1.2
Elastase 2	2.0	1.6
Smad4	2.0	1.0
akt-1	2.0	1.5
STAT 6	2.0	1.2
TIMP-1	2.0	1.8
MAPK14	2.0	1.6

Transcription of most, but not all, IFN-inducible genes are inhibited in cells containing dengue replicons, but, there are certain subsets of genes that appear to be up-regulated.

Table 4.2 Subset of ISGs that are up-regulated in the presence of the dengue replicon. Cells with and without the presence of the dengue replicon were treated with 100IU/ml IFNα2a for 6 and 24 hours before extracting total RNA and analysed using a type I specific macro-array. Genes with a greater than two fold induction in the presence of the dengue replicons are shown.

ISGs	24h no replicon	24h with replicon
SOCS2	1.2	3.8
Smad 5	1.9	3.5
MAP3K5	1.4	2.9
MAPK10	1.5	2.9
Folate receptor	1.3	2.5
5' nucleotidase	1.6	2.4
P70 56 Kinase	1.7	2.3
CCR1	1.8	2.3
PDK2	1.9	2.3
Interferon omega 1	1.9	2.2
IL-10R beta	1.3	2.2

These experiments indicate that some ISGs can be up-regulated by IFN, although the fold induction is relatively slight. The gene of most interest in this set is SOCS2, (Suppressors of cytokine signalling 2). SOCS2 is one of 8 family members: cytokine-inducible SH2 protein (CIS) and SOCS-1 to SOCS7, all of which share a central SH2 domain and a C-terminal SOCS box. SOCS2 came to prominence when a SOCS2 knockout mouse displayed dramatic gigantism and has since been found to play a role in the negative feedback of cytokine signalling and could be important for the suppression of type I interferon signalling seen in the dengue replicon cells.

4.2.7 Immunoblotting for total and phosphorylated STAT1 and STAT2.

During the course of these experiments, a paper was published pinpointing STAT1 as the area in which the dengue exerts its effect (Munoz-Jordan *et al*, 2003). STAT (signal transducer and activator of transcription) proteins are transcription factors located in the cytoplasm that become activated by tyrosine phosphorylation. The activated forms dimerize and translocate to the nucleus to activate specific genes. In the case of type I interferons STAT1 and STAT2 form a heterodimer to activate the ISGs and for IFNγ STAT1 forms homodimers.

Although most of the immunoblots worked according to the manufacturer's instructions for the antibodies, STAT2 blots proved to be problematic and required optimisation. Two different primary antibodies (Epitomics and BD) were tested at various concentrations and using different blocking conditions, but the results were inconsistent. Therefore another method of extracting the protein was tested. Cells were lysed using whole cell lysis (WCL) buffer with added PMSF and protease inhibitor cocktail and incubated on ice for 10 minutes before centrifuging at 13,000rpm for 15 minutes at 4°C. The DNA pellet was removed and 20μl of the supernatant was added to 2.5μl DDT [1M] and 7.5μl SDS loading buffer (Invitrogen) before being heated to 60°C for 10 minutes and loaded onto a gel. The protein was then transferred onto a nitrocellulose membrane (the new blocking buffer 5%PVP was not compatible with PVDF membrane) and blocked for an hour with PVP blocking buffer (see materials and methods) before incubating overnight with 1:500 dilution of the BD αSTAT2 antibody. The next day the membrane was washed 4 times in 0.2%Tween/TBS before incubating for an hour with Dako αmouse secondary antibody at a 1:5000 dilution, before detection with ECL+. For summary of optimisation techniques see table 4.3

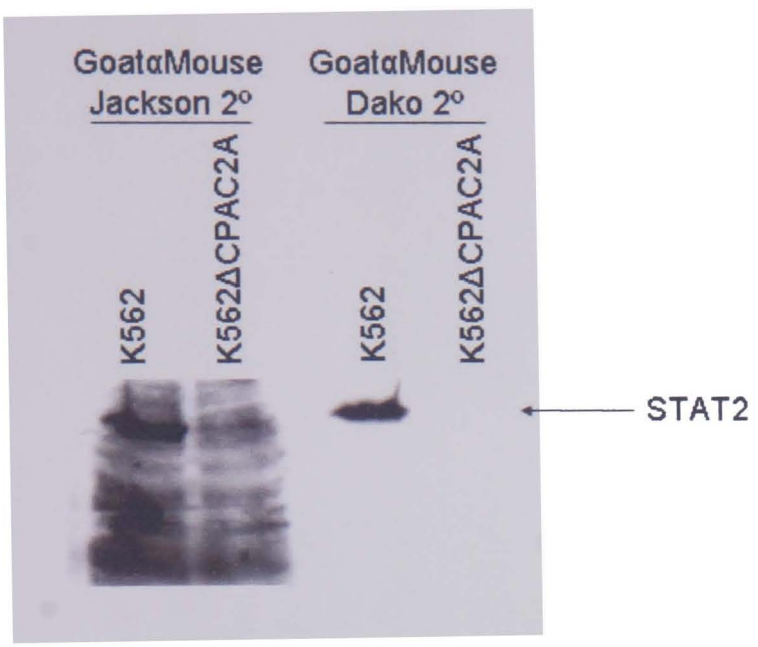


Fig 4.12 STAT2 Immunoblot optimisation. To optimise the STAT2 western different secondary antibodies were tested and as shown in this figure the DAKO secondary Antibody gave cleaner blots.

Protein extraction	Block	1º	2º	Result
Cells lysed in SDS-PAGE sample buffer and heated to 60ºC before loading	5% Milk powder in PBS O/N @ 4ºC	αSTAT2 Mouse mAb, BD transduction lab. 1:500 in 5%Milk/PBS 2hrs @ RT	GoatαMouse Jackson Laboratories 1:10,000	Unsuccessful, dirty blots.
Cells lysed in SDS-PAGE sample buffer and heated to 60ºC before loading	5% Milk powder in PBS for 2hrs @ RT	αSTAT2 Mouse mAb, BD transduction lab. 1:500 in 5%Milk/PBS left O/N @4ºC	GoatαMouse Jackson Laboratories 1:10,000	Variable (worked approx 30% of the time). Weak signal.
Cells lysed in SDS-PAGE sample buffer and heated to 60ºC before loading	5% Milk Powder in PBS O/N @ 4ºC	αSTAT2 Rabbit mAb, Epitomics 1:500 in 5%Milk/PBS	DonkeyαRabbit IgG-HRP Jackson Laboratories 1:10,000	Unsuccessful, 30min for weak signal.
Cells lysed in WCL buffer with added Protease inhibitor and PMSF, DNA pellet removed (see materials and methods)	1xTBS, 0.2% Tween20, 5% w/v PVP and 0.05% w/v FBS for 1hr	αSTAT2 Mouse mAb, BD transduction lab. 1:500 in 1xTBS, 0.2% Tween20, 5% w/v PVP and 0.05% w/v FBS and left O/N @ RT	GoatαMouse Jackson Laboratories 1:10,000	Unsuccessful, dirty blots.
Cells lysed in WCL buffer with added Protease inhibitor and PMSF, DNA pellet removed (see materials and methods)	1xTBS, 0.2% Tween20, 5% w/v PVP and 0.05% w/v FBS for 1hr	αSTAT2 Mouse mAb, BD transduction lab. 1:500 in 1xTBS, 0.2% Tween20, 5% w/v PVP and 0.05% w/v FBS and left O/N @ RT	GoatαMouse Dako Laboratories 1:5,000	Successful (worked approx 90% of the time). Clean blots.

Table 4.3 Optimisation of STAT2 Immunoblotting. Several conditions and combinations of Antibodies were tested and a summary of the optimisation experiments are shown above.

K562 cells (2.5 x 10^5 per reaction) that did and did not contain replicon were stimulated with 100 IU/ml IFN-α2a or IFN-γ (R&D Systems) for 30 min. Unstimulated cells were included as a negative control. Cells were harvested and lysed in 250 µl SDS loading buffer (0.0625 M phosphate, pH 7.0, 10% glycerol, 2% SDS, 0.001% bromophenol blue) pre-warmed to 60 °C, 10µl of each sample was separated by SDS-PAGE electrophoresis, and proteins were then transferred to a polyvinylidene difluoride membrane (Hybond-P, Amersham). Mouse monoclonal antibodies to either STAT1 or phosphorylated STAT1 (Tyr-701) (both Zymed) were used as primary antibodies. Detection was performed using a goat anti-mouse-horseradish peroxidase conjugate (Jackson Immunochemicals) and enhanced chemiluminescence reagents (ECL+, Amersham).

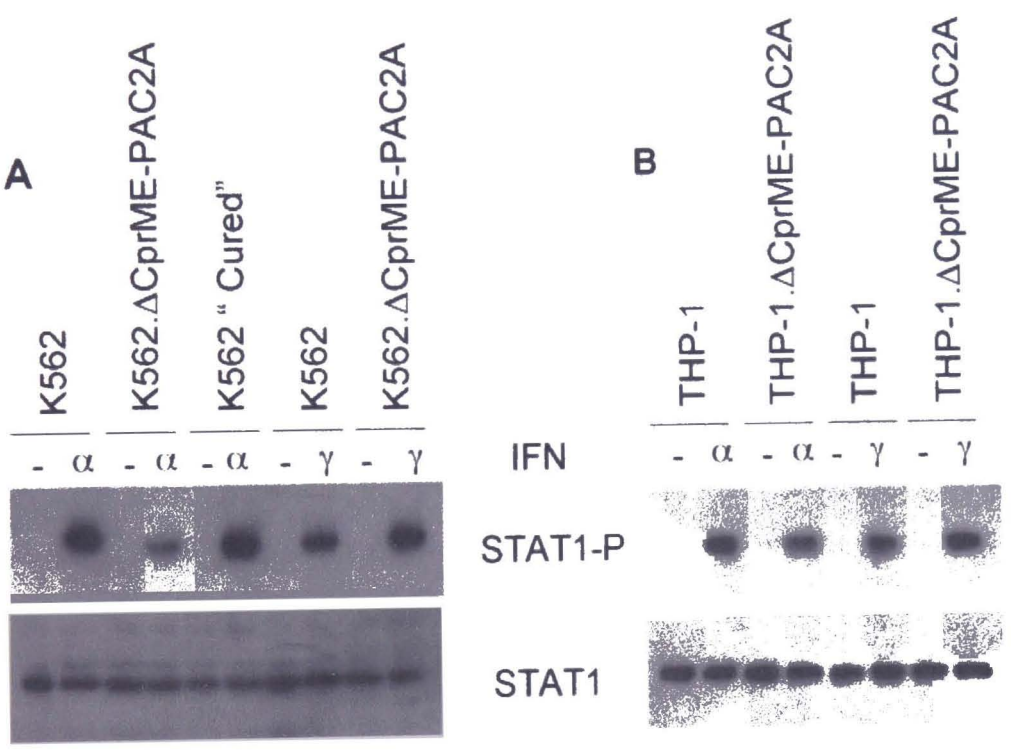


Fig 4.13 Dengue replicon inhibits STAT1 phosphorylation in response to IFNα. A. K562 cells, K562.ΔCprME-PAC2A and "cured" cells or **B.** ThP1 cells and ThP1.ΔCprME-PAC2A cells were treated with and without 100IU/ml IFNα or 100IU/ml IFNγ for 30 min, and then lysed in SDS loading buffer. Proteins were separated by SDS-PAGE and then analysed by immunoblotting using specific antibodies for STAT1 and phosphorylated STAT1.

Cells containing the dengue replicon are shown to inhibit the STAT1 phosphorylation in response to IFNα treatment, but no apparent change can be seen at the unactivated STAT1 level. Dengue RNA replication did not reduce STAT1

phosphorylation in response to IFNγ treatment. Indeed in comparison to cells without the replicon a slight increase in STAT1 phosphorylation is seen with IFNγ treatment.

In the IFN type I signalling pathway the STAT2 is phosphorylated by Tyk2 after binding to the Tyk2-phosphorylated IFNAR2, which then leads to the phosphorylation of STAT 1 by JAK1. To determine whether the observed defect in STAT1 phosphorylation is due to a defect in STAT2 activation we studied STAT2 activation in dengue replicon containing cells.

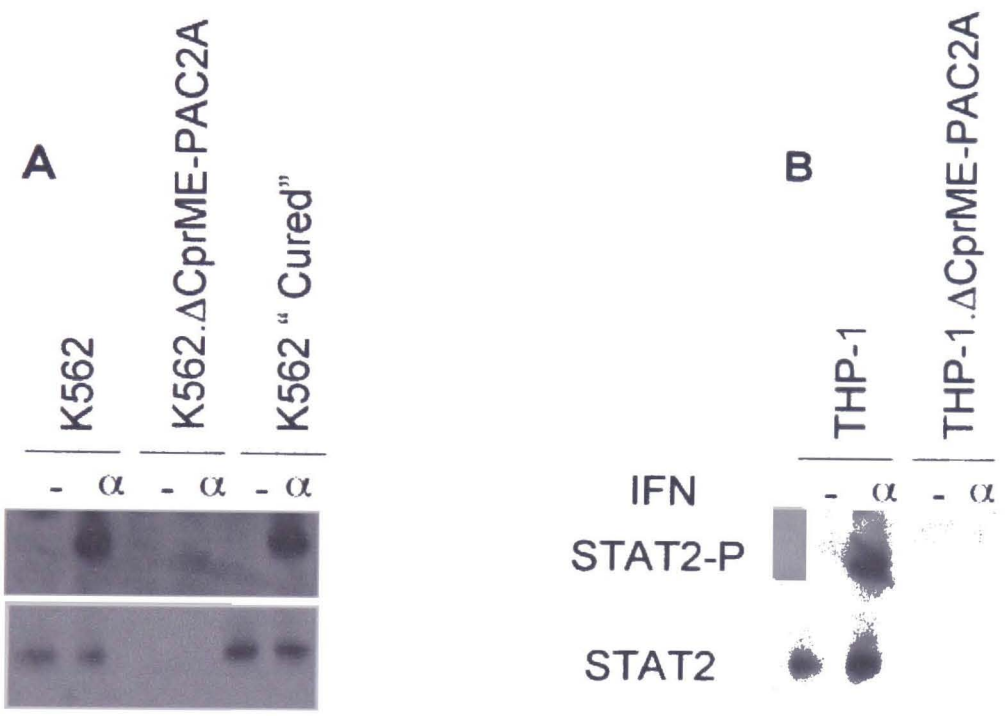


Fig 4.14 Dengue replicon diminishes STAT2 steady state levels. A. K562 cells, K562.ΔCprME-PAC2A and "cured" cells or **B.** ThP1 cells and ThP1.ΔCprME-PAC2A cells were treated with and without 100IU/ml IFNα 30 min, and then lysed in SDS loading buffer. Proteins were separated by SDS-PAGE and then analysed by immunoblotting using specific antibodies for STAT2 and phosphorylated STAT2.

4.2.8 Phosphorylated STAT1 and STAT2 levels in Dengue Infection

To ensure that our results correlated with those of the infectious dengue virus, K562 cells were infected with the virus and STAT1 and STAT2 levels assessed by western blotting. STAT2 expression was analysed in individual cells using dual-label

immunofluorescence. [Experiments done in collaboration with Andrew Davidson (Bristol)].

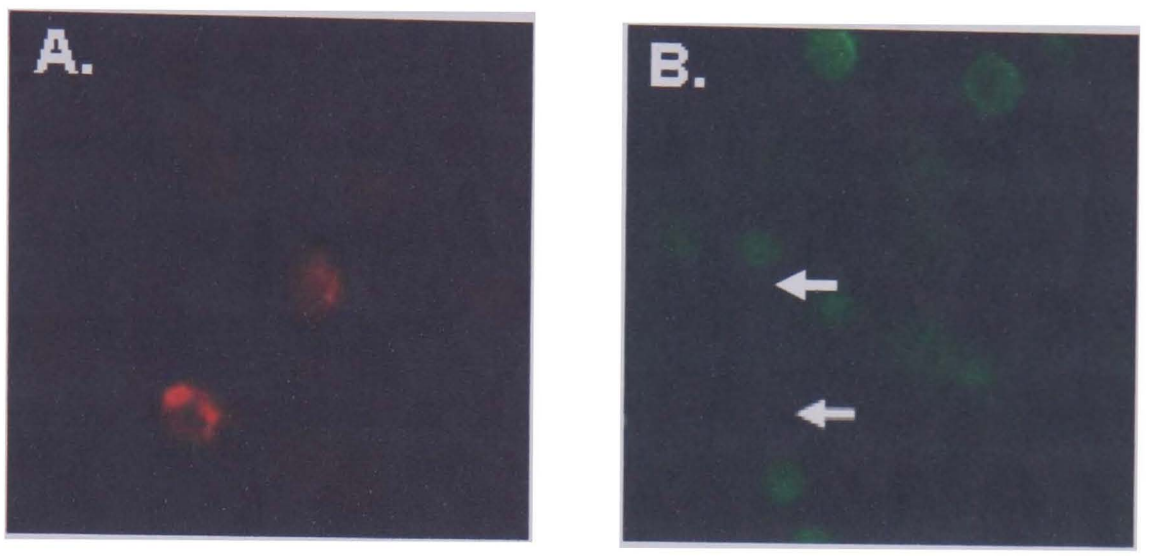


Fig 4.15 Dengue virus infection reduces STAT2 levels. K562 cells were infected with dengue virus for 48 hours and then dual stained with **A.** anti-dengue NS1 mouse monoclonal antibody followed by Texas Red-labelled secondary to detect dengue-infected cells, and **B.** anti STAT2 rabbit polyclonal antibody followed by fluorescein-labelled secondary antibody to detect STAT2. Cells were visualised by confocal microscopy. The arrow shows an infected cell. (This work was done in A. Davidson and M. Jacobs labs). **C.** Cell lysates were separated by SDS-PAGE and then analysed by immunoblotting using specific antibodies for dengue NS1, STAT1 and STAT2. Mock infected cells were included for comparison.

The dengue infected cells showed a marked decrease in steady state STAT2 staining in comparison to the neighbouring, uninfected cells (fig 4.13 A and B). Whole populations of cells analysed by immuno-blotting where 30-50% of the cells

were infected as shown by staining for dengue NS1 protein, the STAT2 levels were diminished in comparison to the mock infected control.

3.2.9 Receptor levels

To ensure that the dengue replicon acts at the STAT2 and does not suppress the type I interferon receptors in our chosen cells, the IFNAR1 and IFNAR2 levels were measured by Western blotting and FACS scanning.

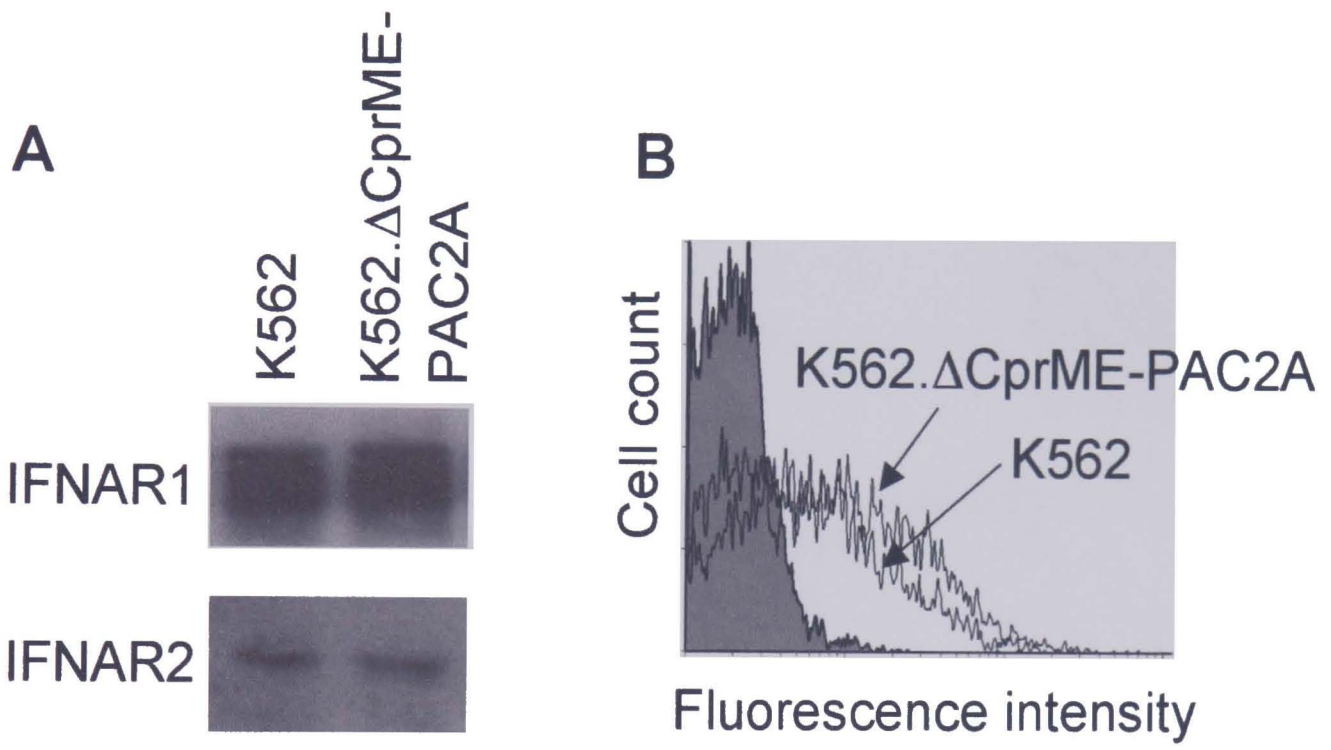


Fig 4.16 Dengue RNA replication does not affect IFNAR protein levels. A. Cell lysates from K562 cells and K562.ΔCprME-PAC2A cells were separated by SDS-PAGE and then analysed by immunoblotting using specific antibodies for IFNAR1 and IFNAR2. **B.** K562 cells and K562.ΔCprME-PAC2A were stained with specific anti-IFNAR2 antibodies and analysed by flow cytometry. Cells stained with secondary antibody alone (grey-filled) were included as a negative staining control.

No distinguishable difference could be seen between the dengue replicon cells and the K562 controls. We therefore concluded that the dengue replicon acts upon the steady state levels of STAT2 to inhibit the type I interferon signalling pathway.

4.2.10 Steady state STAT2 mRNA

To ensure that the dengue replicon was degrading STAT2 at the translational and not at a transcriptional level, steady state STAT2 mRNA levels were measured by quantitative PCR in K562 cells with and without the dengue replicon.

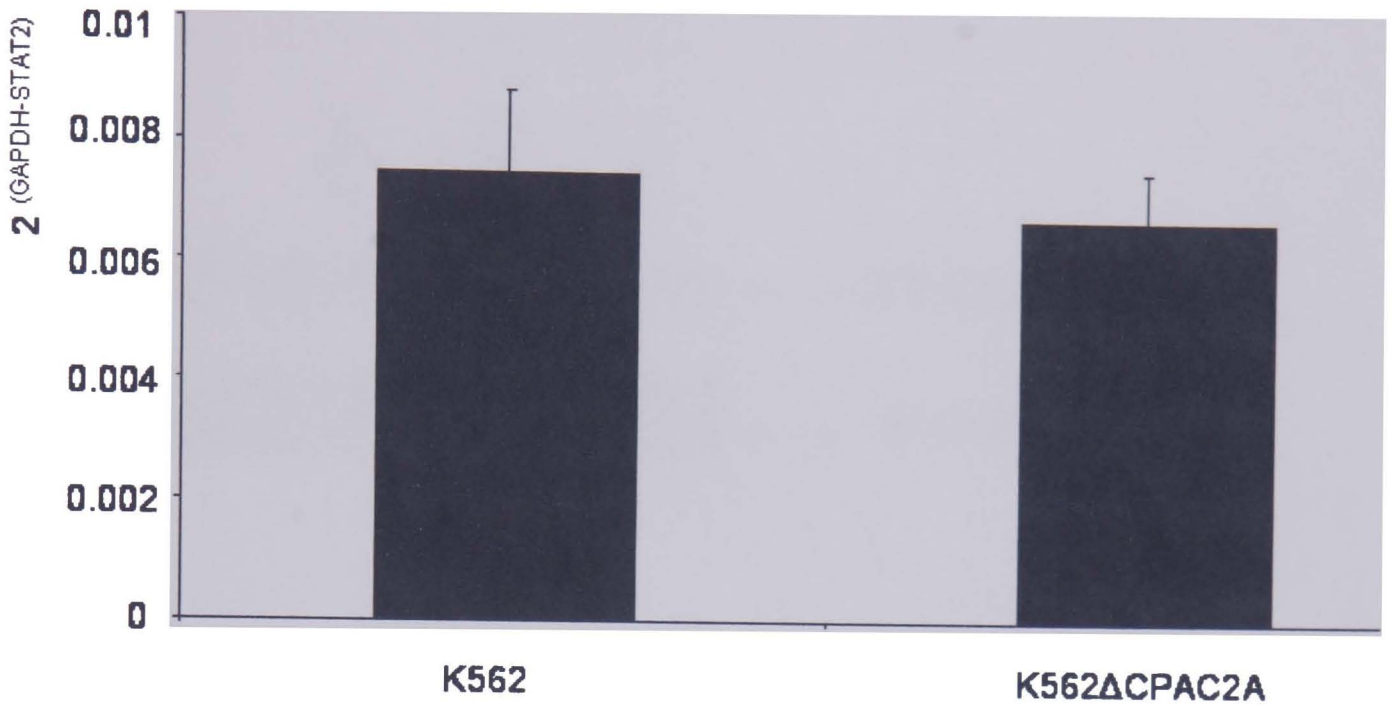


Fig 4.17 Dengue replicon does not affect transcriptional levels of STAT2. STAT2 mRNA levels were measured in K562 and K562 ΔCPAC2A cells by real time PCR

4.2.11 Restoring STAT2 expression

To determine how the dengue replicon degrades STAT2 protein, K562ΔCPAC2a cells were treated with a novel proteasomal inhibitor, Bortezomib (PS-341) (Richardson *et al*, 2003). Protein degradation by the proteasome is a highly selective process which is essential to maintain cellular homeostasis. Generally, proteins are targeted for destruction by the attachment of a small protein marker such as ubiquitin to a lysine side chain, leading to the attachment of further ubiquitin molecules and creating a poly-ubiquitin chain. The proteasomes recognise the poly-ubiquitin chain and degrade the targeted protein to small peptides which are then further hydrolysed by other cellular proteins. K562 and K562ΔCPAC2A cells were treated with 10nM Bortezomib for 24 hours, cells were then counted by trypan blue

exclusion and an equal amount of viable cells were lysed in SDS lysis solution before being separated by SDS-PAGE.

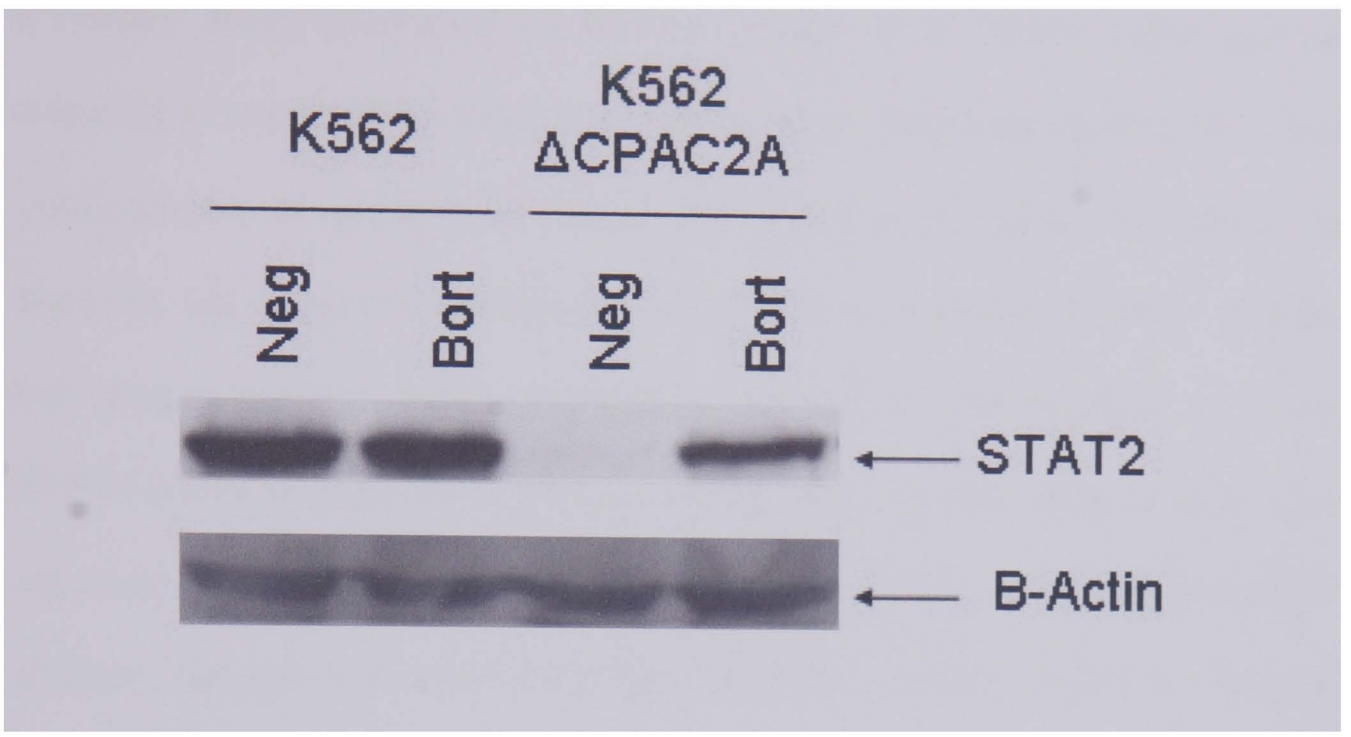


Fig. 4.18 The degradation of STAT2 by dengue is reversed using a proteasomal inhibitor. K562 and K562ΔCPAC2A were grown in the absence or presence of the proteasomal inhibitor, Bortezomib [10nM] for 24hrs. Cells were then harvested in SDS lysis buffer and proteins were separated by SDS-PAGE and analysed by Immunoblotting using a specific antibody for STAT2, a β-Actin control was included as a loading control.

The above figure suggests that STAT2 is targeted for proteasome-mediated degradation in the presence of the dengue replicon and treatment with a proteasomal inhibitor leads to an accumulation of the STAT2 protein. The lack of STAT2 in the dengue replicon cells is not as a result of a transcriptional (Fig. 4.17) or a translational block.

4.3 DISCUSSION

Our results show that the presence of dengue replicons blocks early events in IFN-α signal transduction not only by reducing phosphorylated STAT1 levels [as shown in a related study published by Munoz-Jordan *et al* (2003) whilst our work was ongoing] in response to IFNα, but also reduces phosphorylated STAT2 levels as a consequence of diminished steady state levels of STAT2. As STAT1 activation depends upon the phosphorylation of STAT2 in response to IFNα, it appears that the dengue replicon acts specifically on STAT2 as the replicon has no effect on STAT1 levels in response to IFNγ which utilizes a distinct cell-surface receptor and a separate but overlapping signal transduction pathway. Munoz-Jordan *et al* also showed that the most profound affect on STAT1 phosphorylation was by the NS4b protein, but the inhibitory effect was enhanced when NS4b was co-expressed with other NS proteins. Using the replicon system it will be possible to examine the effect of NS4b upon the IFN signalling pathway by introducing mutations.

Whilst this work was on-going there have been several publications on other viruses of the *Flaviviridae* that inhibit the signal transduction pathway of type I interferon. For example, West Nile virus, which infects migratory birds and can cause human meningoencephalitis has been shown to prevent phosphorylation of STAT1 and STAT2 by blocking the phosphorylation of JAK1 and Tyk2 (Guo *et al*, 2005).

Our data shows that STAT2 is targeted for proteasomal-mediated degradation by dengue infection, which may occur by direct interaction between one or more of the dengue non-structural proteins with STAT2. The non-structural proteins of the *Flaviviridae* could directly interact with JAK1 and TYK2 to inhibit their phosphorylation in a similar way to the V protein of human parainfluenza virus, which induces the proteasome-dependent degradation of STAT proteins (Parisien *et*

al, 2001) or the degradation may be an indirect effect through a cellular pathway that modulates protein degradation such as the PIAS proteins which specifically target the activated STATs for ubiquitination. So far most of the work has concentrated on PIAS1 which specifically targets STAT1 homodimers and no known PIAS has yet been found for STAT1/2 heterodimers. Although this area is of interest to this researcher it is beyond the scope of this thesis and due to limitations of time shall not be explored further at this moment.

This chapter has focused primarily on how the dengue virus counters the antiviral action of type I IFN. However, we also found evidence of increased IFNγ signalling in replicon containing cells (Fig. 3.12) which was later confirmed in a study by Ho *et al*. They also found that dengue virus inhibits IFNα signalling but enhances IFNγ signalling in human dendritic cells. It has previously been suggested that IFNγ plays a key role in the immunopathogenesis of DHF through enhanced activation of dendritic cells resulting in excessive amplification of the cellular immune responses and cytokine production. Although STAT2 expression is not involved in the IFNγ signalling pathway it is plausible that the reduced levels of STAT2 may affect the functional activity and/or levels of components that are common to both pathways, such as STAT1 resulting in enhanced IFNγ responses.

Although we have shown that the dengue virus replicon specifically inhibits IFNα signalling by down-regulating the expression of STAT2, different serotypes of the dengue virus are differentially sensitive to the effects of IFNα and differences in the amino acid sequence within a serotype have also been shown to lead to DHF. The ability of the different proteins of the dengue virus to antagonise the interferon system was compared in a recent study by Munoz and colleagues. The dengue proteins were expressed individually in A549 cells with a GFP labelled NDV

(Newcastle disease virus). Enhanced replication of the NDV-GFP was seen in cells expressing NS2, NS4a and NS4b with NS4b being the best antagonist. Although there are several different strains of each dengue serotype, DHF did not occur in the Americas until the emergence of the South East Asia strain of Den 2. Although there are several amino acid differences between these two strains, a notable one from serine to histidine is in the NS4b region, which would be one of the candidates to be mutated and tested to see if the interferon sensitivity is altered.

In conclusion the dengue replicon down-regulates the type I Interferon responses by degrading STAT2, a major component of the signalling pathway and therefore is not a suitable reporter assay for this thesis.

5. Results: Using a BVDV Replicon as a IFN reporter system

Although the dengue replicon was not suitable as a reporter system, another replicon derived from the Flaviviridae family, BVDV was recently shown to be sensitive to the effects of type I interferon and therefore was tested.

5.1.0 Background

In the 1940s an outbreak of gastroenteritis and severe diarrhoea was noted in a herd of dairy cattle in New York State. It was later described as a disease with two conditions; subacute and acute. The subactute form had probably been circulating in the region for a few years, affecting a couple of animals in a herd, whilst the acute form was characterised as a 7-10 day illness in young animals leading to their death. This also caused more severe disease in more mature cattle, but led to their death within 3-4 days. The new disease was termed bovine viral diarrhea virus which was later classified into the Pestivirus family.

The Pestiviruses are another genus of the *Flaviviridae,* whose members include classical swine fever virus (CSFV), border disease virus (BDV) of sheep and bovine viral diarrhea virus (BVDV). They are animal pathogens of major economical importance which are responsible for a spectrum of disease in animals. BVDV is found worldwide and can replicate in wild ruminants such as camels, deer, elk and bison to maintain a viral reservoir (Sandvik *et al*, 1997;Vilcek *et al*, 1994), but the economic importance of BVDV is due to its effect on the beef and dairy industries. The virus is widespread; about 60-70% of adult cattle have experienced BVDV infection by 4 years of age and animals persistently infected with BVDV represent up to 1% of the cattle population. BVDV infection has a detrimental economic effect on the herd by reducing milk production, reduced reproductive performance, growth retardation and increased mortality among young stock. The estimated losses in

individual herd outbreaks have varied from a few thousand up to $100,000 per herd (Duffell *et al*, 1986).

5.1.1 Clinical Manifestation

The crucial outcome of the pathogenesis of BVDV infection is the transplacental infection of the foetus. The outcome of a BVDV infection depends on the gestation stage of the infected animal; when a foetus is infected at a later stage in gestation, the foetus will produce antibodies and therefore have an active immunity to BVDV (Howard, 1990), probably leading to a weakened newborn calf. However, infection in the first trimester, prior to immunocompetence, can result in a persistent infection of the foetus.

Persistently infected animals are normally antibody negative to BVDV, but can produce antibodies to other BVDV isolates. Persistently infected animals are key transmitters of the infection as they continually shed virus in large quantities. Perisistently infected animals can have an impaired immune system, leaving them susceptible to other infections. Also, only persistently infected animals will succumb to fatal mucosal disease. Mucosal disease is a rare manifestation of BVDV infection which occurs when the persistently infected animal becomes superinfected with an antigenically similar but different biotype of virus. BVDV exists as two biotypes, but only one of these can cross the placenta and establish a persistent infection.

5.1.2 Biotypes and genotypes

The two biotypes of BVDV are; cytopathic (cp) and non-cytopathic (ncp). They are classified according to their lytic effect on cultured cells; the non-cytopathic form being non-lytic. Only the non-cytopathic version of the virus can establish persistent infection which is characterized by immunotolerance. The ncp form arises as a

result of infection of the embryo early in its development, usually if infected between 125-150 days. The calf may be born normally but will remain persistently infected for their lifetime, usually being asymptomatic or causing only mild clinical symptoms.

The cp biotype of BVDV arises through mutation from the ncp form. The mutation generated via RNA recombination events are numerous and can arise from insertion of cellular sequences, gene duplication, deletion or single nucleotide changes. The cp virus is unable to establish a persistant infection (Brownlie *et al*, 1989)

Not only does the virus separate into two biotypes but it recently has also been divided into two genotypes; 1 and 2. Although both genotypes have been known about for a while, new developments in diagnostic tests were required so that they could be differentiated. The basic differenece between the genotypes is the disease severity; type 2 BVDV being responsible for more severe disease within the last few years.

5.1.3 Structure and Genome

BVDV is a small virus of approximately 50nm, consisting of a single capsid protein, surrounded by an envelope containing three glycoproteins. The genome is similar to that of the Flaviviridae with a few notable differences. The genome consists of a single-stranded RNA of about 12.3Kb and consists of a single large ORF flanked by untranslated regions at the 5' and 3' end (Meyers & Thiel, 1996). The 5' NTR contains an internal ribosome entry site that initiates translation of BVDV mRNA in a cap-independent manner. The secondary structure of the 5' NTR is involved in the regulation of both translation and genome replication. Similar to HCV, the BVDV IRES has been shown to bind to ribosomal 40S subunit, independent from the translation initiation factors eIF4A, eIF4B and eIF4F. The BVDV proteins are translated from genomic RNA as a a large single polypeptide of ~4000 a.a. which is

co- and post-translationally cleaved into mature proteins by both viral and host proteases (Grassmann *et al*, 2001).

5.1.4 Structural Proteins

BVDV consists of 4 structural proteins; the capsid protein (C) and three envelope proteins; E^{RNS} (riblonuclease, soluble), E1 and E2. The C protein is the virion nucleocapsid a conserved, highly basic protein. The E^{RNS} glycoprotein is heavily glycosylated, does not contain a potential membrane-spanning domain and is found associated with released virus particles. The other two E proteins are E1 and E2, which can either associate together as a heterodimer or E2 can form homodimers. Monoclonal antibodies to E2 and E^{RNS} can neutralize virus infectivity and mutations in the E^{RNS} give rise to viruses that are attenuated *in vivo*.

5.1.5 Non-Structural Proteins

One of the major differences between the Pestiviruses and the Flaviviruses is the addition of a non-structural protein, N^{pro} on the 5' end of the genome. The N^{pro} is an autoprotease which cleaves itself from the rest of the genome at a highly conserved Cys-Ser site, but it is dispensable for autonomous RNA replication. The other non-structural proteins follow the E2 protein as found in the Flaviviruses.

The NS2 protein is present as the N-terminal portion of NS2/3 and is found as a mature cleavage product only for some cpBVDV strains. Isolates of ncpBVDV do not proceed with NS2/3 cleavage but in cpBVDV biotypes the NS2/3 cleavage is efficient but incomplete so that both NS2/3 and NS3 proteins are found.

The NS3 protein, similar to its function in the *Flaviviridae,* is a RNA helicase which and also contains a protease activity, requiring NS4 as a co-factor, but the NS3 protease in the pestiviruses has an additional function.

Although the cp biotype can be caused by different mechanisms, the biotype depends on the production of NS3; in the cp-BVDV both the cleaved NS3 and the un-cleaved NS2/NS3 can be detected. The last 27 nucleotides in the NS2 coding region are sufficient to mediate the NS2/NS3 cleavage and are therefore essential for the cytopathicity of the BVDV. This raises the possibility that NS3 is directly cytopathic to the cells and that the relative abundance of NS3 would be important to mediate this effect, or the greater RNA replication efficiency of the cp-BVDV form might arise as a consequence of the NS2/NS3 cleavage, leading to greater cytopathicity.

Except for the co-factor function of NS4a little is known about either NS4a or the NS4b proteins. They are similar in size, composition and hydropathicity to the corresponding proteins in the *Flaviviridae*.

The NS5a and NS5b proteins of BVDV can be found as individual proteins (~58KD and ~75KD respectively) or as a large uncleaved protein (133KD). The NS5b protein contains the motifs characterisitic of RdRp and has been characterized *in vitro* and found to extend template-primed RNAs into double stranded copy back products or to catalyse *de novo* inititation from short synthetic RNA or DNA templates.

5.1.6 RNA Replication

The RNA replication is similar to that of the flaviviruses. Negative and positive stranded RNA are detected early in the replication cycle followed by an accumulation of positive strand later on.

5.1.7 BVDV and Interferon

The mechanism of immune suppression in persistently infected animals by BVDV is still unclear, but the effects are multiple:

Table 5.1 Effects of *in vitro* infection on bovine macrophage function (Peterhans *et al*, 2003)

Effect	Ncp BVDV	Cp BVDV
IFN type I synthesis	=*	↑
Induction of apoptosis	=	↑
Prostaglandin E2 synthesis	=	↑
NO synthesis after LPS or S. Dublin treatment	↑	↓
TNF-α synthesis after LPS treatment	↓	↓
Superoxide production induced by PMA	↓	↓
Procoagulant activity induced by S. Dublin	=	=
IL-1 inhibitor activity induced by LPS	↑	↑
Cytokine-induced chemotaxis	↓	↓

=; no effect compared to mock, ↑; enhanced, ↓; reduced

The differences in IFN synthesis between the two biotypes of BVDV could be a possible mechanism for the virus to establish a persistent infection.

The cytopathic form of BVDV has been shown to induce a type I interferon response in bovine macrophages (Adler *et al*, 1997) whereas ncp-BVDV infected macrophages do not produce type I interferon and have been shown to block the induction of IFNα/β by dsRNA (Rossi & Kiesel, 1980). Although viral replication was detected in the spleen of foetuses infected with either ncp or cpBVDV, type I IFN was only detected in the amniotic fluid of the foetuses infected with cpBVDV. The interferon inducible gene Mx was reduced in the ncpBVDV infection, suggesting that the failure of ncpBVDV to induce IFNα/β enables the virus to establish a persistent infection in the early foetus (Charleston *et al*, 2001).

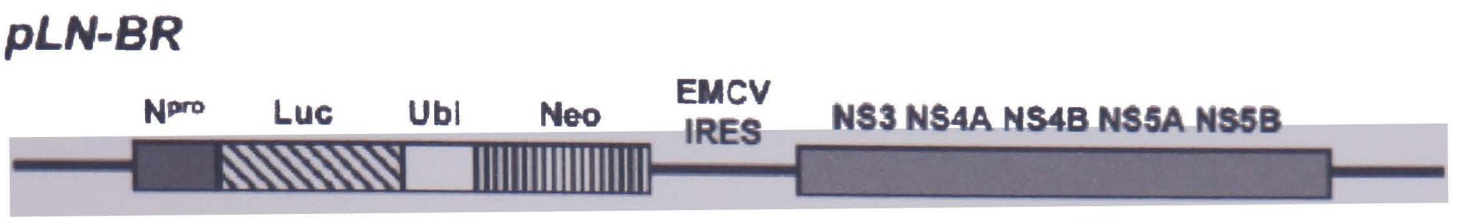
5.1.8 BVDV Replicon System

It has been shown that when a full length complementary RNA of the BVDV is transfected into eukaryotic host cells, a subgenomic RNA molecule D19c, [previously described as a defective interfering particle (DI)] functions as an autonomous replicon. D19c comprises the 5' and 3'UTR, the autoprotease N^{PRO} and the NS proteins NS3 through to NS5b, which is sufficient to support complete RNA replication.

Fig 5.1 Schematic drawing of the D19c BVDV replicon.

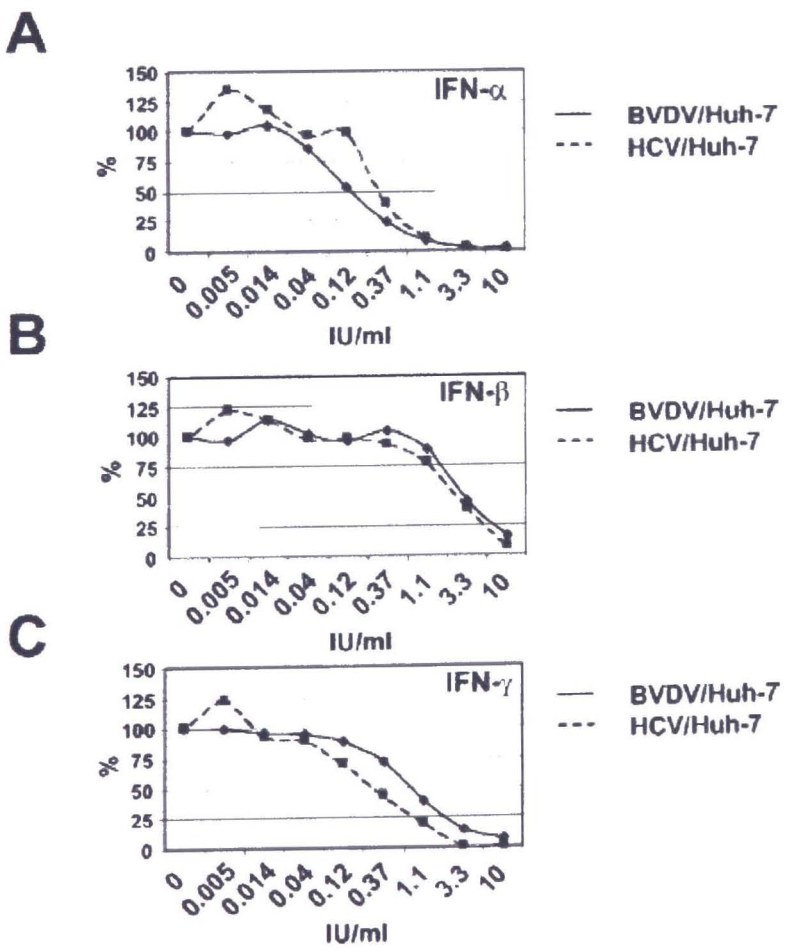
The selectable, bi-cistronic, subgenomic BVDV replicon was obtained from Dr Zhong (Horscroft *et al*, 2005). The structural genes of the BVDV were replaced with a luciferase-ubiquitin-neomycin phosphotransferase cassette and the expression of the non-structural genes was driven by an EMCV IRES.

Fig 5.2 Schematic representation of the BVDV replicon containing the luciferase reporter cassette and Neomycin selection gene.



After showing that the BVDV replicon efficiently replicates in Huh7 cells and that no adaptive mutations were seen in the replicon, Halcroft et al. demonstrated that the replicon was sensitive to interferon treatment. The group treated the Huh7-BVDV cells with up to 10IU/ml IFNα, β or γ for 48 hours before determining the effects upon the RNA replication. The results were found to be comparable to the Huh7-HCV cells with IFNα being the most effective with an EC_{50} value of 0.2IU/ml.

Fig 5.3
Effects of interferons on BVDV and HCV replicon RNA replication in Huh7 cells.
Luciferase expressing BVDV/Huh7 or HCV/Huh7 cells in 96 well plates were treated with indicated amount of IFNα (A), β (B) or γ (C) for 48 hours. (Horscroft *et al*, 2005)



The BVDV replicon pLN-BR (luciferase Neomycin-BVDV replicon) is therefore suitable as a reporter system for this thesis.

5.2 BVDV Results

5.2.1 *In vitro* transcription of the BVDV replicon

PCR primers were designed to amplify the BVDV replicon containing the T7 promoter of the plasmid required for the *in vitro* transcription (see Table 2.5). Two different forward PCR primers were designed to accommodate the T7 promoter site, which requires upstream nucleotides for efficient initiation.

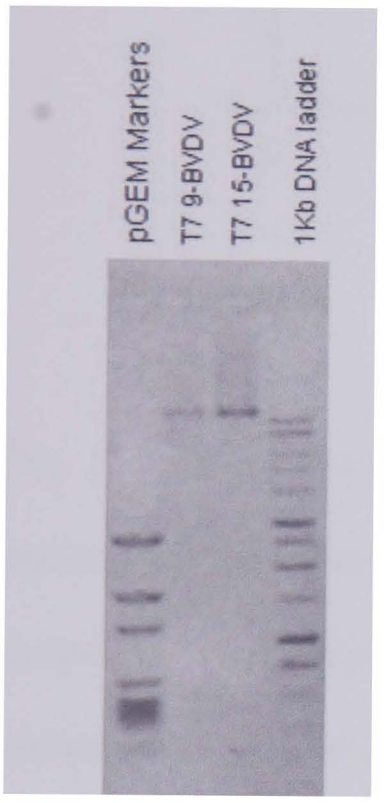


Fig 5.4 Amplification of the BVDV replicon. The T7 promoter sequence included in the forward primer to allow direct priming from the PCR product.

The T7 15-BVDV PCR product was cleaned using a Qiagen PCR purification kit and used to make a full length RNA copy by *in vitro* transcription using a T7 MegaScript kit (Ambion) and transfected into Huh7 cells using electroporation. Whilst this experiment was ongoing, Huh7 cells containing the BVDV replicon (Huh7/LN-BR) were kindly received by Zhong from Valeance Pharmaceuticals and these were used for all subsequent experiments.

5.2.2 IFN sensitivity of the Huh7/LN-BR cells

The Huh7/LN-BR cells were first tested to ensure that our results duplicated the results obtained by Horscroft et al (2005). Huh7/LN-BR cells were plated out in a 96

well plate and left to grow overnight before being treated with IFNα2a for 24 hours. The cells were then lysed and luciferase activity measured.

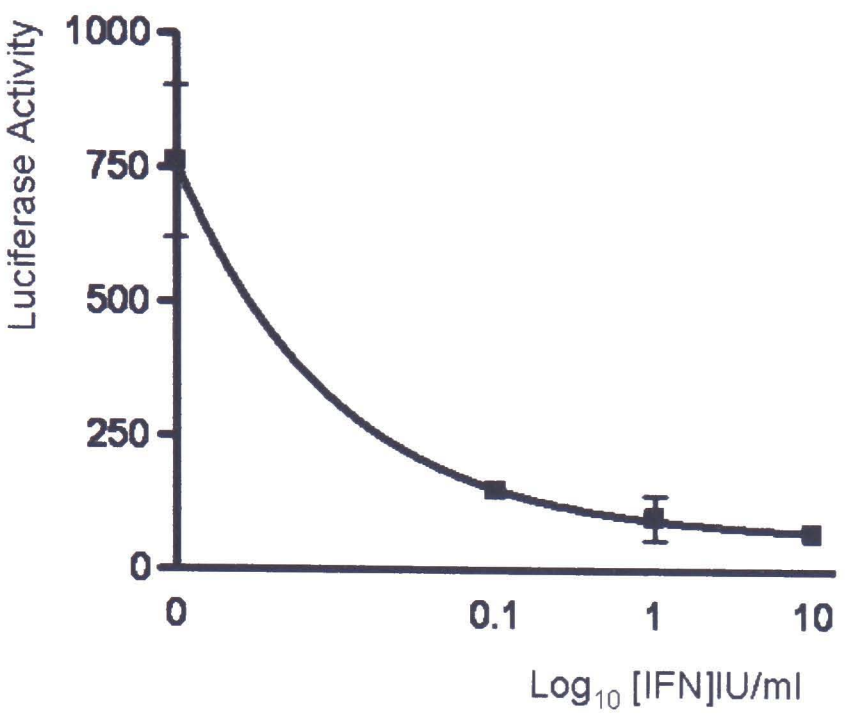


Fig 5.5 Interferon decreases BVDV replicon replication as measured by luciferase activity. $1x10^3$ cells were plated out and left to grow to 70% confluence before being treated with 10 fold dilution of IFNα2a (Roferon). The cells were lysed using PLB (Promega) and Luciferase measured (Promega).

Fig. 5.5 shows that the BVDV replicon is sensitive to the effects of IFNα2a as previously described by Horscroft et al. and therefore could be suitable for our purposes. The inhibitory effects of IFNβ and IFNγ have already been reported by Horscroft et al, although the effects of the IFNα subtypes have not. The above experiment was repeated with the IFNα subtypes (Wellcome) and the percentage inhibition of the luciferase activity plotted against the IFN concentration.

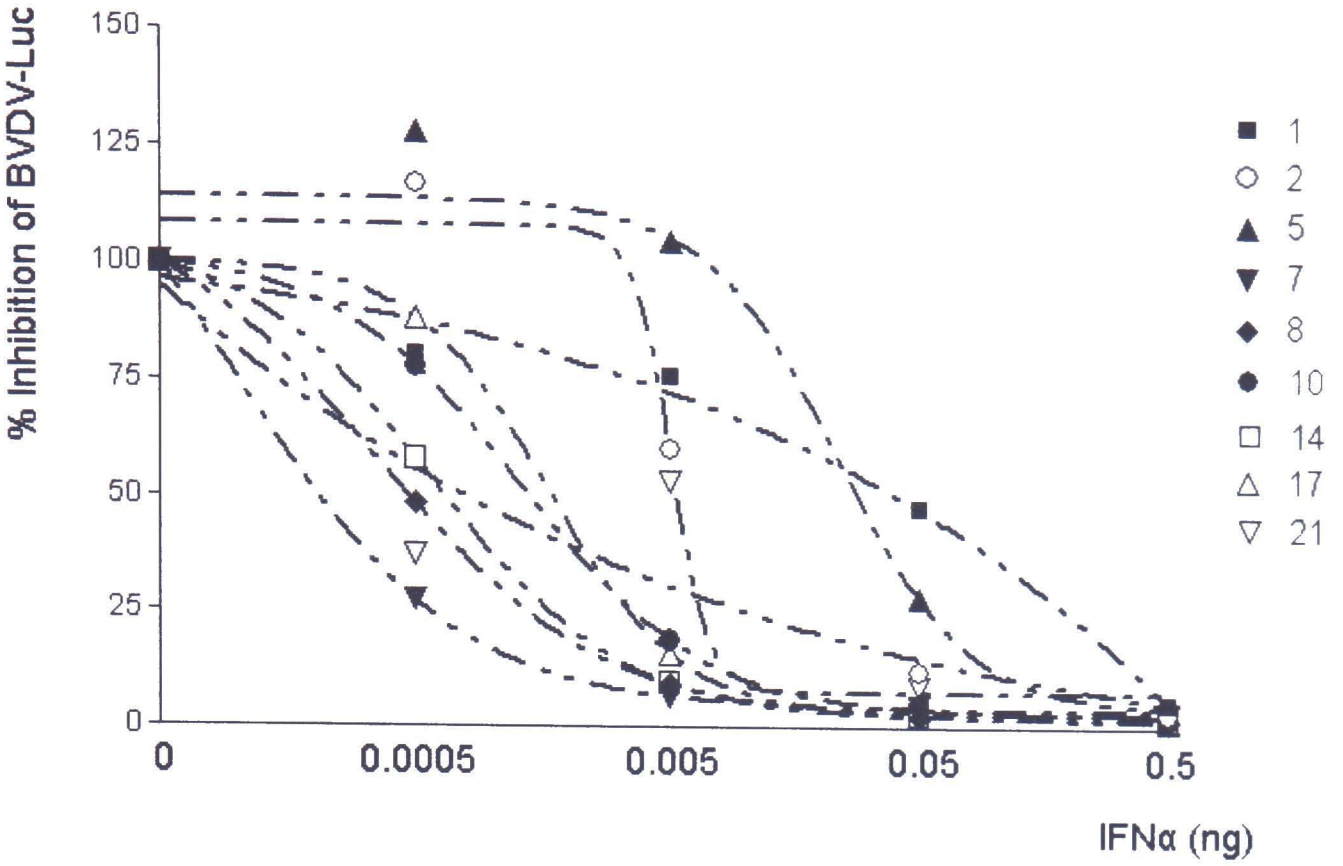


Fig 5.6 Effect of IFNα subtypes on the replication of the BVDV replicon. Huh7/LN-BR cells were plated and left to grow overnight before being treated in triplicate with a 10 fold dilution of the IFNα subtypes (Wellcome). The cells were lysed using PLB (Promega) and the luciferase activity assessed. The average of the triplicates was taken and the percentage inhibition from the negative control (arbitrarily set as 100%) calculated and plotted against IFN (ng).

Although all the subtypes show a decrease in the luciferase activity with increasing amounts of interferon, there is a kinetic difference, with IFNα7 being the best and IFNα5 and IFNα1 being the worst. Interestingly the antiviral activity of IFNα1 has already been reported to be poor against several other viruses (Foster *et al*, 1996).

5.2.3 Interferon stimulated genes

The effect of interferon on the BVDV replicon was further investigated in respect to the interferon stimulated genes using real-time PCR as used in previous experiments with the dengue replicon. Huh7/LN-BR cells were plated in 6 well plates and left to grow overnight. The following day the cells were stimulated with IFNα2a for 6 hours before extracting total RNA. Real time PCRs for ISGs were performed as previously described in chapter 4.

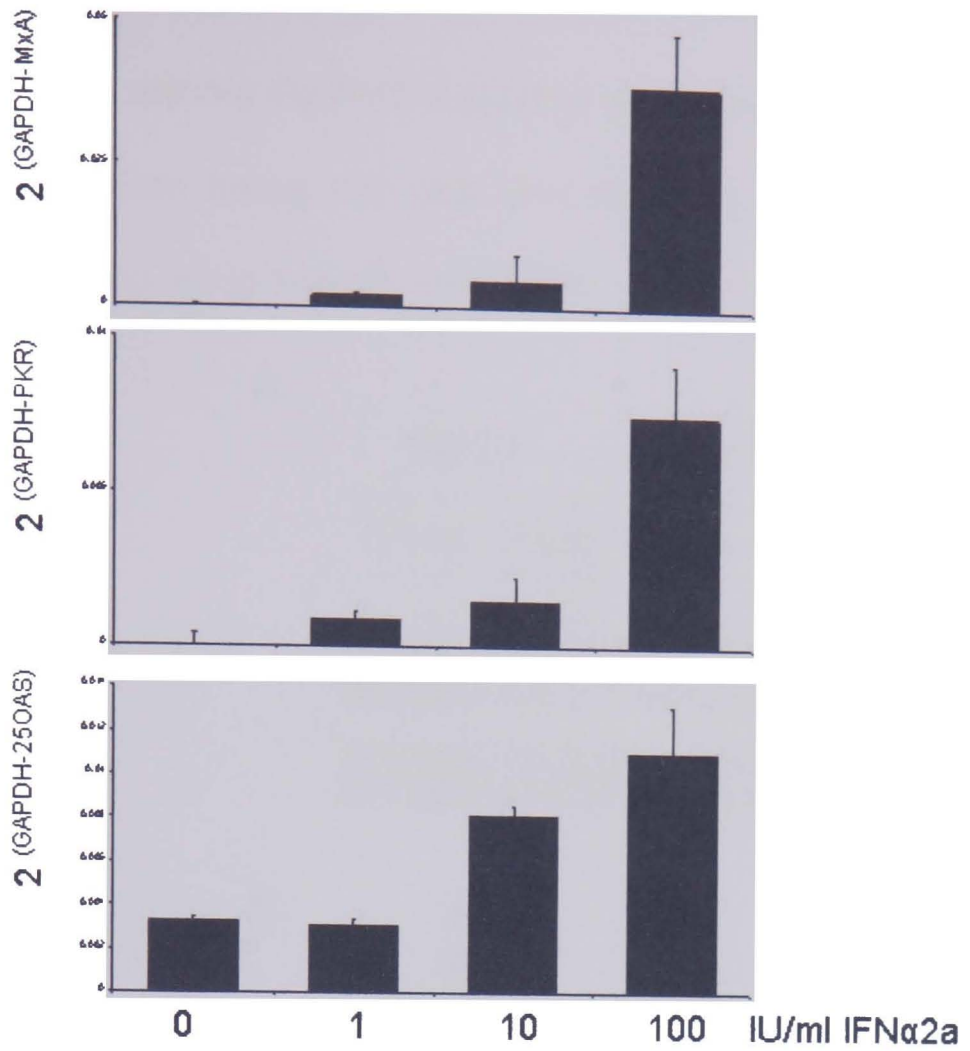


Fig 5.7 Real time PCR to measure the effects of the BVDV replicon on Interferon inducible genes. Huh7/LN-BR cells were treated with 100IU/ml IFNα2a for 6 hours before measuring the interferon inducible genes by real time PCR. **A.** Induction of MxA **B.** PKR gene induction and **C.** 2'5'OAS gene induction

BVDV does not appear to inhibit the induction of interferon stimulated genes as demonstrated by real-time PCR (fig. 5.7). BVDV does not completely abrogate the effects of IFNα as seen earlier with the dengue replicon and therefore the BVDV replicon shall be further tested for use as a reporter for interferon inhibition.

5.2.4 Validating the BVDV replicon as a reporter assay.

To confirm that the BVDV replicon would be suitable as a reporter assay for interferon antagonists, it was tested using the known IFN inhibitor SV5-V (kind gift from Prof. R. Randall). SV5-V has been shown to be one of the most powerful inhibitors of the type I interferon system which works by targeting STAT1 for proteasomal degradation (discussed previously in 1.2.3). To ensure that the SV5-V plasmid was functional, it was transfected into K562 cells using Fugene6 and stable

cells selected using 500μg/ml G418. After approx 2 weeks in G418 selection the cells with and without the SV5-V plasmid were treated with 100IU/ml IFNα2a or IFNγ for 15min before lysing the cells and detecting STAT1, STAT1P and SV5-V by Immunoblotting using specific antibodies.

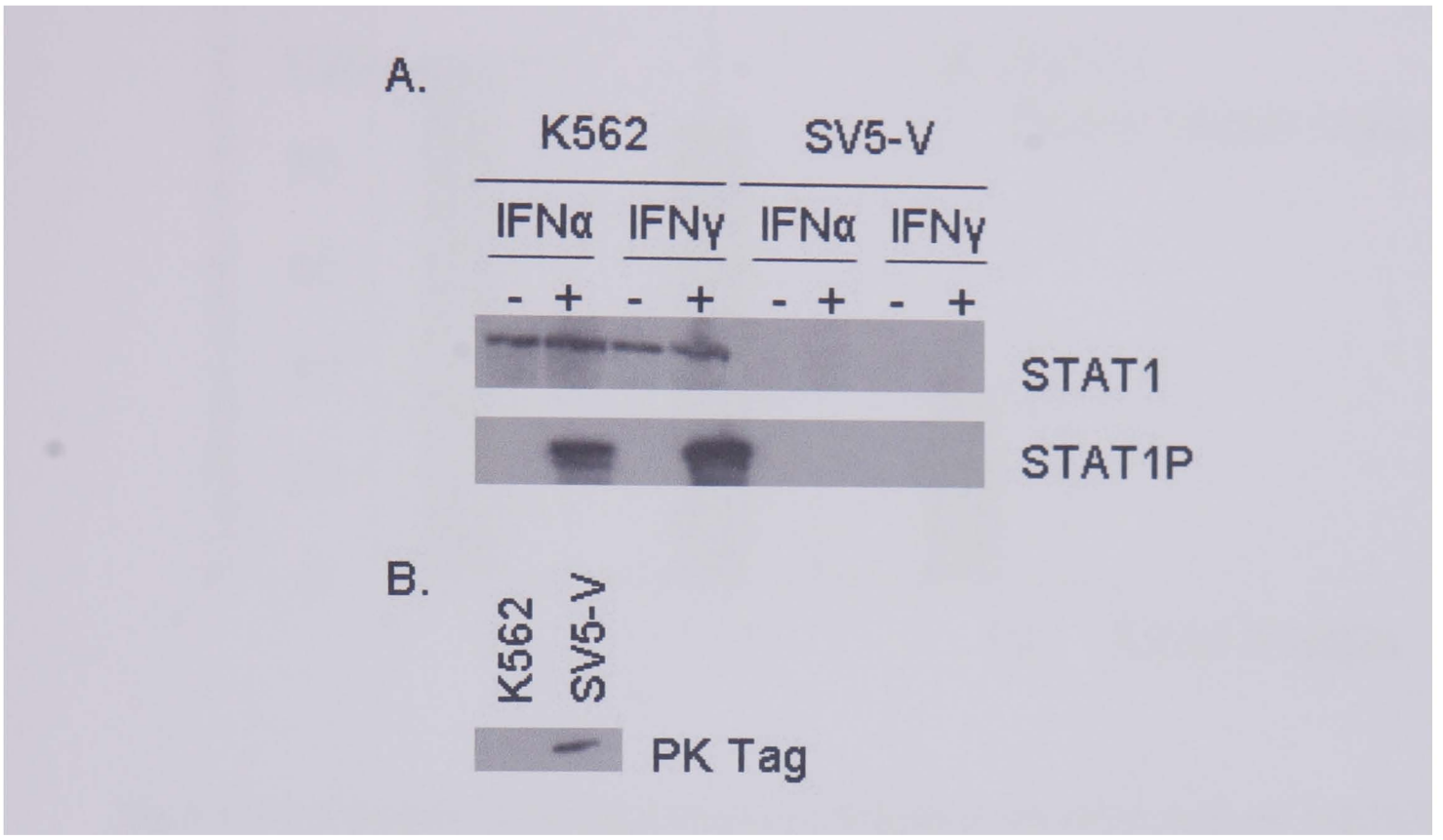


Fig 5.8 SV5-V inhibits IFNα/γ signalling by degrading STAT1. A. K562 cells (negative control) and K562 cells transfected with the SV5-V plasmid were treated with and without a 100IU/ml IFNα and IFNγ for 15 min and then lysed in SDS loading buffer. Proteins were separated by SDS PAGE and analysed by Immunoblotting using specific antibodies for STAT1 and STAT1P. **B.** The presence of the SV5-V proteins was confirmed using a specific antibody agains PK Tag, which is present in the plasmid.

After confirming that the SV5-V plasmid was functional it was tested as an IFN inhibitor using the BVDV assay. Huh7/LN-BR cells were seeded at a density of $1x10^3$ cells per well in a 96 well plate and left overnight until ~70% confluent. The cells were then transfected in triplicate using 5μl of a mixture of Fugene6 with 2μg SV5-V, and 0.1μg pSV-β-Galactosidase. After 24 hours, cells were treated with IFNα2a and approximately 24 hours later, cells were harvested for analysis of luciferase and β-gal production. The results are displayed as percentage inhibition of the luciferase activity, normalised to β-gal.

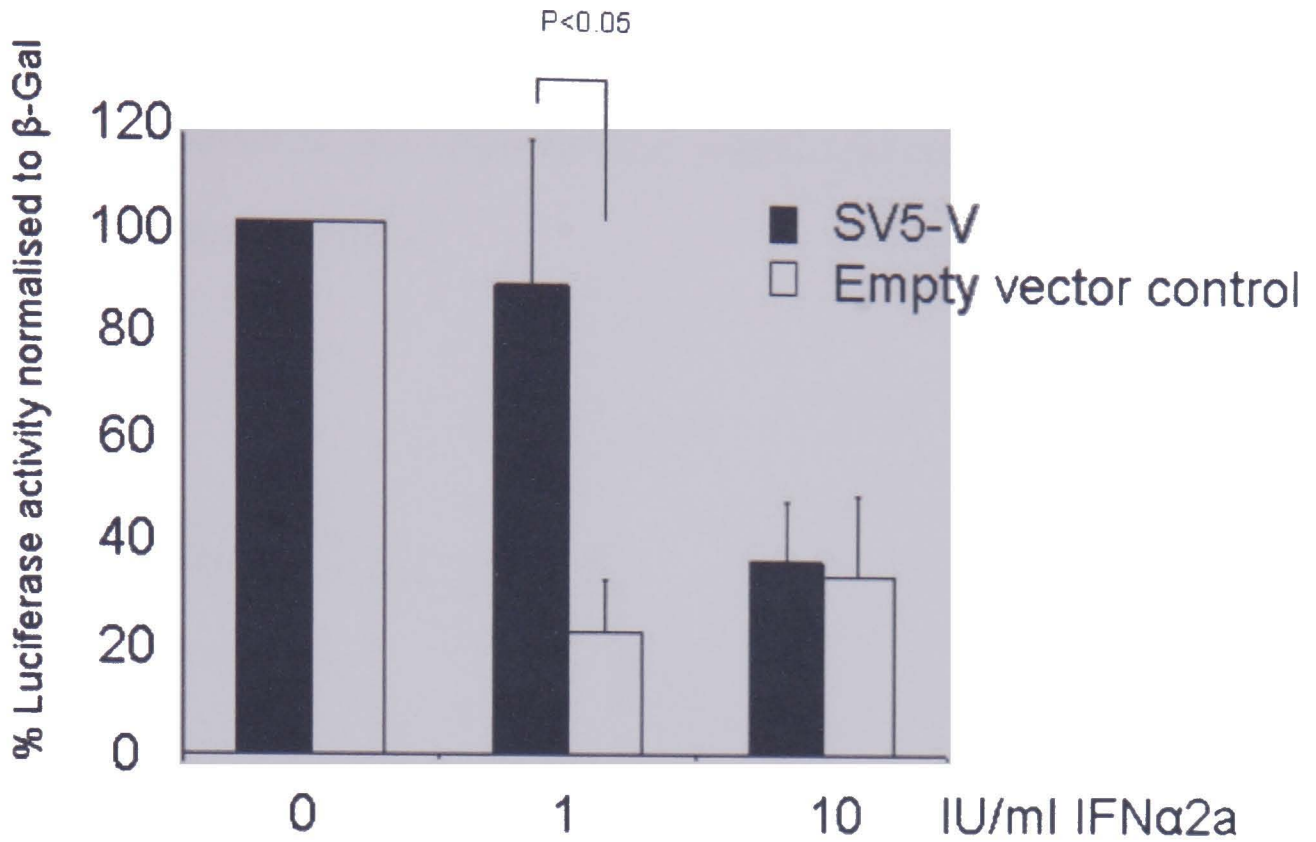


Fig. 5.9 SV5-V inhibits the antiviral action of interferon on the BVDV replicon. Huh7/LN-BR cells were transfected in triplicate with SV5-V protein (a known viral inhibitor of type I IFN) and an empty vector control. Cells were grown overnight before being treated with IFN for a further 24hrs. Cells were lysed and luciferase expression measured and normalised to Beta Gal.

The BVDV-luciferase replicons stably expressed in Huh7 cells have previously been shown to be highly sensitive to the antiviral effects of IFNα (Fig. 5.5). This effect can be inhibited by transient expression of a known IFN antagonist. The V protein of simian virus 5 (fig 5.9) at a concentration of 1IU/ml IFNα but this effect is not seen with higher IFNα concentrations, as the higher concentration overcome the inhibition of the SV5-V protein.

5.2.5 Using the BVDV replicon as a reporter assay

After validating the BVDV reporter system using a known IFNα antagonist it was further tested using two NS5a proteins derived from patients whose treatment outcome was known. The Huh7/LN-BR cells were plated out and transfected in

triplicate as previously mentioned using 2μg of the NS5a-CR or the NS5a-NR plasmids and 0.1μg of the β-gal plasmid as a transfection control. The cells were treated with IFNα for 24 hours before being lysed and the luciferase activity measured and analysed to the β-gal activity, results are shown as percentage reduction of the luciferase activity.

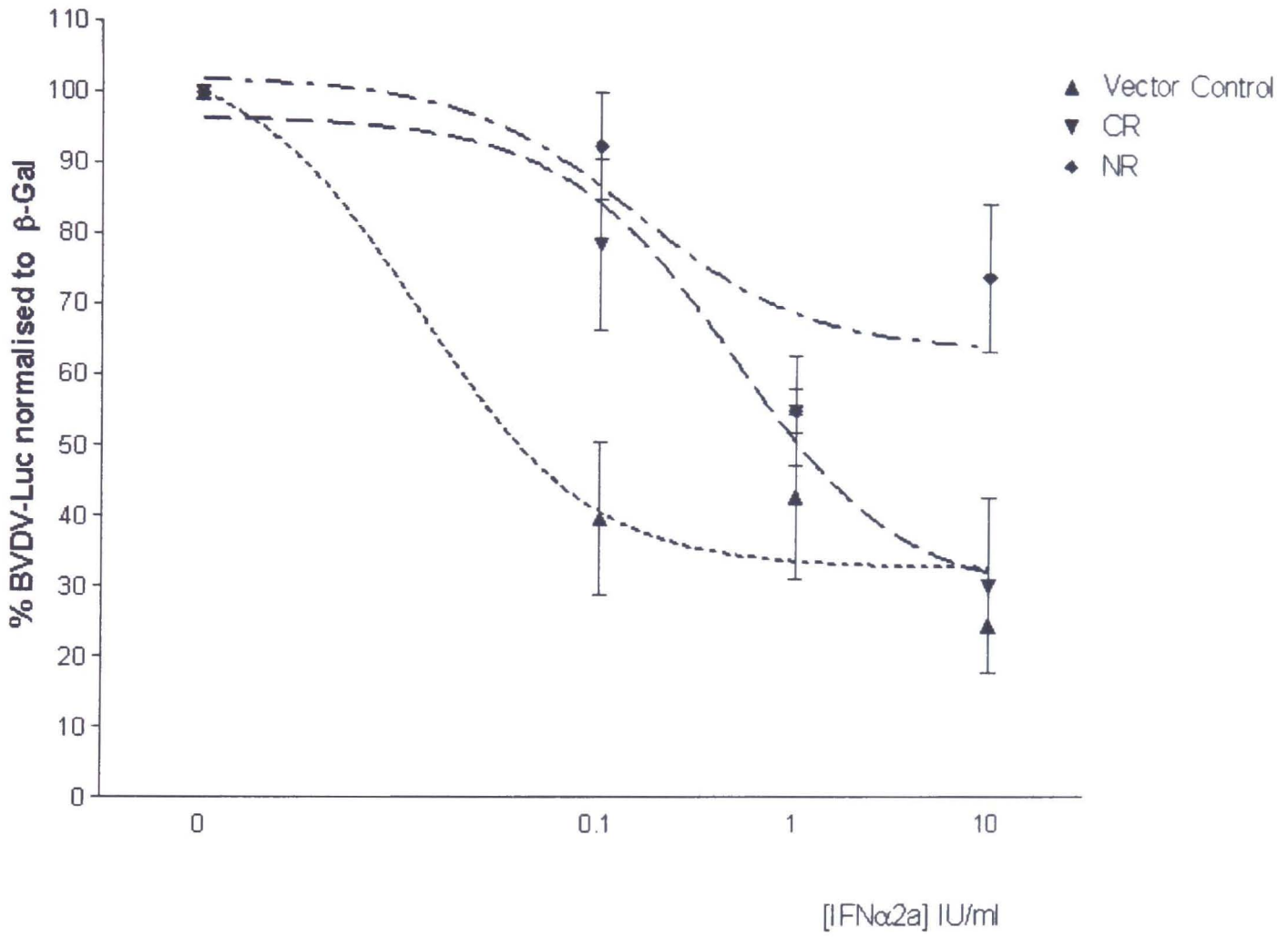


Fig. 5.10 Different NS5A proteins vary in their effect on the action of interferon. NS5a protein derived from an interferon non-responsive (NR) patient reversed the reduction in luciferase activity but this was not the case in the NS5a protein derived from an interferon responsive patient (CR).

5.2.6 Optimising the BVDV reporter assay

Although there appears to be an inhibition of the antiviral effect of IFNα in the presence of SV5-V at 1IU/ml this is not seen at higher IFNα concentrations, SV5-V is a strong inhibitor of type I IFN and more subtle effects by weaker inhibitors could

be missed due to the lack of sensitivity in this assay. To overcome this we have altered the assay so that the results can be analysed by flow cytometry.

The luciferase reporter within the BVDV replicon was replaced by a commercially available destabilised green fluorescent protein (dsGFP) (Clontech). The dsGFP displays a rapid turnover therefore making it ideal for use in a reporter assay. The dsGFP is fused to a portion of a mouse ornithine decarboxylase (MODC) which contains a PEST domain that targets the protein for degradation. This allowed us to analyse the BVDV replicon in individual cells using flow cytometry and improve the sensitivity of the assay.

5.2.7 Overlapping PCR strategy

To replace the luciferase cassette with the dsGFP an overlapping PCR strategy was employed; primers were designed to incorporate SacI and PmeI restriction sites to allow the introduction of the cassette back into the BVDV replicon. The first PCR used the pLN-BR DNA as a template, the 5' primer contained the SacI restriction site and the 3' primer included an overhang which would overlap with the second PCR product from the pZsGreenDR1 (Clontech). The second PCR used the pZsGreenDR1 plasmid as a template and incorporated a PmeI site at the 3' end and an overhang at the 5' end to overlap with the first PCR.

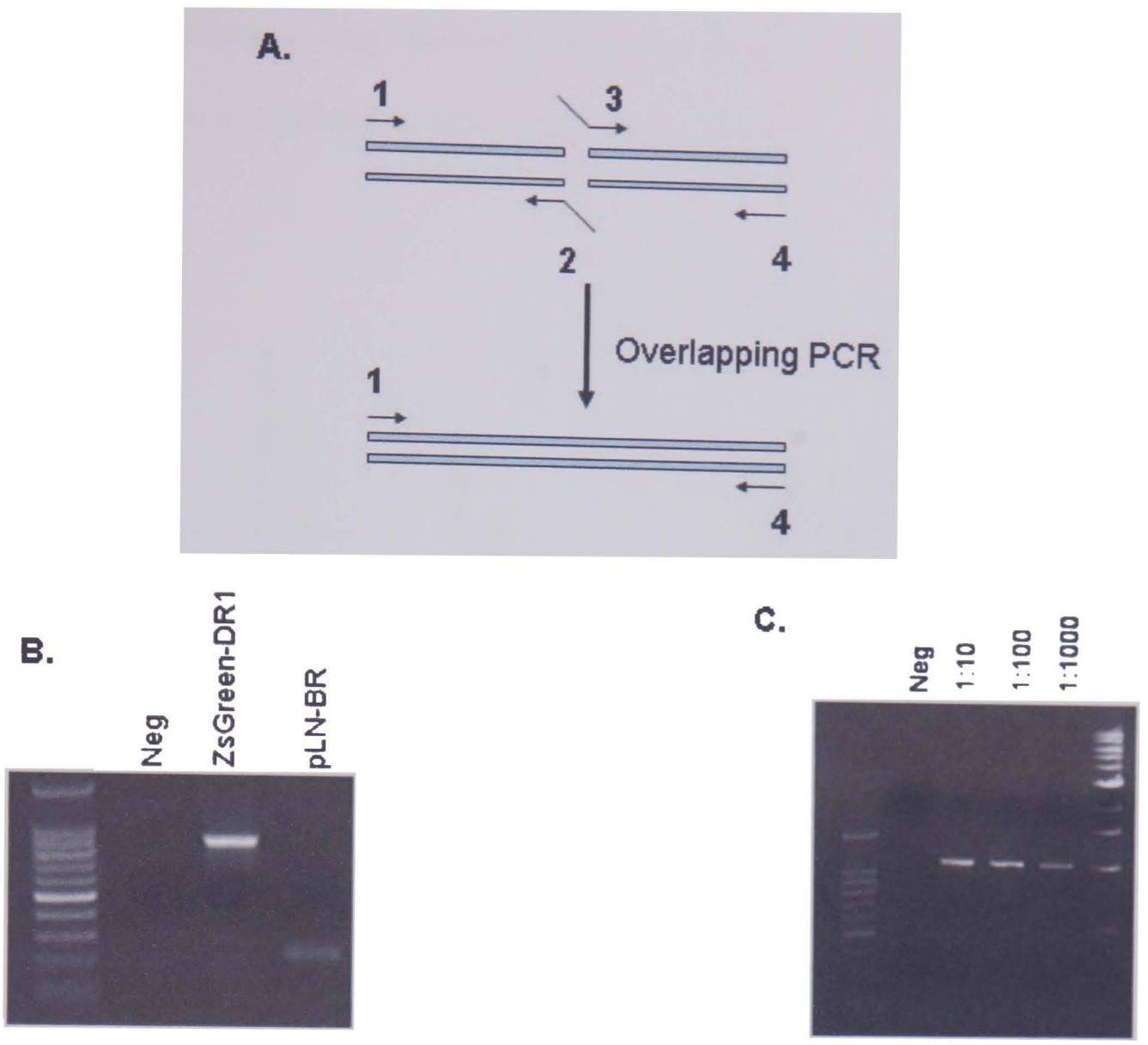


Fig 5.11 Overlapping PCR strategy to replace the luciferase cassette in pLN-BR with a destabilised GFP. A. Schematic representation of the overlapping PCR. **B.** Both PCR products were amplified individually. **C.** PCR products from B. were mixed in an equimolar ratio before being diluted to be used as template for the final PCR reaction.

The overlapping PCR fragment was then A-tailed and sublcloned into pGEM-T and checked by sequencing before being digested with SacI and HpaI (HpaI replaced PmeI, they are both restriction enzymes that produce blunt end products and could subsequently be ligated with one another) to release the fragment and insert into the correspondingly digested and de-phosphorylated pLN-BR plasmid.

5.2.8 Vector and insert preparation

Although several attempts were made to release the fragment from the pGEM-T vector, none were satisfactory and therefore a PCR was performed using SP6 and

T7 primers that flanked the required fragment which was then subsequently digested to release a fragment of the correct size (1042bp).

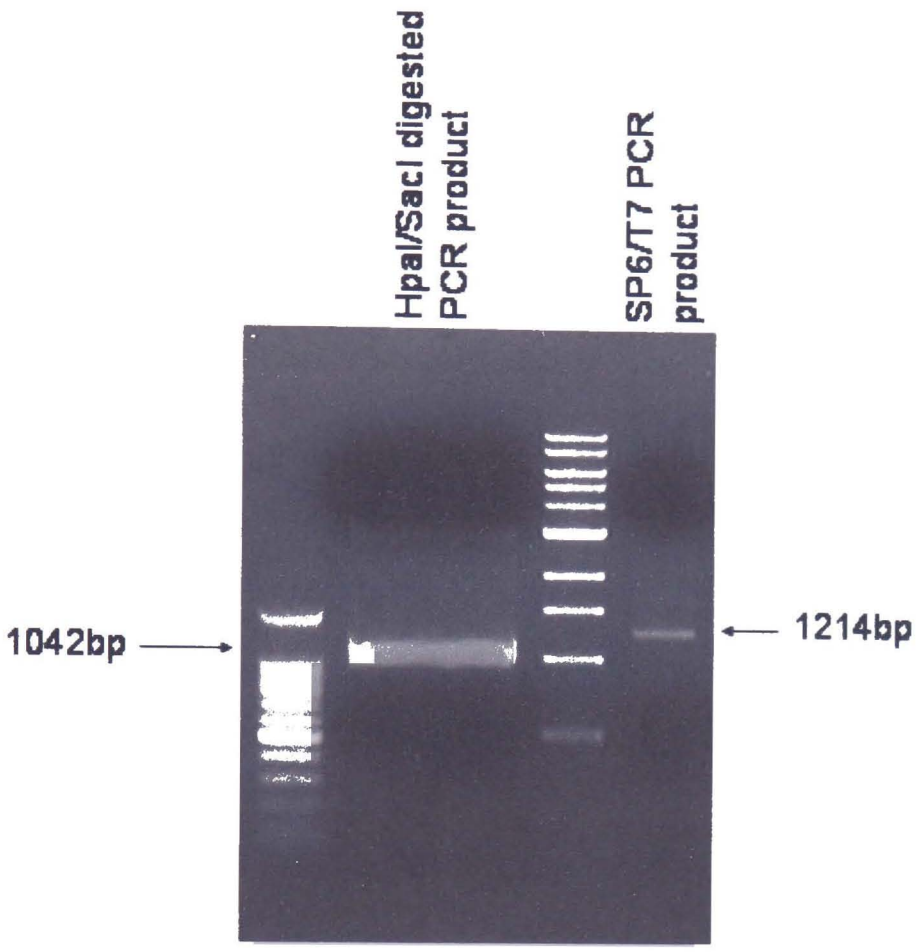


Fig 5.12 Preparation of green insert from pGEM-T. Using the SP6 and T7 primer sites present in the pGEM-T vector, the insert with the flanking vector regions was amplified. This PCR product was then digested with HpaI and SacI and gel purified form the above agarose gel.

The digested insert was then gel purified to be inserted into the prepared pLN-BR vector. The vector was digested with PmeI and SacI before being dephosphorylated to prevent self-ligation. Due to the size of the pLN-BR vector, single digests were performed sequentially to ensure that both restriction digests were carried out to completion.

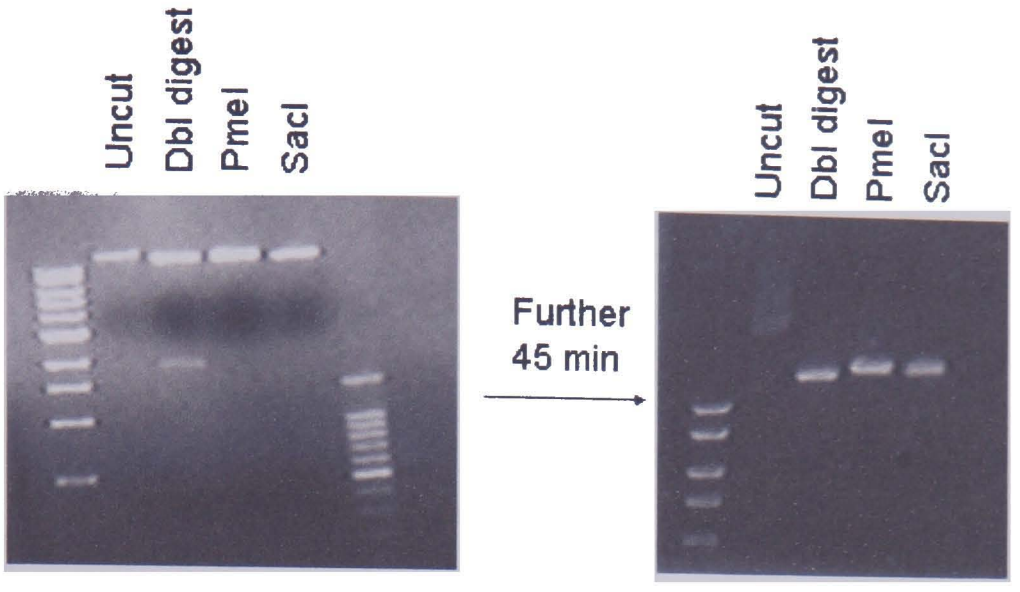


Fig 5.13 Vector preparation. pLN-BR was digested with PmeI restriction enzyme overnight at 37°C before being heat inactivated at 65°C for 20 minutes and adding SacI enzyme for a further 24 hrs.

After dephosphorylation, to ensure that only the digested vector was present, pLN-BR PmeI/SacI was gel purified using QIAEX II beads.

5.2.9 Screening for the green insert

Although both insert and vector had been purified and several attempts were made the new pGN-BR (Green Neomycin – BVDV replicon) was proving difficult to ligate, resulting routinely in a 3Kb band of unknown origin, possibly as a result of gene re-arrangement with the *E. coli*. To overcome this, a new T4 rapid ligase kit from Roche was used and the correct clone was made and tested for the presence of the green insert by PCR.

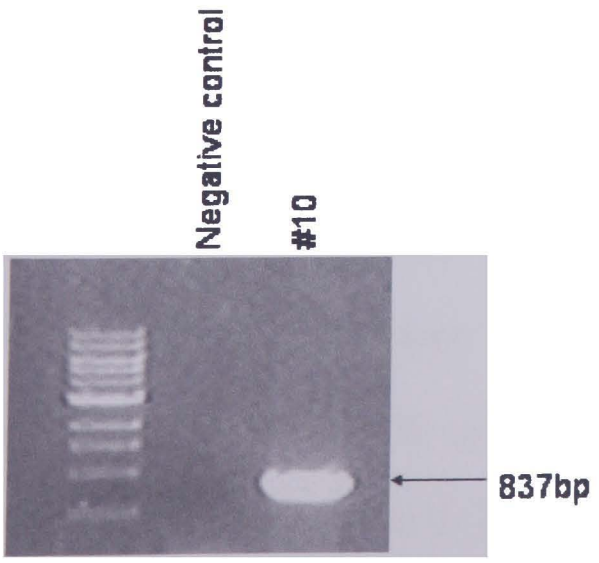


Fig 5.14 PCR screening for the presence of the green insert. Using the primers designed to make the overlap fragment, a PCR reaction was carried out on a potential clone.

After showing that the green insert was present in the clone, it was grown up further before being checked again by sequencing.

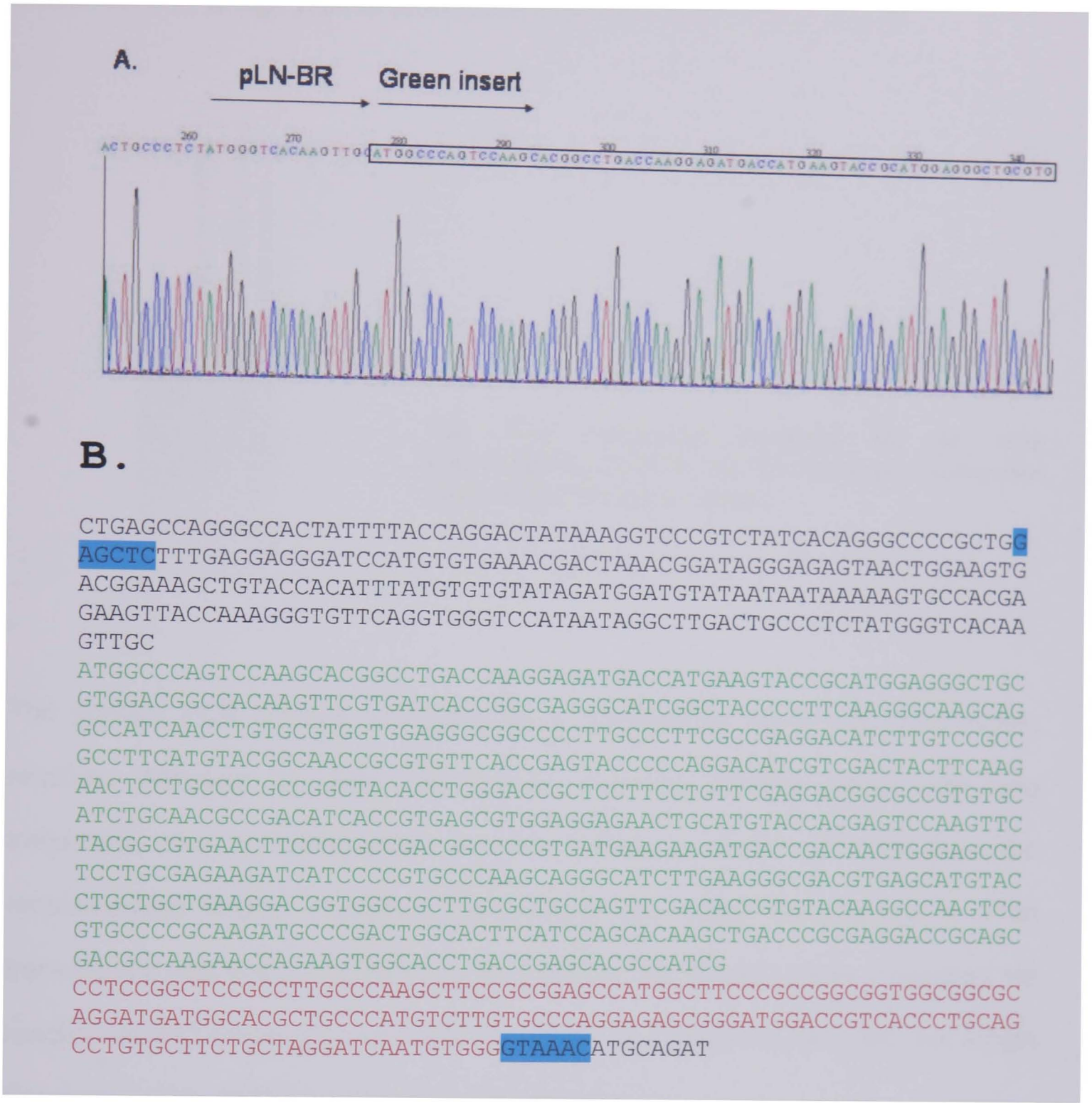


Fig 5.15 Sequence confirmation of the presence of the dsgreen insert in the BVDV replicon. A. Chromatogram showing the area where the BVDV replicon is ligated with the dsGreen insert. B. The forward and reverse sequences were aligned using Blast2, restriction sites used during the cloning are highlighted in blue. The black is the original pLN-BR vector, green is the insert from the pZsGreen vector and the red is the PEST sequence also obtained from the pZsGreen vector.

5.2.10 *In vitro* transcription of the BVDV green replicon

After sequence confirmation the pGN-BR (Green, Neo, BVDV, Replicon) was used as a template for *in vitro* transcription to obtain RNA to transfect into Huh7 cells using a forward primer that contained the T7 promoter site. After confirmation that

the PCR worked, the reaction was scaled up 25 times and the resulting products pooled before being purified using a Qiagen PCR purification column in order to obtain not only a high yield of product but also without any contaminants.

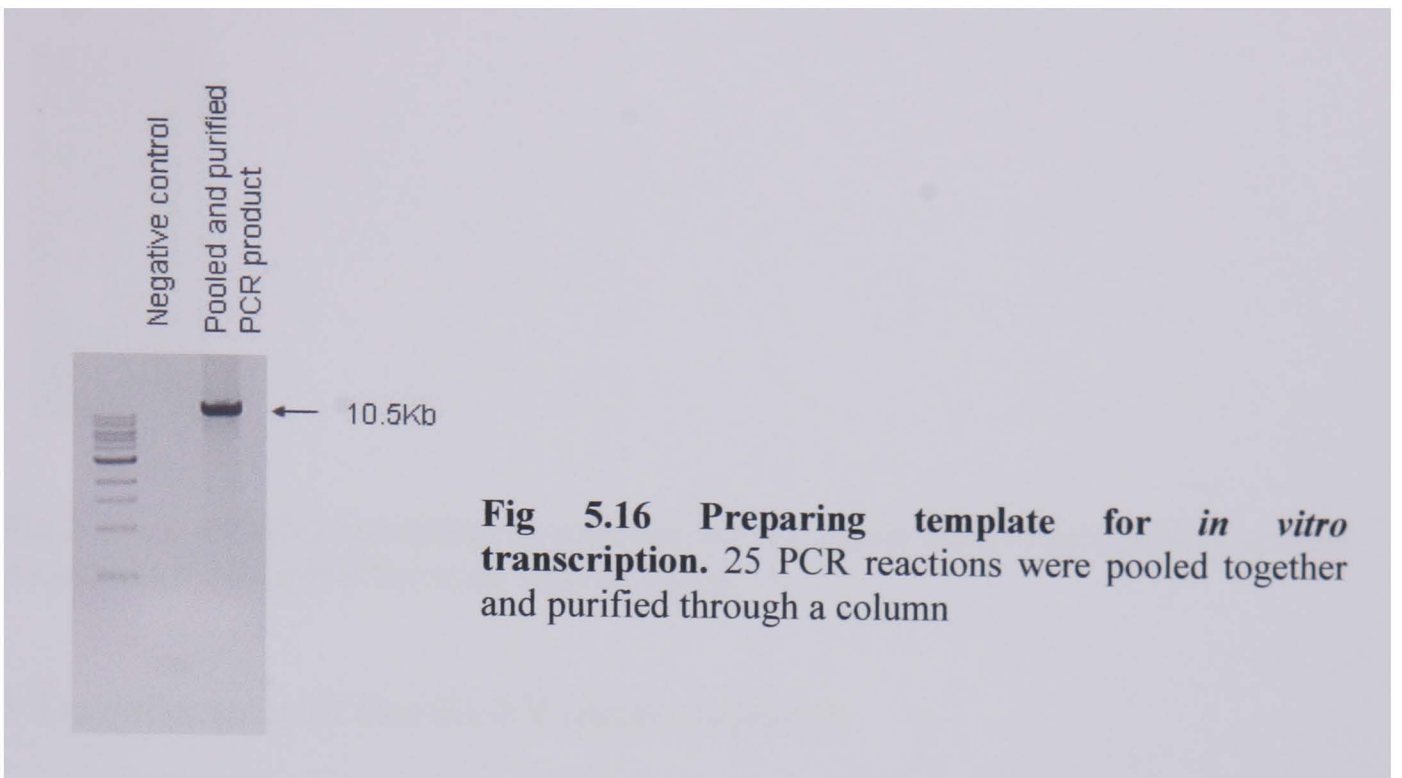


Fig 5.16 Preparing template for *in vitro* transcription. 25 PCR reactions were pooled together and purified through a column

The purified PCR product was used as a template for an *in vitro* transcription reaction. Although *in vitro* transcriptions normally function better with long transcripts, due to transcription initiation events, a 10.5Kb is more than the recommended length. Therefore, the protocol was scaled up and due to short transcripts it was also modified to a 37°C step for 15 minutes before incubating the reaction at 4°C overnight. Although this significantly lowered the yield, full length transcripts of the BVDV green replicon were achieved.

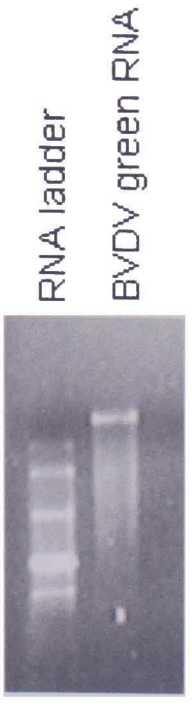


Fig 5.17 *In vitro* transcription to produce BVDV green RNA. Purified PCR product was used as a template for an *in vitro* transcription.

5.2.11 Transfection of the BVDV green replicon

The prepared RNA was then transfected into Huh7 cells either by TransMessenger or by electroporation and left for 48 hours before visualising the GFP by IF. Although numerous attempts have been made to transfect Huh7 cells with the RNA so far they have not been positive by IF. There are several possible explanations for this: 1. The PEST sequence is normally used in the C terminus of the protein. Here we have the BVDV replicon following on from the PEST sequence, which could have rendered the GFP protein unfunctional. 2. The insertion of the PEST sequence might render the BVDV replicon unstable and unable to replicate.

5.3 Discussion

The BVDV reporter assay has several potential advantages: the whole of the signalling pathway of the antiviral action of IFN could be tested and a positive strand RNA virus closely related to HCV was used. Although clear differences between the vector control and the putative IFN inhibitors were seen, the variability within the assay did not allow subtle differences to be interpreted. Replacement of the luciferase reporter from within the BVDV replicon with a dsGFP, will improve the sensitivity of the assay. The putative IFN inhibitors are cloned into a bicistronic expression vector encoding a red fluorescent protein (pIRES2DsRed2, see chapter 3) and transiently transfected into the Huh7 cells stably expressing the BVDV-dsGreen. A two colour flow cytometry will be used to quantify the antiviral effects of IFNα on the BVDV replicon (green FL1 channel) in cells also expressing the putative inhibitors (red FL2 channel) whilst untransfected cells (not red) will act as an internal control.

Although it was demonstrated that BVDV could be used as a reporter for IFN inhibitors, in the long term, the analysis of the putative inhibitors would be greatly facillitated by being analysed by flow cytometry. Unfortunately within the time constraints of this thesis it was not possible to transfect the BVDV-Green replicon RNA into Huh7 and tests its viability. Other cell lines, which have previously been shown to support BVDV replication should be tested to ensure that the replicon is viable.

In conclusion, it was shown that a BVDV replicon was a feasible reporter assay for putative IFN inhibitors but more work is required to develop the assay in terms of sensitivity and ease of use.

Reporter assays for interferon stimulated genes.

6.1 Background

The effects of the HCV genes on the proteins mediating the antiviral effects of IFNα were investigated in human hepatoma cells. Since the discovery of interferon, several reporter systems have been constructed. In this chapter, a reporter system for the MxA promoter was used to investigate the effects of various patient derived NS5a plasmids.

6.2 MxA

As previously described, MxA is a key component of the antiviral action of interferon. The Mx1 protein was first identified in an A2G inbred strain of mouse that showed resistance to otherwise lethal doses of mouse-adapted influenza virus (Haller *et al*, 1981). The Mx1 localises to the nucleus suggesting exertion of its antiviral activity by inhibiting virus transcription. In contrast the human homologues are cytoplasmically located. These differences in sub-cellular localization suggest different mechanisms of antiviral action.

The association between MxA and HCV infection is vague at best; MxA proteins are not found in the whole blood of HCV patients pre-treatment (Chieux *et al*, 1998), even those with a high viral load, nor is MxA expressed in the PBMCs during the acute phase of HCV infection (Jakschies *et al*, 1994). In contrast, high levels of MxA expression were found after IFNα treatment (Chieux *et al*, 1998) and also in the PBMCs of patients with chronic HCV (Antonelli *et al*, 1999;Giannelli *et al*, 2004). However, it hasn't been conclusively shown whether the increase in MxA expression is due to the IFNα regime or if it is dependent upon the virus (Meier *et al*, 2000). Although MxA expression in liver biopsies taken from patients prior to IFN treatment

showed elevated MxA expression levels in hepatocytes in >80% of the samples, the antiviral effect of MxA on HCV replication is unclear (MacQuillan *et al*, 2002;MacQuillan *et al*, 2003). HCV RNA replication within the replicon system was not affected when MxA was transiently overly expressed, and a dominant negative mutant of MxA did not interfere with the antiviral activity of IFNα against HCV RNA replication (Frese *et al*, 2001). Although these results imply that the antiviral effect of IFNα on HCV replication is MxA independent, it has been shown that a single nucleotide polymorphism (SNP) in the promoter region of the MxA was associated with the IFN response of HCV patients (Hijikata *et al*, 2000), [later shown to be true only for patients with a low viral load (Suzuki *et al*, 2004)] and that the expression of MxA was significantly lower in patients who responded to interferon treatment (Giannelli *et al*, 2004).

6.2.1 Assay development

The MxA promoter linked to a luciferase construct as previously described (Ronni *et al*, 1998) was kindly donated by Dr. Ilkka Julkunen.

The level of luciferase (reporter gene product) expression was used to measure transcription from the MxA promoter. Firefly luciferase is a monomeric enzyme of 61KD that catalysed a two step oxidation reaction to yield light. The luciferyl carboxylate is activated by ATP to give a reactive mixed anhydride which then reacts with oxygen to create a transient dioxetane that breaks down to oxyluciferin and CO_2.

Transfection based experiments are notoriously variable; to control for experimental variation, a transfection control of a pSV-β-Galactosidase vector control was included at a 1 in 20 of the concentration of the luciferase vector in each transfection. The SV40 early promoter and enhancer drive transcription of the *lacZ*

gene, which encodes the β-Galactosidase which can be assayed quickly in cell extracts using spectrophotometric, fluorescent or chemiluminescent techniques.

Using Fugene6 the MxA plasmid was transfected into Huh7 cells, with a beta-gal plasmid (Promega) for a normalisation control to determine the optimum experimental conditions. The cells were treated with IFNα2a (Roferon) for 24 hours before the MxA activity was measured and normalised.

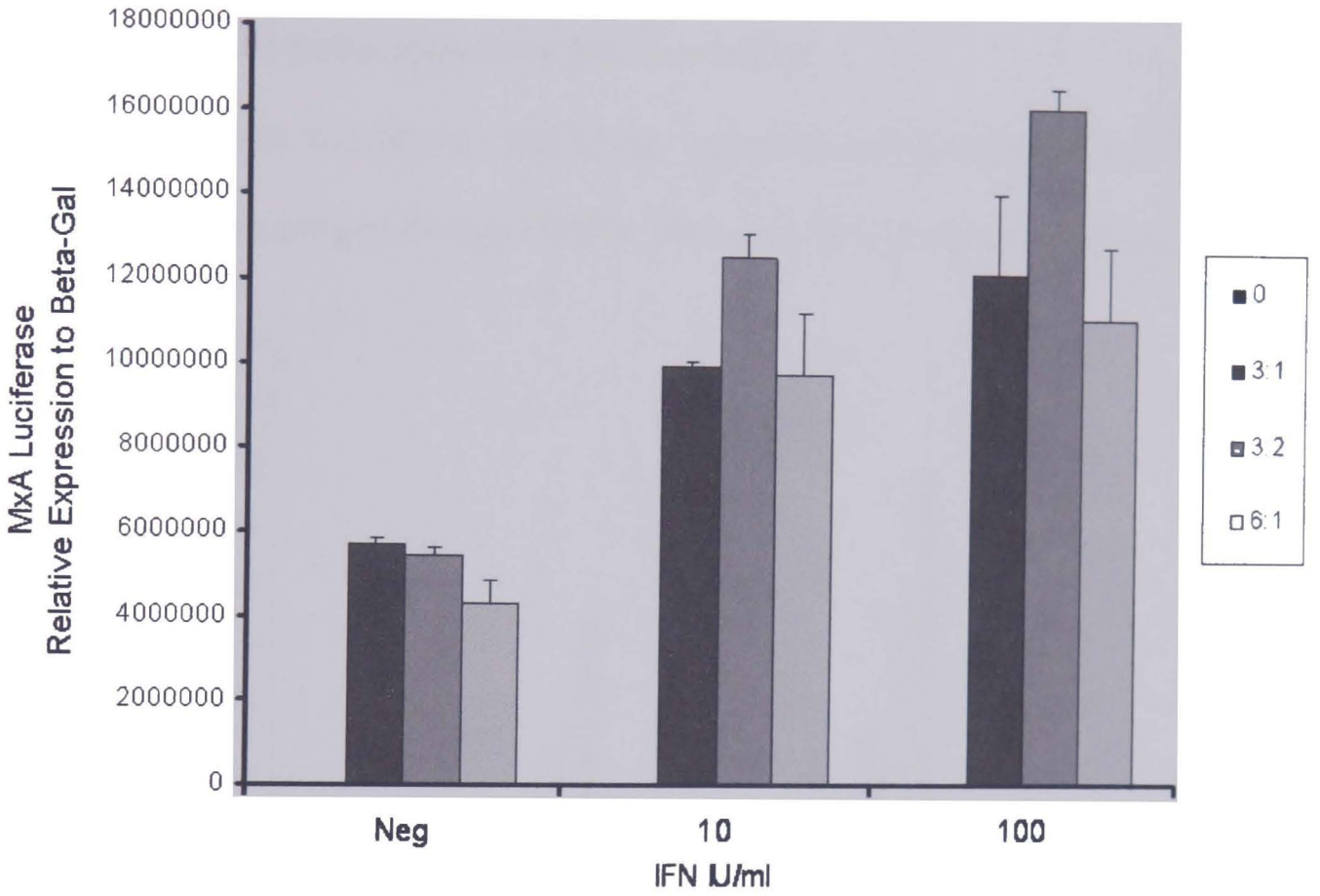


Fig 6.1 Optimisation of transfection: MxA into Huh7 cells. $1x10^3$ cells were plated overnight in a 96 well plate before being transfected with MxA-luc and β-Gal using a variety of Fugene6:[DNA]. The cells were left for another 24 hours before stimulating with IFNα2a. After a further 24 hours, the cells were lysed using PLB and luciferase and β-gal activity measured.

From fig 6.1, a ratio of 3:2 Fugene6:[DNA] was decided upon as the optimum condition for subsequent experiments. Although the background luciferase reading was high, changing the timing of the experiments, i.e. to shorter and longer exposure to IFNα did not significantly lower the background levels of MxA-luciferase. Therefore for convenience sake, a 24 hour stimulation was chosen.

6.2.2 Summary of Optimised Method

Huh7 cells were seeded at a density of $1x10^3$ cells per well in a 96 well plate and left overnight until ~70% confluent. The cells were then transfected using a mixture of Fugene6 with 2μg MxA-luc, and 0.1μg pSV-β-Galactosidase, 5μl of the mix was added to each well. After 24 hours, cells were treated with IFNα2a and approximately 24 hours later, cells were harvested for analysis of luciferase and β-gal production.

6.2.3 Effect of patient derived NS5a on MxA

Using the above conditions, luciferase analysis was performed on Huh7 cells co-transfected with patient derived NS5a, MxA-Luc and β-gal and treated with IFNα2a.

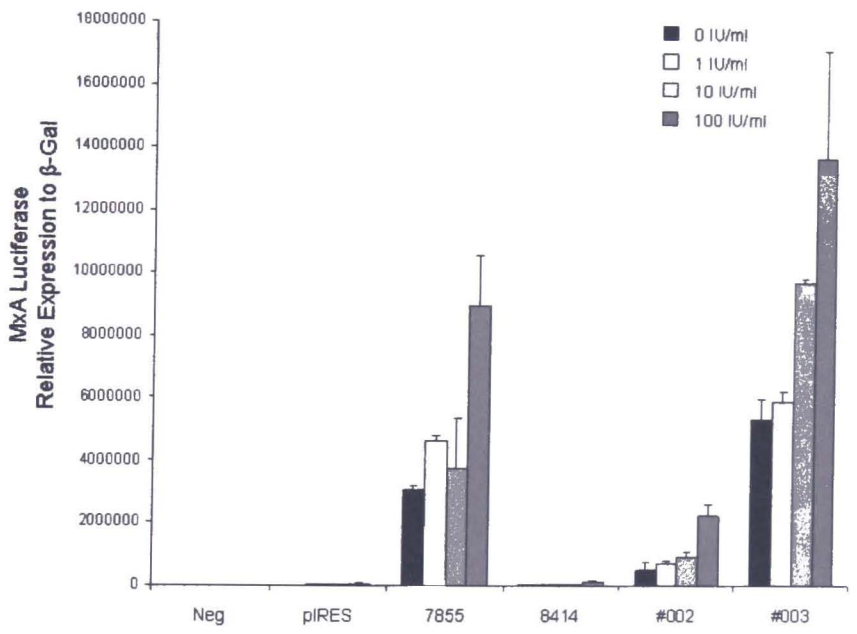


Fig 6.2 The effect of patient derived NS5a on IFNα stimulated transcription from MxA promoter, measured using a luciferase reporter. Growing Huh7 cells were transfected with MxA-Luc, pSV-β-Gal and patient derived NS5a. After 24 hours, cells were treated with IFNα2a and harvested after a further 24 hours and luciferase and β-gal production measured. Assays were performed in duplicate and mean data shown.

The above Fig (6.2) shows the level of MxA promoter activity with different NS5a samples using increasing concentrations of Interferon. The experiment was repeated several times and the above figure illustrates a typical result.

It was observed that NS5a samples from patients 7855, #002 & #003 superinduced the luciferase activity i.e the MxA promoter activity, in the absence of IFNα2a, but that the luciferase activity also increased with increasing IFNα2a concentration. This finding is against the perception that the HCV NS5a gene inhibits the MxA protein in order to evade the antiviral response and achieve chronicity. In comparison the luciferase levels from clone 8414 were comparable to the negative and vector control.

6.2.4 Superinduction of MxA promoter activity; measured with the luciferase assay

The above results were used to gain the fold induction of MxA promoter with increasing concentrations of IFNα2a

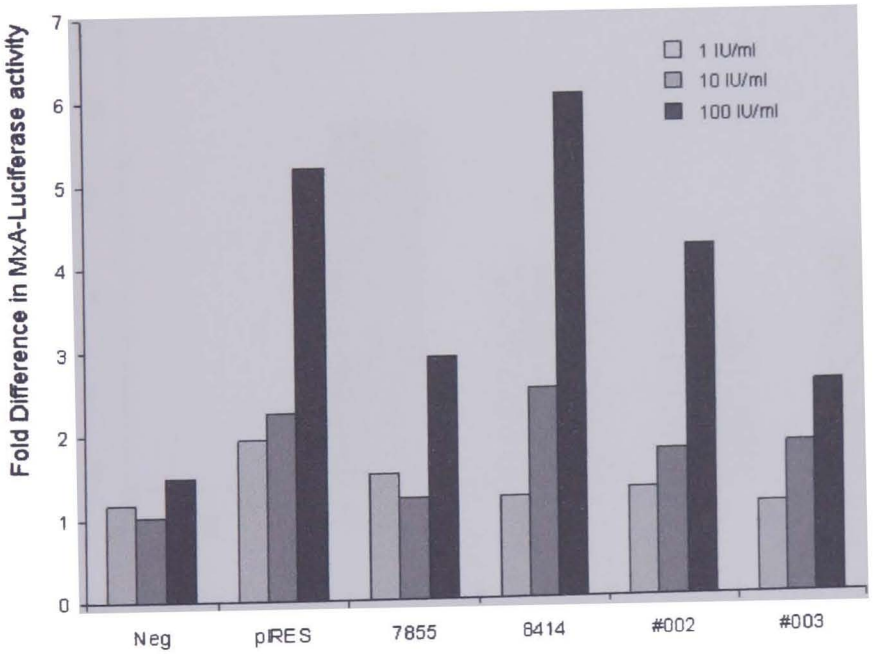


Fig 6.3 Fold induction of MxA-luciferase activity in the presence of NS5A. The fold induction was measured from the negative IFNα2a control.

Although when luciferase activity was measured, no detectable difference was seen in sample 8414 and only low activity was detected in sample #002, when analyzing the fold induction with increasing IFNα2a concentration, it was clear that the fold induction of MxA promoter activity in the 8414 cells was comparable if not greater

than in the other NS5a transfected cells. It appears that although samples 7855 and #003 show greater luciferase activity in the absence of IFNα2a, the fold induction shows converse results with samples 8414 and #002 at higher IFNα concentrations inducing greater MxA promoter actitivty.

6.2.5 Superinduction of MxA in the presence of NS5a patient derived samples.

To further investigate the effect of the different patient derived NS5a proteins on the MxA promoter, the endogenous MxA was measured in the presence and absence of NS5a. Huh7 cells were transfected with the patient derived NS5a for 24 hours and total RNA was extracted, MxA gene transcription was analysed by quantitative RT-PCR and normalised to the Neomycin resistance gene found in the vector.

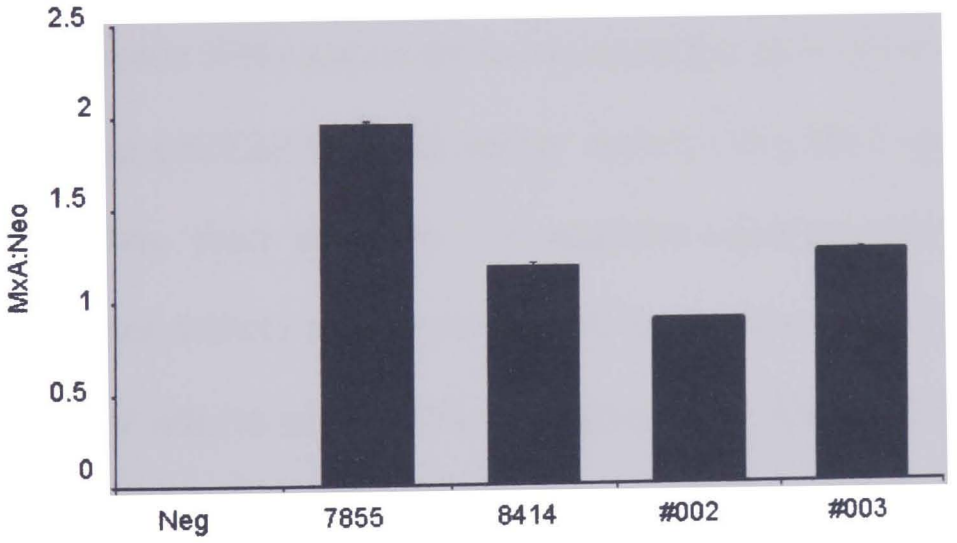


Fig 6.4 Endogenous MxA induction by patient derived NS5a. Patient derived NS5a were transfected into Huh7 cells for 24hours before extracting total RNA and measuring MxA induction by real time PCR, normalized to Neomycin. Experiments were performed in triplicates and the mean data shown ± standard error of the mean.

In correlation with the previous data, patient derived NS5a was found to induce MxA, basal MxA was not detectable in non-transfected Huh7 cells. In previous experiments, the amount of basal MxA activity induced in the 8414 sample was significantly less than the others, but although the induction of endogenous MxA in

the 8414 was less than in the 7855 sample, it did not represent such a significant difference.

Discussion

MxA has been reported to be one of the most specific parameters for IFNα/β action, but as shown in this chapter, using the MxA promoter linked to the luciferase cassette may not have been the best way of evaluating the effects of different HCV proteins on the interferon system. Several of the HCV proteins appear to activate the MxA promoter in the absence of exogenous IFNα, and the promoter activity was increased further after the addition of exogenous IFNα. The data was re-analysed, using a fold-increase of the MxA promoter activity from the samples without IFNα, thereby negating the effect of the samples on the promoter without IFNα. The samples which had previously been shown to activate the MxA promoter without exogenous IFNα appeared to increase the MxA promoter activity less in comparison with the pIRES2-DsRed2 vector control. The MxA reporter assay has raised more questions than answered. It appears counter-intuitive that NS5A induces MxA promoter activity and thereby MxA expression when the virus is trying to evade the antiviral effects of the IFNα/β system. The difference in the basal induction of MxA with NS5a derived from different patients needs to be explored further. It is especially interesting to note the differences between the plasmids that have previously been described as being derived form a non-responding and a responding patient. Further samples from well-defined patients are necessary to explore the interaction between the MxA promoter and the HCV proteins and whether or not this leads to an inhibition of the IFNα/β system. As a reporter system, the MxA is of limited value until these questions are answered.

7.1 Discussion

The Hepatitis C Virus which was first identified in 1989 (Choo *et al*, 1989) , is transmitted via blood to blood contact. Of those who become infected, approximately 85% develop chronic infection and about a quarter of these patients go on to develop cirrhosis of the liver and are also at risk of developing hepatocellular carcinoma. HCV infection is currently the leading cause worldwide for liver transplantation.

The current standard treatment for HCV infection is pegylated IFNα in combination with ribavirin. Although clinical trials with combination therapy have demonstrated high rates of sustained virological response amongst chronic HCV patients, 24% of patients with genotype 2 or 3 and 54% of patients with genotype 1 fail to achieve sustained virological response. Poor response to IFNα therapy, particularly amongst patients infected with HCV genotype 1 is a major consideration for clinicians starting patients on a costly course of IFNα therapy, a treatment that also includes several adverse effects for the patient. Predictive markers of response to IFNα therapy could be of benefit in this decision making process.

HCV has clearly developed strategies to evade antiviral immune reponses. In this thesis, three different systems have been investigated as a potential reporter assay for testing the ability of individual HCV proteins to inhibit the antiviral effects of IFNα.

Although the Dengue replicon did not prove to be a suitable reporter assay due to its insensititivity to type I IFN, the mechanism by which it subverted the effects of interferon were further investigated. The replicon system was used to pinpoint STAT2 as being the target of the dengue virus and its degradation was instrumental in the way the dengue virus avoids the interferon pathway. As the viral load of

dengue early in disease is an important marker for progression any mechanism that the virus can use to increase its peak viral titre has severe consequence. By evading the effects of interferon by reducing steady state STAT2 levels, the dengue virus has tipped the balance of virus vs immune system in its favour.

STAT2 is an important target in the type I IFN signalling pathway and several viruses have used different mechanisms to block its function. Human parainfluenza virus 2 (HPIV2) has been demonstrated to act on STAT2 by degradation via the proteaseone. As demonstrated in this thesis with dengue virus, Parisien *et al* (2002) showed that expression of HPIV2 V protein in cultured cells does not influence STAT2 mRNA levels but reduces STAT2 steady state levels by targeting the protein for degradation.

In conjunction with our studies, several other members of the flaviviruses have recently been shown to inhibit the interferon signalling pathway by acting on the STAT proteins e.g. Best *et al* (2005) showed that Langat virus (LGTV) prevented the phosphorylation of STAT1/2 in response to IFNα and IFNγ.

In 2003, Munoz-Jordan et al suggested that the viral proteins NS2a, NS4a and NS4b were responsible for the inhibition of the IFN signalling pathway. In this study, NS4b appeared to block both IFNα/β and IFNγ signalling by acting on STAT1. The study was performed in a monkey kidney cell line, but STAT2 is not well conserved among species. It will be interesting to study the individual non-structural proteins of the dengue virus within the replicon system by either mutational analysis or individually expression to assess their individual effects on STAT2 in a human cell line. Although studies were attempted to pinpoint which of the dengue non-structural proteins was responsible for the degradation of STAT2 using immunoprecipitation, the lack of antibodies against all the non-strucutral proteins and the difficulties in

reaching a balance between cell death and an effective proteasomal inhibitor proved too problematic.

Another system investigated within this thesis was the BVDV replicon system which could potentially be used as an effective reporter system. We have shown that it is sensitive to the effects of interferon and cells containing the reporter can still respond to exogenous type I IFN. The reporter was then tested using a known interferon inhibitor, SV5-V and shown to be effective at low interferon concentrations, but that higher concentrations of IFNα masked the effects of the inhibitor on the reporter system. Using two different and well established HCV-NS5a clones the BVDV reporter system showed differences in their inhibition of the reporter assay. The BVDV reporter system needs to be examined further with known IFN antagonists, before its usefulness as a reporter system is verified.

Although the assay works in principle, a lack of sensitivity of the luciferase reporter will not allow us to study subtle changes in inhibition, therefore the assay needs to be modified. The luciferase cassette has been replaced with a destabilised GFP which will allow us to study the inhibitors in a FACS based assay that will improve the sensitivity. Although a cloning strategy was designed for the removal of the luciferase cassette with the destabilised GFP so far it has not been possible to transfect this construct into Huh7 cells. Other cells lines that support BVDV replication should be utilised as well as detecting the BVDV replicon using specific antibodies and not relying on the GFP.

The MxA reporter assay has raised more questions than answers. It appears counter-intuitive that NS5A induces MxA expression and the difference in the basal induction of MxA with NS5a derived from different patients needs to be explored

further. As a reporter system, the MxA is of limited value until these questions are answered.

7.2 Future work

1. HCV cloning and expression

Although it was shown that the NS5a protein derived from different patients could be expressed *in vitro* by using specific antibodies against the protein, the pIRES2-Dsred2 vector was unsatisfactory. Expression of the RFP could be detected in BHK cells by FACS but, the same could not be achieved in more relevant human liver cells such as Huh7 and HepG2. This could be due to the EMCV IRES, present in this bicistronic vector, which has been shown to be susceptible to the introduction of mutations during the cloning process. Several others groups have reported problems with the expression of RFP in human cells using the bicistronic vector. To overcome this problem, additional vectors should be explored. Lentiviral vectors containing the MESV leader sequence, have been shown capable of transducing several mammalian cell lines and express the protein of interest at a high enough level level to be detected by Western blots (Mazzon and Jones, unpublished data). The lentiviral vector is a dual promoter vector, containing GFP. For use with the BVDV replicon system, the GFP would be changed to RFP to allow both the replicon and the individual protein to be detected within the same cell by FACS. Historically, viral vectors were avoided when studying the IFN system as they were thought to induce IFN. Recent evidence suggest that the IFNα/β induced by these viral vectors was below the level of detection using an antiviral assay (Hibbert *et al* unpublished data). Another way of ensuring that exogenous IFNα/β does not interfere with the experiment would be to use K562 cells which although responsive to type I IFN they are unable to produce IFNα/β.

2. Dengue virus and its inhibition of the IFNα/β system

In this thesis it was shown that STAT2 was targeted for proteosome-mediated degradation in dengue replicon-containing cells. As the replicon consists only of the non-structural proteins, we thereby excluded the requirement for dengue structural proteins for this effect. Work is ongoing to determine which of the non-strucutral protein/s of the dengue virus is responsible for this phenomenom. Using a lentiviral system, the individual non-structural proteins of dengue have been transduced into K562 cells and currently no individual protein alone can be shown to be responsible for the degradation of STAT2 (Jones and Mazzon, unpublished data). A combination of the non-strucutral proteins will be tested in the future, concentrating on the effects of NS4b. This had been pinpointed by Munoz-Jordan *et al* and using bioinformatic analyses we have identified a putative Elongin BC motif within this gene. We are currently mutating this site by overlapping PCR with the aim of testing the mutation within the replicon system for its affect on STAT2 degradation. Another avenue to be investigated, is the re-constitution of wild type STAT2 into the cells containing the dengue replicon. Results so far indicate that exogenous STAT2 only partially restores IFN mediated signal transduction and after IFNα treatment does not appear to inhibit the RNA replication of the dengue replicon (Jones and Mazzon unpublished data). It is possible that dengue virus, like some other viruses, inhibits the IFN system by more than one way, which will be investigated further.

3. The BVDV reporter system

We have developed a novel BVDV reporter replicon containing a dsGFP which will enable us to study the effect of individual non-structural proteins on host innate immune responses. This has potential as a dynamic reporter of whole cell IFN function, and may allow increased-throughput screening of potential IFN antagonists using FACS. So far, the luciferase cassette of the BVDV replicon has been replaced

by a destabilised GFP and verified by sequencing, but we have been unable to establish cell lines expressing the pGNBR. This could be due to the presence of the PEST, which could limit our ability to detect GFP by FACS or that by altering the sequence of the replicon we have inadvertently changed the secondary structure thereby rendering it replication incompetent. Further investigastion is needed before being able to use the BVDV system as a reporter for IFNα/β antagonists.

The overall aim of this thesis was to identify IFNα/β antagonists by developing a reporter assay. This in turn could lead to the development of novel antiviral drugs, preventing the inhibition of IFNα/β antiviral system. The BVDV system, with several modifications, could be used as such a reporter.

Dengue and Dengue Haemorrhagic fever. 2002.
Ref Type: Report

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Dengue virus inhibits interferon-α signaling by reducing STAT2 expression

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ABSTRACT

Dengue is a mosquito-borne viral disease of immense global importance. Intensity of viral replication in the first days of infection determines clinical outcome, which ranges from benign febrile illness to life-threatening disease (dengue haemorrhagic fever). During this critical early period, innate immunity serves to limit viral replication. The interferon (IFN) system, a key component of innate antiviral responses, has been shown to inhibit early events in dengue replication but have little effect on subsequent steps in the virus cycle. We hypothesized that the dengue virus, in common with several other RNA viruses, has evolved countermeasures to subvert the IFN response.

METHODS

In order to investigate events downstream of input strand translation, we developed human cell lines that continuously express dengue replicons (self-replicating subgenomic fragments). Cells with and without dengue replicons were treated with IFNα and the events studied.

RESULTS

Fig. 1

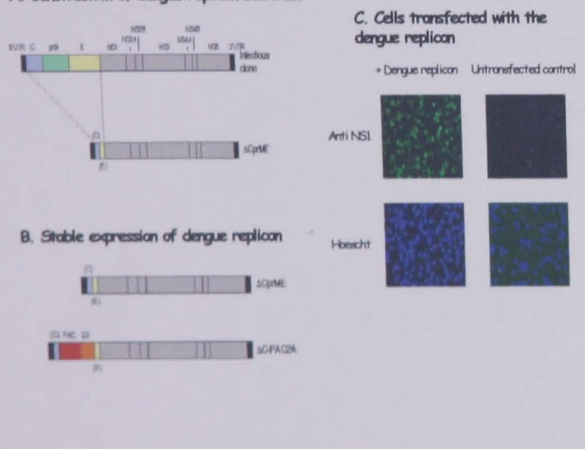

Fig 1. Construction and expression of the dengue replicon.

A-B. The dengue replicon was generated by introducing a large in-frame deletion within the dengue structural genes and substituting an antibiotic selection cassette (puromycin N-actetyl transferase (PAC) and FMDV 2A).

C. Expression of dengue NS1 protein in stably transfected K562 cells detected by indirect immunofluorescence.

Fig. 2

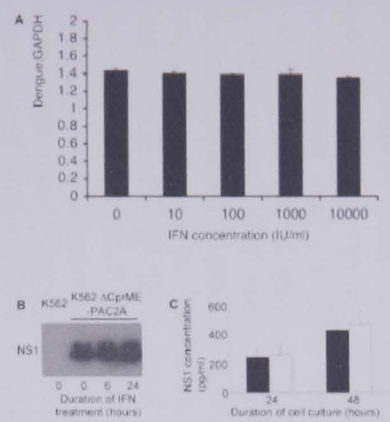

Fig. 3

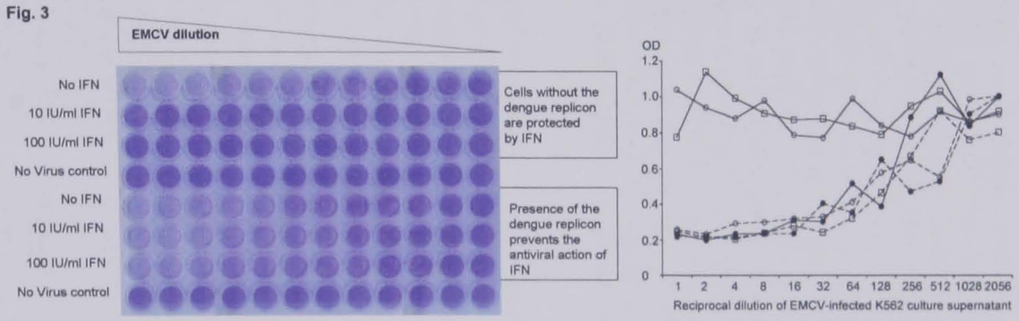

Fig. 3 EMCV replication in replicon-containing cells is not inhibited by IFNα. Cells with and without dengue replicons were treated with IFNα for 24hours before adding 0.5pfu EMCV for 1hour.The cells were then cultured for another 24hours before harvesting the supernatants. The supernatants were plated in a serial dilution onto a A549 monolayer for 1hour before replacing with fresh medium and grown overnight. The cells were stained for viability the next day and the OD measured at 570nm

Fig. 4

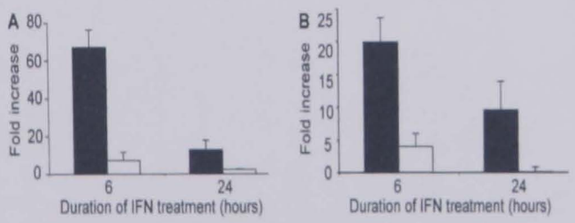

Fig. 4. Induction of classical ISGs by IFN-α is inhibited in dengue replicon containing cells. K562 cells (black bars) and K562.ΔC-prME-PAC2A cells (white bars) were stimulated with 100IU/ml IFN-α2a for 6 and 24 hours. A. MxA and B. PKR gene transcription were measured by real time PCR and normalised to GAPDH.

Table 1. ISG transcription in response to IFN-α in cells that do and do not contain dengue replicons. ISGs up regulated more than 4 fold in K562 cells are shown in comparison to K562.ΔCprME-PAC2A cells as studied using a specific type I IFN macroarray.

Table 1.

	Fold induction	
	K562	K562.ΔCprME-PAC2A
IFN-alpha inducted protein 27	55.8	11.5
VCAM-1	30.6	2.6
MxA	17.9	1.8
IFN-alpha inducted protein (clone IFI-6-16)	12.9	1.1
nerve growth factor (hepatocyte growth factor)	7.0	1.8
PSMB9	7.0	0.4
IFN-inducted protein 17	7.5	1.5
Viperin (cig5)	7.2	1.3
IL-15	6.5	1.1
9-27nerve	6.4	1.7
STAT1	6.2	1.1
STAT+	6.2	1.3
IFIT1	6.1	1.7
KIAA0284	5.8	1.3
STAT1 (91kDa)	4.8	1.8
IFN inducted transmembrane protein 3	4.8	1.4
DNDO	4.8	0.8
IL-6	4.8	0.8
IFN inducted transmembrane protein 2	4.5	1.1
MAP3K4	4.3	1.0
IFI35	4.4	1.1
Mouse caprese STAT	4.1	1.0

Fig. 2
Dengue replicon RNA is resistant to IFN-α

A. K562.ΔCprME-PAC2A cells were grown in the in the presence of up to 10000IU/ml IFN-α2a for 24 hours. Dengue replicon RNA levels were measured using quantitative PCR and normalised to GAPDH mRNA.

B. K562.ΔCprME-PAC2A cells were grown in the presence of 100IU/ml IFNα2a for 0, 6 and 24 hours. Cell lysates ($2x10^5$ cells per reaction) were separated by SDS-PAGE and then the dengue NS1 protein was analysed by immunoblotting. K562 cells were included as a negative control. **C.** K562.ΔCprME-PAC2a cells were grown in the absence (black bars) or presence (white bars) of 100IU/ml IFN-α2a for 24 and 48 hours. The cumulative concentrations of NS1 in the culture supernatants at each time point were measured by ELISA.

Fig. 5

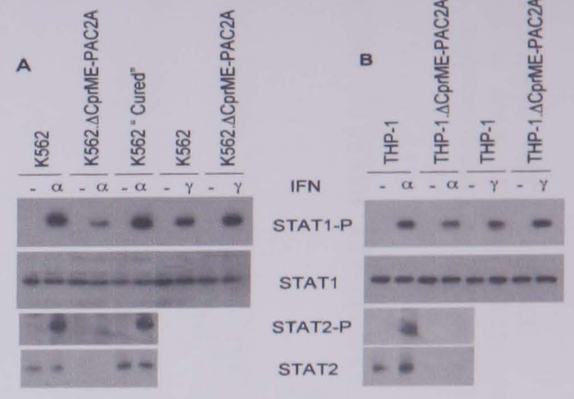

Fig. 5 Steady-state STAT2 levels, and phosphorylation of STAT1 and STAT2 in response to IFN-α, are inhibited by the dengue replicon. Cells that did and did not contain replicons were treated with IFN-α and IFN-γ for 30 minutes. STATs and phosphorylated STATs were analysed by immunoblotting as labelled. (K562 "cured" are cells that previously expressed replicons; these cells were treated for 6 months with glycirrhyzic acid, which eliminated replicon expression)

Fig. 6

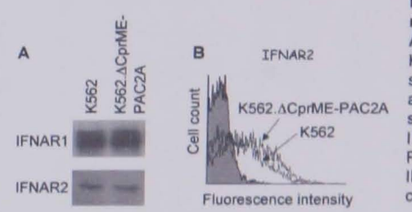

Fig. 6. Dengue RNA replication does not affect IFNAR protein levels.
A. Cell lysates from K562 and K562.ΔCprME-PAC2A cells were separated by SDS-PAGE and then analysed by immunoblotting using specific antibodies for IFNAR1 and IFNAR2. **B.** K562 and K562.ΔCprME-PAC2A were stained with specific anti-IFNAR2 antibodies and analysed by flow cytometry.

Fig. 7

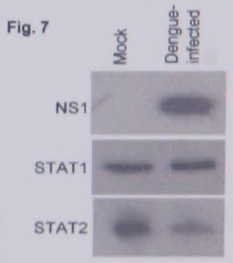

Fig. 7 Dengue virus infection reduces STAT2 levels. K562 cells were infected with dengue virus (type 2 NGC) for 48 hours; mock infected cells were included for comparison. Cell lysates were separated by SDS-PAGE and then analysed by immunoblotting using specific antibodies for dengue NS1, STAT1 and STAT2.

CONCLUSION

Dengue virus specifically inhibits IFN-α, but not IFN-γ, signal transduction by reducing expression of STAT2

BIOLOGICAL SIGNIFICANCE?

- Understanding the molecular basis of the race between dengue virus replication and the IFN response would provide critical insight into disease pathogenesis
- Differences in IFN antagonism may contribute to differences in pathogenicity observed between dengue strains
- Potential to engineer dengue viruses with enhanced sensitivity to IFN, in order to generate safer and more cost-effective vaccine candidates

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Dengue Virus Inhibits Alpha Interferon Signaling by Reducing STAT2 Expression

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Alpha/beta interferon (IFN-α/β) is a key mediator of innate antiviral responses but has little effect on the established replication of dengue viruses, which are mosquito-borne flaviviruses of immense global health importance. Understanding how the IFN system is inhibited in dengue virus-infected cells would provide critical insights into disease pathogenesis. In a recent study analyzing the ability of individual dengue virus-encoded proteins to antagonize the IFN response, nonstructural (NS) protein 4B and possibly NS2A and NS4A were identified as candidate IFN antagonists. In monkey cells, NS4B appeared to inhibit both the IFN-α/β and IFN-γ signal transduction pathways, which are distinct but overlapping (J. L. Munoz-Jordan, G. G. Sanchez-Burgos, M. Laurent-Rolle, and A. Garcia-Sastre, Proc. Natl. Acad. Sci. USA 100:14333–14338, 2003). For this study, we examined the effects of dengue virus on the human IFN system, using cell lines that were stably transfected with self-replicating subgenomic dengue virus RNA (replicons) and that expressed all of the dengue virus nonstructural proteins together. We show here that in replicon-containing cells dengue virus RNA replication and the replication of encephalomyocarditis virus, an IFN-sensitive virus, are resistant to the antiviral effects of IFN-α. The presence of dengue virus replicons reduces global IFN-α-stimulated gene expression and specifically inhibits IFN-α but not IFN-γ signal transduction. In cells containing replicons or infected with dengue virus, we found reduced levels of signal transducer and activator of transcription 2 (STAT2), which is a key component of IFN-α but not IFN-γ signaling. Collectively, these data show that dengue virus is capable of subverting the human IFN response by down-regulating STAT2 expression.

Dengue viruses are mosquito-borne flaviviruses of immense global public health importance, causing tens of millions of human infections worldwide each year (11). The intensity of viral replication in the first days of infection determines the clinical outcome, which ranges from benign febrile illness to life-threatening disease (dengue hemorrhagic fever) (39). During this critical early phase, prior to the full recruitment of antigen-specific defenses, innate cellular antiviral mechanisms mediated by alpha/beta interferon (IFN-α/β) are potentially the most important pathways of the host defense limiting viral replication. Virus infection classically induces the secretion of IFN-α/β, which binds to cell surface IFN-α receptors (IFNAR, comprising IFNAR1 and IFNAR2 subunits) on infected and nearby cells. The binding of IFN-α/β to IFNAR leads to the activation of Jak1 and Tyk2 kinases via tyrosine phosphorylation (4). In turn, signal transducer and activator of transcription 2 (STAT2) and then STAT1 are phosphorylated and form heterodimers, which then associate with p48/IRF-9 to form ISGF3 complexes (12). ISGF3 complexes translocate to the nucleus and initiate the transcription of interferon-stimulated genes (ISGs) by binding interferon-stimulated response elements, leading to the transcriptional up-regulation of hundreds of cellular genes and the induction of an antiviral state (35).

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Experimental evidence suggests that the IFN system plays an important role in limiting dengue virus replication, since knockout mice that lack IFN-α/β receptors develop severe infections after a challenge with dengue virus (15, 34). Also, the pretreatment of cultured cells with IFN-α/β dramatically reduces dengue virus replication (5, 6). This occurs primarily through the inhibition of translation of input-strand dengue virus RNA by an unknown mechanism (5). In contrast, IFN-α/β has little effect on dengue virus replication after viral replication has been established (5, 6), suggesting that the IFN system cannot fully engage in dengue virus-infected cells. In keeping with this observation, dengue virus can achieve high titers ($<10^9$ infectious doses per ml) in humans despite the induction of high levels of circulating IFN-α (21, 36, 39). It therefore seems likely that dengue virus has evolved mechanisms to counter the IFN response, although not absolutely, which is a characteristic that may be shared by many pathogenic viruses (9, 42).

Muñoz-Jordan and colleagues recently published an in vitro study that analyzed the ability of individual dengue virus proteins to block the IFN system, in which they concluded that NS4B and possibly NS2A and NS4A act as IFN signaling inhibitors (25). They showed that NS4B and dengue virus infection blocked signal transduction in response to both IFN-β and IFN-γ in a monkey kidney cell line, suggesting that the target for NS4B-mediated inhibition of IFN signaling may be a component (possibly phosphorylated STAT1 [STAT1-P]) that is common to these distinct but overlapping signal transduction

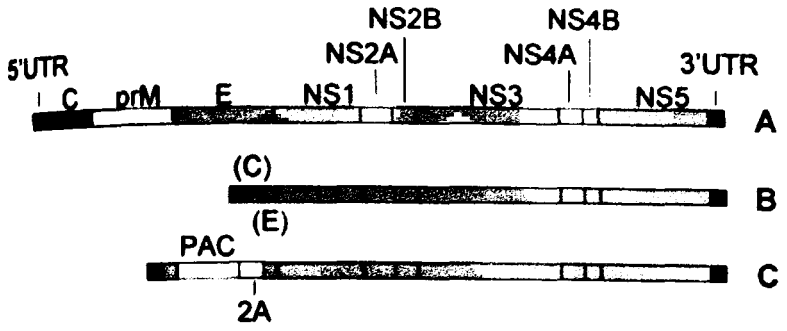

FIG. 1. Schematic showing construction of plasmid pDENΔ CprME-PAC2A. (A) Dengue virus type 2 infectious clone cDNA (in plasmid pDVWS601 [29]), showing a single open reading frame carrying three structural genes (C, core; prM, premembrane; E, envelope), seven nonstructural (NS) genes, and flanking 5′ and 3′ untranslated regions (UTR). (B) pDENΔCprME. A large in-frame deletion was introduced within the region carrying the structural genes. (C) pDENΔCprME-PAC2A. An antibiotic selection cassette encoding PAC and the foot-and-mouth disease virus protein 2A was cloned in place of the deleted structural genes.

pathways (25). We adopted a complementary experimental approach specifically aimed at studying the effect of dengue virus replication downstream of the translation of input-strand RNA on the human IFN system. We first established human cell lines that continuously expressed self-replicating subgenomic dengue virus RNA (replicons). Flavivirus replicons express all of the viral nonstructural proteins together in a way that mimics expression during authentic viral infection, and they have proved to be powerful tools for studying the functional roles of nonstructural proteins in RNA and virus replication (16–18, 24). We show here that the presence of dengue virus replicons in human cell lines inhibits the antiviral effect of IFN-α by blocking early events in IFN-α signal transduction, resulting in reduced levels of STAT1-P. In contrast, STAT1-P levels in replicon-containing cells are increased rather than reduced in response to IFN-γ. We show that steady-state levels of STAT2 are reduced in cells containing dengue virus replicons, which is consistent with the observed responses to IFN-α and IFN-γ. Reduced STAT2 levels are also found in cells infected with dengue virus, suggesting that dengue virus is capable of subverting the human IFN response by down-regulating STAT2 expression.

MATERIALS AND METHODS

Cell lines stably expressing dengue virus replicons. A series of cell lines that continuously express dengue virus replicons have been established in our laboratory (unpublished data). For this study, K562 (human chronic myeloid leukemia) and THP-1 (human monocytic) cell lines stably expressing the dengue virus replicon ΔCprME-PAC2A were used (designated K562.ΔCprME-PAC2A and THP-1.ΔCprME-PAC2A, respectively). The plasmid pDENΔCprME-PAC2A was used for in vitro transcription of ΔCprME-PAC2A replicon RNAs. pDENΔCprME-PAC2A was derived from pDVWS601 (29), which contains a genome-length dengue virus type 2 (New Guinea C strain) cDNA clone, by the introduction of a large in-frame deletion in the structural region, retaining only the first 27 codons of the C gene and the last 24 codons of the E gene. In addition, pDENΔCprME-PAC2A contains an antibiotic selection cassette encoding puromycin *N*-acetyltransferase (PAC) followed by an artificial protein cleavage site (foot-and-mouth disease virus protein 2A) in place of the deleted structural genes (Fig. 1). Cells stably expressing dengue virus replicon RNA were generated by transfection with ΔCprME-PAC2A RNA and then propagation in RPMI containing 10% fetal bovine serum (FBS) and 3 μg of puromycin (Sigma)/ml. Cells were removed from puromycin selection and checked for replicon expression before use by indirect immunofluorescence of the dengue virus NS1 protein with a specific monoclonal antibody (5H5.4) (7). K562 and THP-1 cells without replicons were continuously maintained in the same medium without puromycin.

Cured K562 cell line. K562 cells that stably expressed ΔCprME-PAC2A were removed from puromycin selection and passaged continuously in RPMI containing 10% FBS and 500 μg of glycyrrhizic acid (Fluka Chemicals)/ml, which has activity against RNA viruses through an unknown mechanism (3). At intervals, cells were checked for replicon expression by indirect immunofluorescence of the dengue virus NS1 protein and by reverse transcription-PCR (RT-PCR) for dengue virus RNA (see below). Once the cell line had been cured of the replicon, it was subsequently grown in RPMI containing 10% FBS, without glycyrrhizic acid, and checked for the continued absence of replicons as described above.

Analysis of dengue virus RNA levels and NS1 expression. K562.ΔCprME-PAC2A cells were grown in the presence of 0, 10, 100, 1,000, or 10,000 IU of IFN-α2a (Roferon-A; Roche)/ml for 24 h, and then the total cellular RNAs were extracted by the use of Trizol (Invitrogen). Extracted RNAs were treated with RQ1 RNase-free DNase (Promega) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) using random decamer primers. PCRs were performed and analyzed on a Rotorgene instrument (Corbett Research) by the use of custom primers and a fluorescent probe specific for dengue virus NS1 (forward primer, 5′CTGAAGTGTGGCAGTGGGATT; reverse primer, 5′CTTCAAAGCTAGCTTCAGCTATCCA; probe, 5′CACAGA CAACGTGCACACATGGACAGA). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed in the same samples by the use of specific primers (forward primer, 5′ACAGTCCATGCCATCACTGCC; reverse primer, 5′GCCTGCTTCACCACCTTCTTG) and QuantiTect SYBR green (QIAGEN). In parallel experiments, K562.ΔCprME-PAC2A cells were grown in the presence of 100 IU of IFN-α2a/ml, and cell-associated and secreted dengue virus NS1 proteins were analyzed by immunoblotting and an enzyme-linked immunosorbent assay (ELISA), respectively, as previously described (14, 44).

EMCV *trans* rescue assay. K562 cells that did and did not contain dengue virus replicons were grown in RPMI containing 10% FBS with 0, 10, and 100 IU of IFN-α2a/ml for 24 h. The cells were washed in RPMI, and 10^6 cells were then infected with 5×10^5 PFU of encephalomyocarditis virus (EMCV) in RPMI containing 2% FBS for 1 h. The cells were washed and then cultured for a further 24 h in RPMI plus 10% FBS. The culture supernatants were then harvested, and serial dilutions were plated onto confluent A549 cells in a 96-well plate for 1 h before replacing the inoculum with RPMI containing 10% FBS. After a further 24 h, the A549 cells were fixed and stained with methyl violet, and the optical density in each well was read at 570 nm in an automated plate reader (27).

MxA and PKR gene expression. K562 and K562.ΔCprME-PAC2A cells were grown in the presence or absence of 100 IU of IFN-α2a/ml for 6 and 24 h. RNA extraction and reverse transcription were performed as described above. PCRs were performed and analyzed on a Rotorgene instrument by the use of SYBR green as described above, using primers specific for the MxA gene (forward primer, 5′AACAACCTGTGCAGCCAGTA; reverse primer, 5′AAGGGCAAC TCCTGAGAGTG) or the protein kinase R (PKR) gene (forward primer, 5′T CTCTGGCGGTCTTCAGAAT; reverse primer, 5′ACTCCCTGCTTCTGACG GTA). The housekeeping gene GAPDH was analyzed in the same samples as described above.

ISG expression profiling by macroarray analysis. K562 and K562.ΔCprME-PAC2A cells (2×10^7 per reaction) were treated with 100 IU of IFN-α2a/ml for 24 h before extraction of the total cellular RNAs by the use of Trizol. Radiolabeled cDNAs were generated from 20 μg of total RNA by reverse transcription with Superscript II (Gibco) in the presence of [^{32}P]dCTP. Residual RNAs were hydrolyzed by an alkaline treatment at 70°C for 20 min, and the cDNAs were purified through G-50 columns (Amersham Pharmacia). Before hybridization to the macroarrays, the labeled cDNAs were mixed with 50 μg of COT-DNA (Gibco) and 10 μg of poly(A) DNA (Sigma), denatured at 95°C for 5 min, and hybridized for 1 h to minimize nonspecific binding. Preparation of the macroarrays (representing 150 genes, including many that are known to be stimulated by interferon), hybridization of the radioactive cDNAs, and scanning and analysis of the macroarrays were performed as described previously (33).

Immunoblotting. K562 cells (2.5×10^5 per reaction) that did and did not contain replicons were stimulated with 100 IU of IFN-α2a or IFN-γ (R&D Systems)/ml for 30 min. Unstimulated cells were included for comparison. Cells were harvested and lysed in 250 μl of sodium dodecyl sulfate (SDS) loading buffer (0.0625 M phosphate [pH 7.0], 10% glycerol, 2% SDS, 0.001% bromophenol blue) that had been prewarmed to 60°C. Ten microliters of each sample was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were then transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham). Mouse monoclonal antibodies to STAT1, phosphorylated STAT1 (Tyr-701) (both Zymed), or STAT2 (BD Transduction Laboratories), a

rabbit polyclonal antibody to phosphorylated STAT2 (Upstate Biotechnology), a goat polyclonal antibody to IFNAR1 (Abcam), and a rabbit polyclonal antibody to IFNAR2 (PBL Biomedical Laboratories) were used as primary antibodies. Detection was performed by the use of relevant horseradish peroxidase-conjugated secondary antibodies (Jackson Immunochemicals) and enhanced chemiluminescence reagents (ECL$^+$; Amersham).

Fluorescence-activated cell sorting (FACS) analysis. K562 and K562.ΔCprME-PAC2A (10^6 per reaction) cells were stained with an anti-IFNAR2 antibody in RPMI containing 2% FBS at 4°C. Detection was performed by use of a phycoerythrin-conjugated donkey anti-rabbit secondary antibody (Jackson Immunochemicals), and samples were analyzed on a Becton Dickinson FACScan instrument. Data analysis was performed with WinMDI software.

Dengue virus infection of K562 cells. K562 cells were incubated with dengue virus type 2 (New Guinea C strain) at a multiplicity of infection of 4 and then grown in RPMI containing 10% FBS. After 48 h, the cells were air dried on glass slides and fixed in cold methanol-acetone (50:50 [vol/vol]). The cells were dually labeled with a mouse anti-dengue virus NS1 antibody (5H5.4) and a rabbit anti-STAT2 antibody (C20; Santa Cruz Biotechnology). Fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas Red-conjugated horse anti-mouse antibodies (both from Vector Laboratories) were used for detection. Images were analyzed under a Bio-Rad Radiance 2100 confocal microscope. In parallel experiments, cells (2×10^6 per reaction) were lysed and analyzed by immunoblotting as described above.

RESULTS

Dengue virus replicon RNA replication is resistant to IFN-α. Previous studies have shown that the antiviral effect of IFN-α on dengue virus infection in cell cultures is markedly inhibited if the treatment is delayed a few hours after infection (5, 6), suggesting that dengue virus can counter the IFN response once replication has been established. We tested directly whether IFN could inhibit established dengue virus RNA replication in the form of the dengue virus replicon ΔCprME-PAC2A, which is stably maintained in K562.ΔCprME-PAC2A cells (unpublished data). K562.ΔCprME-PAC2A cells were grown in the presence of 0, 10, 100, 1,000, or 10,000 IU of IFN-α2a/ml for 24 h, and dengue virus replicon RNA levels were measured by quantitative RT-PCR. In addition, the effect of 100 IU of IFN-α/ml on the levels of cell-associated and secreted NS1 protein was analyzed by Western blotting and ELISA, respectively. Figure 2 shows that IFN-α had no significant effect on dengue virus replicon RNA levels or NS1 expression. These data confirm previous evidence suggesting that established dengue virus RNA replication is resistant to IFN-α (5).

Antiviral action of IFN-α is blocked by dengue virus RNA replication. We next tested whether the presence of dengue virus replicons inhibits the general antiviral action of IFN in cells. K562 cells that did and did not contain replicons were first treated with IFN-α2a and then infected with an IFN-sensitive virus, EMCV. The basis of this technique is that the inhibition of the antiviral action of IFN by dengue virus replicons results in a rescue of EMCV replication, which is detected in a modified plaque assay on A549 cells. Figure 3 shows that in the absence of IFN-α2a, EMCV replication was equal in cells that did and did not contain replicons. As expected, a pretreatment of K562 cells with either 10 or 100 IU of IFN-α2a/ml dramatically inhibited the replication of EMCV. In contrast, pretreatments of K562.ΔCprME-PAC2A cells with the same concentrations of IFN-α2a had no effect on EMCV replication (Fig. 3). In order to prove that dengue virus RNA replication inhibited the IFN response, we repeated the EMCV rescue assay, using K562.ΔCprME-PAC2A cells that had previously been cured of the replicon by continuous growth in the presence of glycyrrhizic acid. Cured K562 cells were negative for the presence of dengue virus NS1 protein and dengue virus RNA by indirect immunofluorescence and RT-PCR, respectively (unpublished data). Cured K562 cells reverted to the IFN-responsive phenotype of the original K562 cells (data not shown). Taken together, these data show that the antiviral activity of IFN is blocked in the presence of dengue virus replicons.

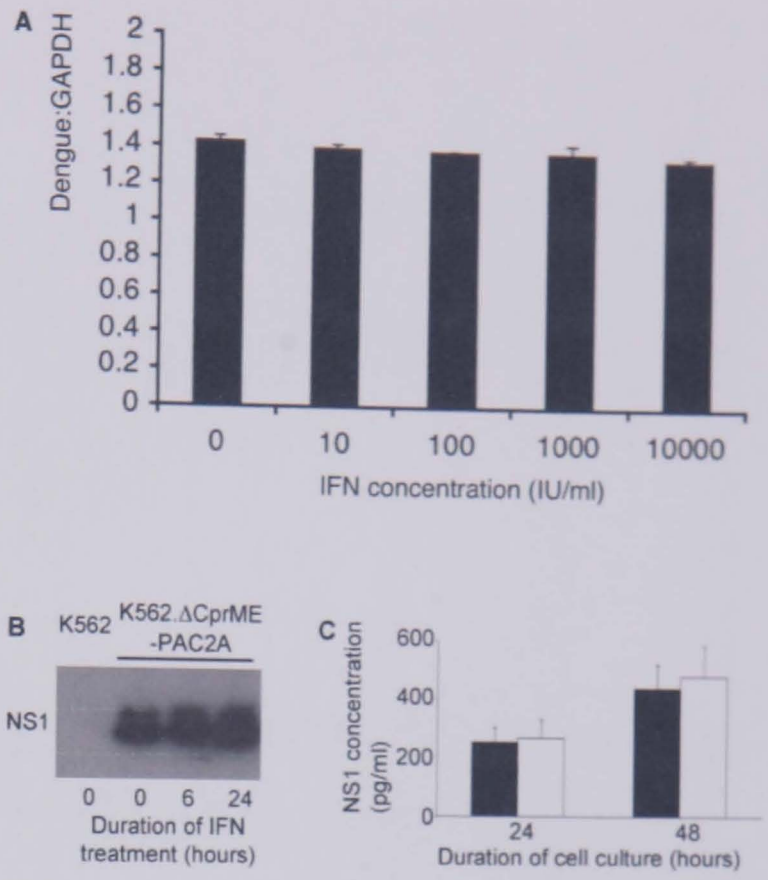

FIG. 2. Dengue virus replicon RNA replication is resistant to IFN-α. (A) K562.ΔCprME-PAC2A cells were grown in the presence of various concentrations of IFN-α2a, as indicated, for 24 h. Dengue virus replicon RNA levels were measured by quantitative PCR and normalized to GAPDH mRNA levels. (B) K562.ΔCprME-PAC2A cells were grown in the presence of 100 IU of IFN-α2a/ml for 0, 6, and 24 h. Cell lysates (2×10^5 cells per reaction) were separated by SDS-PAGE, and then the dengue virus NS1 protein was analyzed by immunoblotting. K562 cells were included as a negative control. (C) K562.ΔCprME-PAC2A cells were grown in the absence (black bars) or presence (white bars) of 100 IU of IFN-α2a/ml for 24 and 48 h. The cumulative concentrations of NS1 in the culture supernatants at each time point were measured by ELISA.

IFN-α-induced gene expression is inhibited in replicon-containing cells. Different viruses have evolved a diverse range of molecular mechanisms that act on different cellular targets to inhibit IFN-mediated antiviral pathways (9, 13). In order to test whether the observed inhibition of the antiviral effect of IFN in replicon-containing cells was due at least in part to an inhibition of IFN signal transduction, we first measured the induction of MxA and PKR gene transcription. These genes are classical ISGs that contain an interferon-stimulated response element within the promoter region (20) and encode proteins that are key mediators of the antiviral effects of IFN (13). K562 cells that did and did not contain replicons were stimulated with IFN-α2a for 6 and 24 h, and MxA and PKR gene transcription was analyzed by quantitative RT-PCR. As expected, IFN induced the transcription of both of these genes

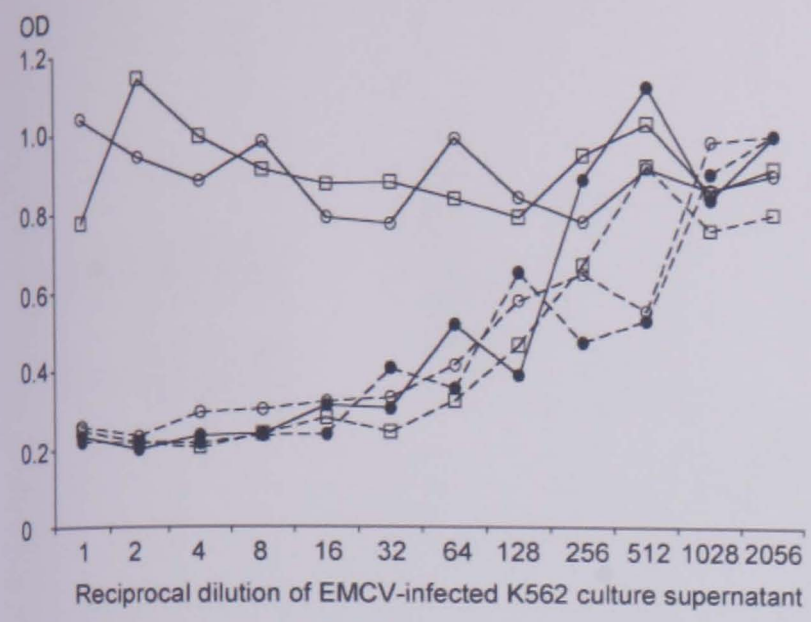

FIG. 3. Antiviral effect of IFN-α is blocked in dengue virus replicon-containing cells. K562 cells (solid lines) and K562.ΔCprME-PAC2A cells (broken lines) were treated with 0 (●), 10 (□), or 100 (○) IU of IFN-α2a/ml for 24 h. The cells were then infected with EMCV, and after a further 24 h, the supernatants were harvested and serially diluted on confluent A549 cells. After 24 h, the A549 cells were fixed and stained with methyl violet. The amount of staining was quantified by measuring the optical density (OD) of each well at 570 nm. More EMCV replication resulted in increased cell death and lower optical density readings.

in K562 cells. In contrast, the IFN induction of MxA and PKR gene transcription was dramatically inhibited in K562.ΔCprME-PAC2A cells (Fig. 4). Having shown that the induction of two genes, MxA and PKR, was inhibited in replicon-containing cells, we examined the global pattern of IFN-inducible gene transcription in cells that did and did not contain replicons by using a custom macroarray. We found that the IFN response was profoundly suppressed in K562.ΔCprME-PAC2A cells compared with K562 cells. Table 1 lists the ISGs that were most up-regulated (more than fourfold) in K562 cells in response to IFN and shows comparative data for K562.ΔCprME-PAC2A cells. These data indicate that IFN-α-induced gene expression is inhibited in cells containing dengue virus replicons, though not absolutely, and suggest that IFN-α signal transduction is inhibited.

Dengue virus RNA replication inhibits early events in IFN-α signaling by down-regulating steady-state STAT2 levels. In order to determine whether early events in IFN signal transduction were inhibited in replicon-containing cells, we performed a Western blot analysis of STAT1 phosphorylation in

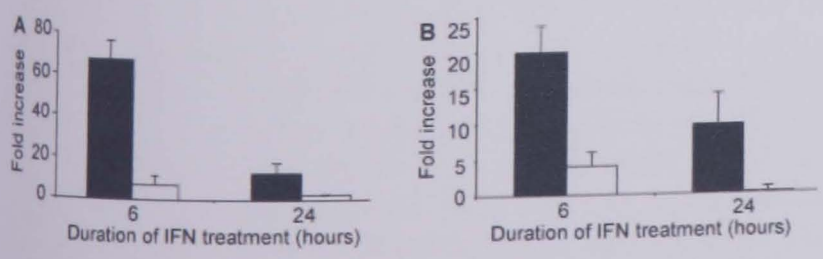

FIG. 4. Induction of classical ISGs by IFN-α is inhibited in dengue virus replicon-containing cells. K562 cells (black bars) and K562.ΔCprME-PAC2A cells (white bars) were stimulated with 100 IU of IFN-α2a for 6 and 24 h. MxA (A) and PKR (B) gene transcription was measured by real-time PCR and normalized to the housekeeping gene GAPDH.

TABLE 1. ISG transcription in response to IFN-α in cells that do and do not contain dengue virus replicons[a]

ISG product	Fold induction	
	K562 cells	K562 ΔCprME-PAC2A cells
IFN-α-induced protein 27	55.8	11.5
VCAM-1	30.6	2.6
MxA	17.9	1.8
IFN-α-induced protein (clone IFI-616)	12.9	1.1
Met proto-oncogene product (hepatocyte growth factor)	7.9	1.8
PSMB9	7.9	0.4
IFN-induced protein 17	7.5	1.3
Vipirin (Cig5)	7.2	1.3
Interleukin-15	6.5	1.1
9-27mrna	6.4	1.2
STAT1	6.2	1.1
STAT4	6.2	1.5
IFIT1	6.1	1.3
KIAA0284	5.6	1.5
STAT1 (91 kDa)	4.9	1.8
IFN-induced transmembrane protein 3	4.9	1.4
INDO	4.8	0.6
Interleukin-6	4.8	0.6
IFN-induced transmembrane protein 2	4.5	1.1
MAP2K4	4.5	1.0
IFI35	4.4	1.1
Homo sapiens STAT	4.1	1.6

[a] ISGs up-regulated more than fourfold in K562 cells compared to K562.ΔCprME-PAC2A cells are shown.

K562 cells that did and did not contain replicons. The steady-state levels of STAT1 were similar in each cell line (Fig. 5A). As expected, a treatment with IFN-α induced STAT1 phosphorylation in K562 cells. The levels of phosphorylated STAT1 (STAT1-P) were significantly lower in K562.ΔCprME-PAC2A

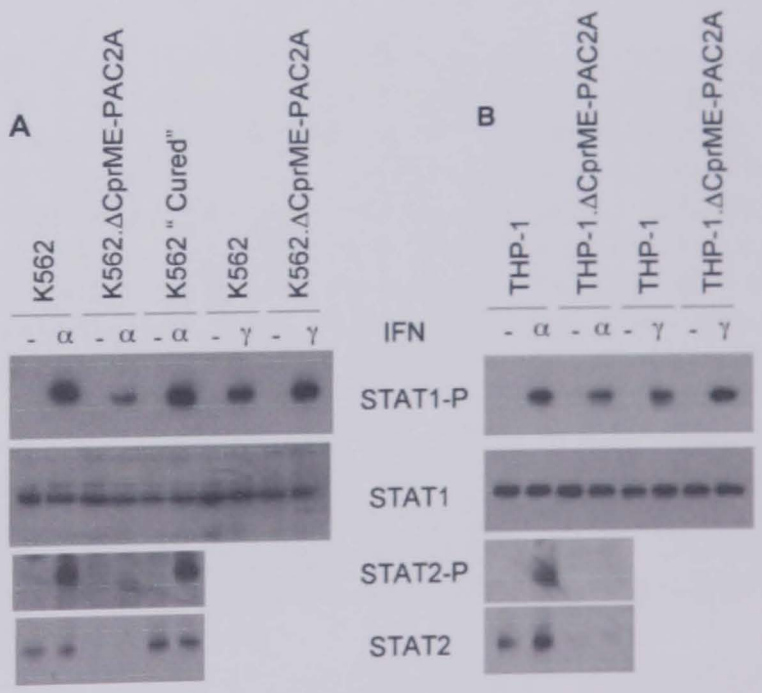

FIG. 5. Dengue virus RNA replication inhibits STAT1 and STAT2 phosphorylation in response to IFN-α and reduces steady-state levels of STAT2. (A) K562, K562.ΔCprME-PAC2A, and cured K562 cells; (B) THP-1 and THP-1.ΔCprME-PAC2A cells. Cells were left untreated or treated with 100 IU of IFN-α or IFN-γ/ml for 30 min and then lysed in SDS loading buffer. Proteins were separated by SDS-PAGE and then analyzed by immunoblotting with specific antibodies for STAT1, phosphorylated STAT1, STAT2, and phosphorylated STAT2, as indicated.

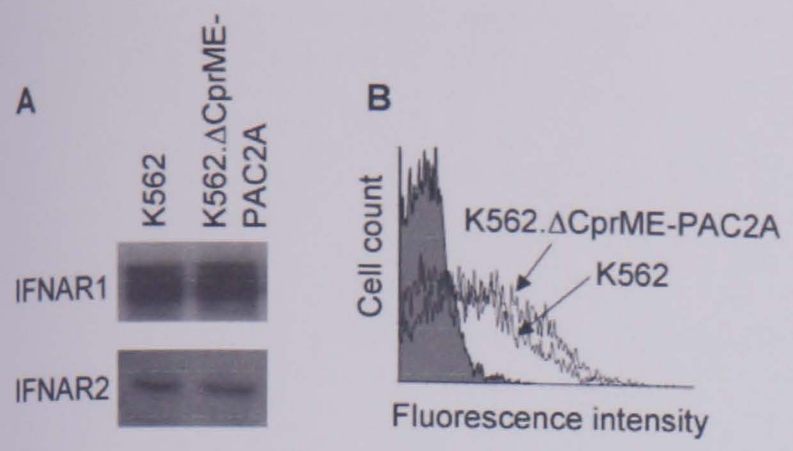

FIG. 6. Dengue virus RNA replication does not affect IFNAR protein levels. (A) Cell lysates from K562 and K562.ΔCprME-PAC2A cells were separated by SDS-PAGE and then analyzed by immunoblotting with specific antibodies for IFNAR1 and IFNAR2, as indicated. (B) K562 and K562.ΔCprME-PAC2A cells were stained with specific anti-IFNAR2 antibodies and then analyzed by flow cytometry. Cells stained with the secondary antibody alone (gray-filled plot) were included as a negative staining control.

cells in response to IFN-α, but the response was restored to normal in cured K562 cells. In contrast, STAT1-P levels were not reduced in K562.ΔCprME-PAC2A cells in response to IFN-γ, which signals through a distinct but overlapping pathway (9); in fact, we observed a consistent increase in STAT1 phosphorylation in response to IFN-γ in K562.ΔCprME-PAC2A cells compared with K562 cells (Fig. 5A). In order to ensure that these observations were not limited to the specific cell type used, we repeated these experiments with THP-1 cells that stably expressed ΔCprME-PAC2A, with similar results (Fig. 5B). These data imply that early components of the IFN-α but not the IFN-γ signal transduction pathway are targets for dengue virus inhibition.

We next performed a Western blot analysis of STAT2 phosphorylation, which is a key step in IFN-α but not IFN-γ signaling (9). Figure 5 shows that the steady-state levels of STAT2 were very markedly reduced in both K562 and THP-1 cells containing dengue virus replicons compared with the parental cells. The levels of STAT2-P in response to IFN-α were also greatly reduced in both replicon-containing cell lines. Cured K562 cells reverted to the phenotype of parental K562 cells, with similar steady-state levels of STAT2 and STAT2-P in response to IFN-α (Fig. 5A). In order to confirm that cells containing dengue virus replicons did not have a generally reduced expression of proteins involved in the first part of the IFN-α signal transduction pathway, we examined the levels of IFNAR1 and IFNAR2. Total IFNAR1 and IFNAR2 protein levels were assessed by Western blotting and were similar in K562 and K562.ΔCprME-PAC2A cells (Fig. 6A). The cell surface expression of IFNAR2 was measured by flow cytometry and was similar in K562 cells that did and did not contain replicons (Fig. 6B); reagents to examine the cell surface expression of IFNAR1 by FACS analysis were not available. Similar levels of IFNAR1 and IFNAR2 were also found in THP-1 cells that did and did not contain replicons (data not shown). Taken together, these data show that the presence of dengue virus replicons specifically inhibits early events in IFN-α but not IFN-γ signal transduction by reducing STAT2 levels.

Dengue virus infection reduces STAT2 levels. In order to determine whether STAT2 expression is also reduced in dengue virus-infected cells, we infected K562 cells with dengue virus type 2 and analyzed STAT2 expression in individual cells by dual-label immunofluorescence. We observed infected cells with markedly reduced staining for STAT2 compared with neighboring, uninfected cells (Fig. 7A and B). In order to assess STAT2 expression in the whole population of cells infected with dengue virus, we analyzed STAT2 levels at 48 h postinfection by immunoblotting. At this time point, approximately 30 to 50% of cells that had been infected stained strongly positive for NS1 protein by immunofluorescence. Figure 7C shows that STAT2 levels were markedly reduced in cells infected with dengue virus compared with mock-infected cells, whereas STAT1 levels were unchanged. Collectively, the data suggest that the down-regulation of STAT2 expression is a key component of dengue virus countermeasures against the human IFN response.

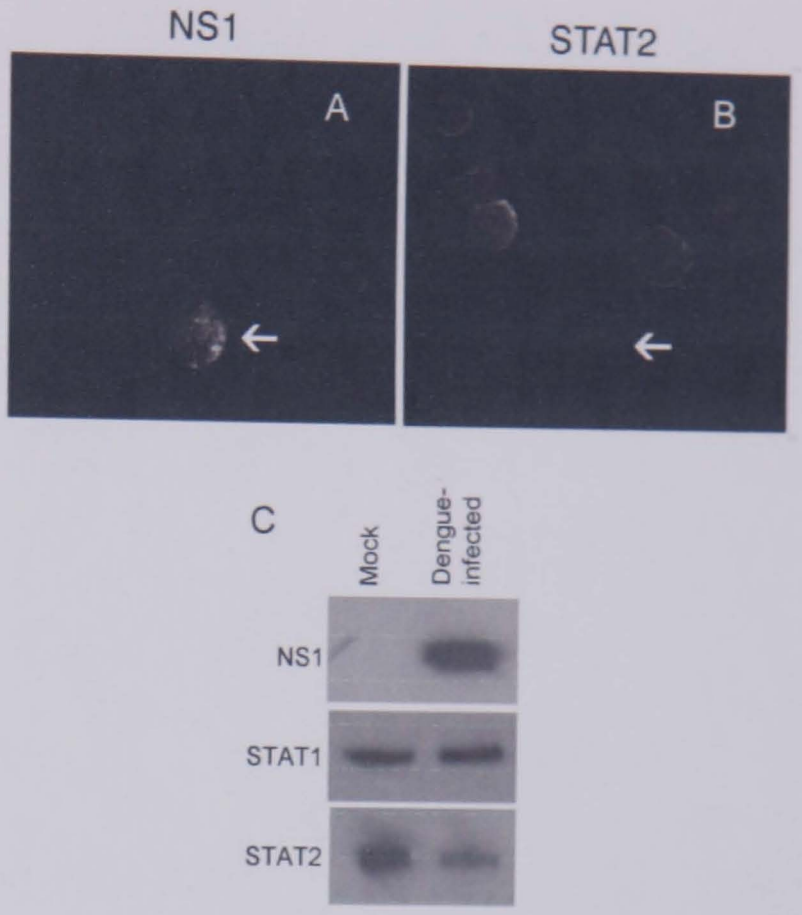

FIG. 7. Dengue virus infection reduces STAT2 levels. K562 cells were infected with dengue virus for 48 h and then dually stained with an anti-dengue virus NS1 mouse monoclonal antibody followed by a Texas Red-labeled secondary antibody to detect dengue virus-infected cells (A) and an anti-STAT2 rabbit polyclonal antibody followed by a fluorescein isothiocyanate-labeled secondary antibody to detect STAT2 (B). Cells were visualized by confocal microscopy. Arrows show infected cells. (C) Cell lysates were separated by SDS-PAGE and then analyzed by immunoblotting with specific antibodies for dengue virus NS1, STAT1, and STAT2, as indicated. Mock-infected cells were included for comparison.

DISCUSSION

For this study, we used human cell lines that stably express dengue virus replicons to show that dengue virus RNA replication inhibits early events in IFN signaling. This work extends a related study published by Muñoz-Jordan and colleagues during the progress of our research showing that dengue virus

proteins NS2A, NS4A, and NS4B have the capacity to inhibit the nuclear translocation of STAT1-P in response to IFN-β (25). We show here for the first time that dengue virus RNA replication not only reduces STAT1-P levels in response to IFN-α but also results in a marked reduction in STAT2-P levels as a consequence of reduced steady-state levels of STAT2. We first made this observation with dengue virus replicon-containing cells and subsequently with cells infected with dengue virus. Since STAT2-P recruits STAT1 for phosphorylation in response to IFN-α (22), generating STAT1/STAT2 heterodimers, all of our data can be explained if dengue virus specifically down-regulates STAT2 levels in order to counter the IFN response. In keeping with this hypothesis, dengue virus RNA replication did not reduce STAT1 phosphorylation in response to IFN-γ, which is independent of STAT2 (22). In fact, dengue virus replicon RNA replication increased rather than decreased STAT1 phosphorylation in response to IFN-γ, consistent with previous data showing increased STAT1 phosphorylation (30) and IFN-γ-mediated gene transcription (1) in the context of reduced STAT2 levels. The mechanism underlying this effect and its biological significance are as yet unknown.

We concluded that dengue virus specifically inhibits IFN-α/β signaling by down-regulating the expression of STAT2. Muñoz-Jordan and colleagues reported conflicting data showing an inhibition of both IFN-α/β- and IFN-γ-mediated STAT1 phosphorylation by dengue virus NS4B and dengue virus infection in LLCMK2 (monkey kidney) cells, and they suggested that common players involved in both signaling pathways (which do not include STAT2) were likely targets for IFN antagonism by dengue virus (25). The differences in our findings may reflect interspecies differences in IFN antagonism. Although the cell tropism of dengue virus in humans is not definitively known, the predominant targets are probably cells of hematopoietic origin, particularly dendritic cells, monocytes, and macrophages, as well as hepatocytes (2, 40, 41). For this study, we used human cell lines (K562 and THP-1) related to cells targeted by dengue virus in vivo, and our data provide the basis for further work to confirm the relevance of our observations to human dengue virus infection.

Despite the fact that IFN-α signaling was not completely blocked in dengue virus replicon-containing cells, supraphysiological concentrations of IFN-α2a had no significant effect on dengue virus replicon RNA replication or protein production. It is possible that dengue virus utilizes more than one mechanism to counter the IFN response, as suggested for other viruses, including another flavivirus, hepatitis C virus (8, 28, 37, 38). Alternatively, the inhibition of IFN signaling by reducing STAT2 levels may be sufficient for dengue virus replication to proceed faster than can be inhibited by the reduced antiviral IFN response. Future work will elucidate whether STAT2 levels are reduced as a consequence of a down-regulation of STAT2 gene transcription or protein synthesis or an enhanced degradation of the STAT2 protein. Further data are also needed to determine if the capacity to reduce STAT2 levels is conserved among all dengue virus strains (including field isolates) and in any other members of the *Flaviviridae* family. Recent data suggest that Japanese encephalitis virus also inhibits IFN-α signal transduction but utilizes a different mechanism that results in reduced levels of phosphorylated Tyk2 (and hence STAT2-P) without altering the expression of STAT2 (23). Two important human pathogens in the *Paramyxoviridae* family of enveloped, negative-strand RNA viruses, *Respiratory syncytial virus* and *Human parainfluenza virus* type 2, have been shown to subvert antiviral IFN responses by reducing STAT2 levels (26, 30). This effect is likely mediated through proteasome-mediated degradation of STAT2. However, other paramyxoviruses do not reduce steady-state STAT2 levels and instead have evolved a variety of different strategies to block IFN-α/β signaling (10, 19, 32, 43), which is a broadly effective strategy for countering the IFN response.

Future work is needed to define the precise interaction between components of the IFN signal transduction pathway and specific dengue virus proteins. Replicon-containing human cell lines are powerful tools for these studies because preliminary evidence suggests that several dengue virus nonstructural proteins may act together to produce strong, species-specific inhibition of IFN (25). We were careful to ensure that our observations did not reflect the selection of an IFN-defective subpopulation of cells during the generation of our stable replicon-containing cell lines. Several lines of evidence mitigate against this conclusion and suggest that our results accurately reflect an important host-pathogen interaction that subverts the IFN response: replicon-expressing cells were propagated from a total population of transfected cells rather than from individual cell clones; our observations were the same for two cell types, K562 and THP-1; and K562 cells that had previously expressed replicons and had been cured with glycyrrhizic acid reverted to the phenotype of parental K562 cells. Most importantly, the key observation made with our replicon model, namely, the reduction in STAT2 levels, led us to the same finding with cells infected with dengue virus. Collectively, the data suggest that the down-regulation of STAT2 expression is an important mechanism by which dengue virus subverts innate antiviral defenses mediated by IFN.

Understanding the molecular basis of the race between dengue virus replication and the IFN response early in infection would represent a critical advance, as the efficiency with which dengue virus evades the IFN response in humans is probably an important factor in early viral replication, and hence, disease pathogenesis. Further data will elucidate whether differences in IFN antagonism contribute to the differences in pathogenicity observed among dengue virus strains (31). Understanding the molecular mechanisms that dengue virus utilizes to subvert innate immune responses mediated by IFN may also inform strategies for rational attenuation in order to generate safer and more cost-effective dengue vaccine candidates.

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