CONTROL OF PROTEIN SYNTHESIS BY

THE

REOVIRUS S4 GENE

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SUMMARY

Mammalian reoviruses have been used as a model to study the control of host cell protein synthesis following infection. The reovirus genome is composed of 10 segments of double stranded RNA each of which, with one exception, encodes a single protein. The three serotypes of the virus show marked differences in their effects on host cell protein synthesis following viral infection. Serotypes 2 and 3 give pronounced inhibition and serotype 1 has little effect. Genetic studies using intertypic reassortants have mapped this property to the S4 gene of the virus which encodes the major outer capsid polypeptide $\sigma 3$. The S4 gene of serotypes 1 and 3 have been sequenced and show 96 % homology at the amino acid level.

Full length cDNA clones of the S4 gene of serotypes 1 and 3 (S4-1 and S4-3) were used to develop *in vitro* and *in vivo* assay systems to study the mechanism underlying the differential effects on host cell protein synthesis of the corresponding $\sigma 3$ proteins. The ability of $\sigma 3$ to inhibit host cell protein synthesis was investigated in two *in vitro* translation systems: the rabbit reticulocyte lysate system and S-10 cell extracts prepared from uninfected and type 1 and type 3 infected L-cells. $\sigma 3$ had no effect on either the translation of β-actin when used as a marker for eukaryotic protein synthesis, or endogenous protein synthesis in either of these systems.

In vivo assay systems made use of the HIVLTR/tat inducible reporter system to drive $\sigma 3$ expression. The effects of $\sigma 3$ on the expression of two reporter genes, chloramphenicol acetyltransferase (CAT) and β-galactosidase were investigated in transient *in vivo* assay systems. $\sigma 3$ expressed from S4-3 cDNA caused a significant stimulation of reporter gene expression. In contrast $\sigma 3$ from S4-1 cDNA had no affect on gene expression, suggesting a domain of S4-3 was responsible for this stimulation. In an attempt to domain map this property two hybrids were constructed, one containing the 5' 800 base pairs of S4-1 and the 3' 400 base pairs of S4-3 (HY-1), and the other, HY-3, the converse of this. $\sigma 3$ synthesised from HY-1 stimulated reporter gene expression whereas that from HY-3 had no effect. $\sigma 3$ was readily detected in cells transiently expressing either the parental type 3 S4 cDNA or the HY-1 hybrid by immunoprecipitation and immunofluorescence. By contrast only very small amounts of $\sigma 3$ were detected when either the S4 cDNA from type 1 or the HY-3 were used. Pulse-chase experiments indicated that the $\sigma 3$ from type 1 virus is less stable than the type 3 protein and that the domain responsible for this stability is in the 3' terminus of the S4 gene. This domain is also that shown by others to be responsible for the ability of $\sigma 3$ to bind dsRNA.

A model is proposed in which $\sigma 3$ is postulated as controlling host cell protein synthesis by inactivating the dsRNA kinase inhibitor (DAI), induced by foreign dsRNAs in the cell, which leads to inhibition of initiation of protein synthesis. In the infected cell $\sigma 3$ either exists free or is associated with protein $\mu 1c$ on the outer capsid shell. The model proposes that unassociated $\sigma 3$ from the type 3 virus is stable and can bind to dsRNA structures produced during viral infection, thereby inactivating DAI and allowing expression of late viral proteins at the expense of cellular proteins. By contrast unassociated $\sigma 3$ from type 1 appears to be unstable and therefore may not be able to promote late viral protein synthesis as efficiently as type 3 virus can. This model also accounts for the differing abilities of the two serotypes to inhibit host cell protein synthesis.

To my family,

Sam, June, Allen and John

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ABBREVIATIONS

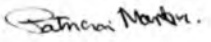
APS	Ammonium persulphate
BSA	Bovine serum albumin
c.p.e	cytopathic effect
CAT	Chloramphenicol acetyl transferase
cDNA	complementary DNA
CIP	Calf intestinal phosphatase
CMV	Cytomegalovirus
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytosine triphosphate
dGTP	deoxy guanosine triphosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ds	double strand
DTT	Dithiothreitol
dTTP	deoxy thymidine triphosphate
EDTA	Diaminoethanetetra-acetic acid, disodium salt
EGTA	Ethyleneglycol-bis (aminoethyl-ether) N'N'- Acid
FCS	Fetal calf serum
HEPES	N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid
HIV	Human immunodeficiency virus
IPTG	Isopropylthio-ß-D-galactosidase
LTR	Long terminal repeat
Mnase	Micrococcal nuclease
m.o.i.	multiplicity of infection

MOPS	3-[N morpholino] propanesulfonic acid
OD	Optical density
ONPG	O-Nitrophenyl-ß-D-galactopyranosidase
ori	origin of replication
PBS	Phosphate buffered saline
pfu	plaque forming unit
PPO	2,5 Diphenyl oxozole
rATP	Ribo-adenosine triphosphate
rCTP	Ribo-cytidine triphosphate
rGTP	Ribo-guanosine triphosphate
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolution per minute
RSV	Rous sarcoma virus
rUTP	Ribo-uridine triphosphate
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
^{35}S-met	^{35}S- methionine
ss	single strand
SV40	Simian virus 40
ß-gal	ß-galactosidase
TCA	Trichloroactic acid
TEMED	N,N,N',N'-tetramethyl ethylenediamine
	tetra-acteic acid
TK	Thymidine Kinase

DECLARATION

The work presented in this thesis was carried out by myself under the supervision of Dr M.A. McCrae. All sources of information have been acknowledged by means of reference.

None of the work contained in this thesis has been used in any previous application for a degree.



Patricia E.M. Martin

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CHAPTER 1
GENERAL INTRODUCTION

1.1 TYPES OF PATHOGENESIS

Viruses are often termed intracellular parasites as they utilise the cellular gene products for DNA and RNA replication, transcription, processing and transport of mRNA, protein synthesis and post-translational modification of polypeptides.

The events that occur when a virus infects an animal can be divided into two major stages:

A. Extracellular pathogenesis

B. Intracellular pathogenesis

A. Extracellular pathogenesis:

This can be used to refer to the steps that the virus takes from entering the host until it reaches and enters the target cell: that is, how the virus reaches and recognises a specific receptor on the surface of the target cell and then enters it.

B. Intracellular pathogenesis:

This can be used to refer to the steps that the virus takes in utilising the host cell systems for its own propagation and in so doing causing, in many cases, the eventual death of the target cell.

One important aspect of intracellular pathogenesis is the inhibition of host macromolecular synthesis, particularly protein synthesis, following viral infection. Before discussing how viruses exert their effects on the host cell translational apparatus, a basic understanding of eukaryotic protein synthesis is required.

1.2 EUKARYOTIC PROTEIN SYNTHESIS

Over the last twenty years there has been considerable work on the regulation of gene expression, both at the level of transcription and of translation. Studies concerning regulation at the translational level have largely been carried out in mammalian reticulocyte lysates. These are non-nucleated cells and hence translational control is the only method by which gene expression can be regulated. The information obtained from reticulocyte lysates forms the basis of our understanding of mammalian protein synthesis and will form the basis for this discussion.

1.2.1 Production of mRNA

mRNA is transcribed in the nucleus where it is also processed to ensure its stability. This processing involves the addition of a modified 5' cap structure and a 3' polyadenylated tail. Most eukaryotic transcripts are also spliced prior to export of the mature mRNA into the cytoplasm where it enters the translational process.

The presence of a 5' terminal 'cap' structure $m^7G(5')ppp(5')N$ is a common feature of eukaryotic mRNAs. The addition of the cap is an early transcriptional modification occurring at the 5' triphosphate ends of nascent nuclear pre-mRNAs. The reaction is catalysed by nuclear guanyl and methyltransferases, thus viruses that replicate exclusively in the cytoplasm must encode their own versions of these enzymes. The cap structure serves as a stabilising element on both pre-mRNAs in the nucleus (Green *et al.*, 1983), and mRNAs in the cytoplasm (Furuichi *et al.*, 1977).

In eukaryotic genes the protein coding sequence (consisting of a number of exons) is often interrupted by one or more intervening sequences (introns). The primary transcripts are therefore spliced to remove these introns and generate the

mature mRNA with an uninterrupted reading frame for translation (Rogers & Wall, 1980; Cech, 1983). The splicing mechanism plays a critical role in the regulation of gene expression (Krämer *et al.*, 1984). Many viral mRNAs also contain introns (Sharp, 1981).

mRNA is also modified at the 3' end by the addition of a long sequence (up to 200 nucleotides) of polyadenylic acid. This poly A tail is thought to serve to stabilise mRNA (Kozak, 1983; Zeevi, 1982;). However this cannot be its exclusive role since other mRNAs lacking poly A are stable. For example, reovirus and histone mRNAs are non polyadenylated (McCrae & Woodland, 1981; Adesnick & Darnell, 1972). Recently Munroe and Jacobson (1990) have reported that the poly A tail may have a role in enhancing initiation of translation.

1.2.2 Protein Synthesis

Mature mRNA enters the cytoplasm and the translation pathways. The translational process can be divided into three stages: initiation, elongation and termination (recently reviewed by Nygård 1990).

1.2.2.1 Initiation

Initiation is one of the most highly regulated steps in translation and it too can be divided into 3 stages:

a). formation of the 43S pre-initiation complex

b). formation of the 48S initiation complex

c). formation of the 80S initiation complex.

Each of these stages involves a number of initiation factors (eIF's), which are listed in table 1.

Table 1: Eukaryotic initiation factors

FACTOR	PUTATIVE FUNCTION
eIF2	Binding met-tRNA to the 40S subunit
[1]GEF	Nucleotide exchange factor
eIF3	Dissociation of 80S ribosomes
eIF4A	ATP dependent unwinding of mRNA
eIF4B	binding of mRNA to 40S subunit
eIF4C	dissociation of 80S ribosomes
eIF4E	cap recognition
eIF4F	cap recognition and mRNA binding
eIF5	GTP dependent subunit joining
eIF6	dissociation of 80S ribosomes

[1]GEF = Guanine nucleotide exchange factor.

The first step in initiation is the dissociation of the 80S ribosome into its two subunits - 40S and 60S. The interaction of the initiation factors eIF-3 and eIF-4C with the 40 S subunit (Lutsch *et al.*, 1986) and eIF-6 with the 60 S subunit (Raychaudhuri *et al.*, 1984) shift the equilibrium of the reaction towards dissociation of the subunits (figure 1).

The initiator tRNA, Met-tRNA$_f$, forms a ternary complex with GTP and initiation factor eIF2. This binds to the 40S ribosome subunit, which is complexed with two initiation factors, eIF-3 and eIF-4C, to form a 43S pre-initiation complex (Proud, 1986) (step A figure 1). The second step of initiation involves the binding of mRNA to the 43S complex to form the 48S initiation complex (step B figure 1). In eukaryotes this process involves several initiation factors, eIF-4B and eIF4F, and the hydrolysis of ATP. Initiation factor eIF-4F is a multi-subunit complex consisting of two initiation factors, eIF-4A and eIF4E, and a 220kDa protein (Rhoads, 1988). eIF-4E is a protein of about 25 kDa (Rychlik *et al.*, 1987), often referred to as CBP1 (cap binding protein 1), and in the eIF-4F complex it recognises the 5' cap structure on the mRNA (Shatkin, 1985). eIF-4A and eIF-4B appear to unfold the mRNA secondary structure, in an ATP dependent process, providing a suitable site for the association of the 43S pre-initiation complex (Tahara *et al.*, 1983). eIF-4B also has an affinity for the codon AUG and may be responsible for identifying the initiation signal. It was recently proposed that the 43S pre-initiation complex associates with the cap structure on the mRNA-factor complex and scans the mRNA from the 5' end to locate the correct initiation codon (Kozak, 1989). The polypeptide p220 is required for many of the factor interactions.

Once the initiator signal is identified the programmed 40S particle is ready to associate with the 60S subunit to form the 80S ribosome (step C figure 1). This requires initiation factor eIF-5 and is accompanied by the hydrolysis of GTP. During the subunit joining the factors associated with the pre-initiation

FIGURE 1: INITIATION OF EUKARYOTIC PROTEIN SYNTHESIS

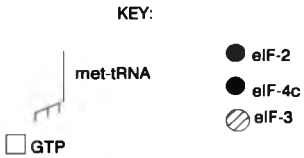
This figure illustrates the major steps in the initiation of protein synthesis in eukaryotes.

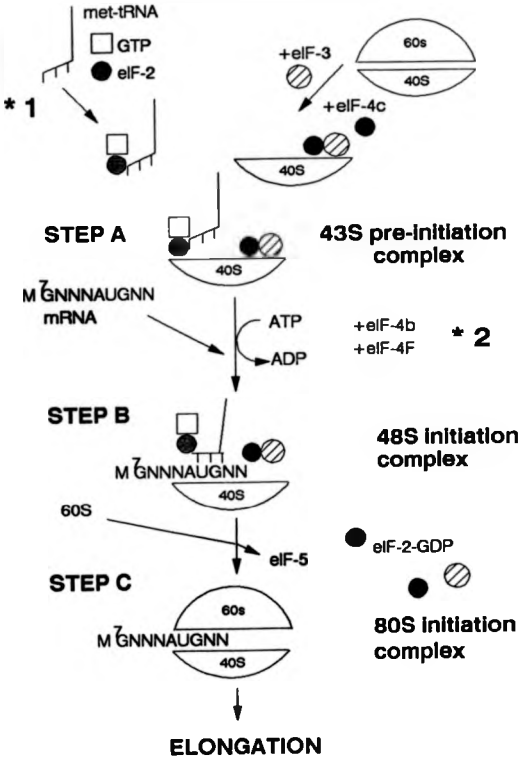
A: met-tRNA binds to GTP and eIF-2 which associate with eIF-3 and eIF-4c to form the 43S pre-initiation complex.

B: mRNA binds to the 43S complex, in an ATP driven process involving initiation factors eIF-4F and eIF-4b. The mRNA scans the area for the initiation complex and moves to this site forming the 48S initiation complex.

C: Formation of the 80S initiation complex: eIF-5 allows the addition of the 60S ribosomal subunit to the complex, resulting in the release of all initiation factors.

*1 and *2 indicate major regulatory areas for control of protein synthesis.




met-tRNA
GTP
eIF-2
* 1
+eIF-3
60s
40S
+eIF-4c
40S
STEP A
43S pre-initiation complex
40S
M 7GNNNAUGNN
mRNA
ATP
ADP
+eIF-4b
+eIF-4F
* 2
STEP B
48S initiation complex
M 7GNNNAUGNN
40S
60S
eIF-5
eIF-2-GDP
STEP C
60s
M 7GNNNAUGNN
40S
80S initiation complex
ELONGATION

complex are released from the ribosome and the 80S initiation complex is ready to enter the elongation cycle.

1.2.2.2 Elongation

The ribosome has two major sites for positioning of mRNA, the P or donor site and the A or acceptor site. At the end of the initiation process met-$tRNA_f$ is bound to the P site and its anticodon is base-paired at that site with the initiator codon AUG in the mRNA. Like initiation, the elongation process is also mediated by soluble cellular factors, which have been termed elongation factors EF1 and EF2. Elongation is a cyclic process which can be divided into 3 basic reaction steps:

1. The cognate aminoacyl-tRNAs are brought to the A site in a ternary complex with EF1 and GTP, in a process which involves the hydrolysis of GTP.

2. When the two ribosomal binding sites are filled a peptide bond is formed between the methionine residue and the incoming aminoacyl moiety. This reaction is catalysed by peptidyltransferase and results in the newly formed dipeptide occupying the A site, leaving the P site with a deacylated tRNA.

3. The A site is the decoding site for chain elongation and movement of the next codon involves elongation factor EF2 promoting the translocation of the elongated peptidyl t-RNA from the A site to the P site with the concomitant hydrolysis of GTP (Moldave, 1985).

1.2.2.3 Termination

Elongation continues until a termination codon is reached (UAA, UAG, UGA) and occupies the A site (Beaudet & Caskey, 1971). When this occurs a protein factor (release factor RF) binds to the A site in the presence of GTP and catalyses the termination reaction. This reaction involves the hydrolysis of the peptidyl tRNA ester bond, the hydrolysis of GTP and the release of the

completed polypeptide chain, the deacylated tRNA and the ribosome from mRNA. It also appears to be catalysed by peptidyltransferase.

1.2.3 Regulation of translation: Recycling of eIF-2b

At the end of initiation all the initiation factors are released from the initiation complex. The initiation factors enter the cellular pool and are ready for the next round of initiation. eIF-2 has a very strong affinity for, and forms a complex with, the GDP generated by the GTP → GDP + Pi hydrolysis which is part of the initiation process. The cycle can only be completed once the GDP has been exchanged for GTP. This is a major regulatory point in eukaryotic protein synthesis.

The factor eIF-2B is responsible for the exchange of bound GDP for free GTP thereby regulating the reentry of eIF-2 into the active pool and allowing the initiation cycle to recommence (Safer, 1983). eIF2 is a trimer of 3 distinct subunits designated alpha, (38K), beta (35K) and gamma (55K). The alpha and beta subunits take part in guanine nucleotide binding and/or exchange mechanism and eIF-2 gamma binds met-$tRNA_f$. This nucleotide binding regulates the rate and extent of met-$tRNA_f$ binding. Hydrolysis of GTP is required for the release of eIF-2 and the other initiation factors from the 48S initiation complex upon 60S ribosomal joining.

The ability of the cell to recycle eIF-2 may be blocked as a means of protecting the cell against viral infection or deprivation of certain vitamins or minerals. This occurs by phosphorylation / dephosphorylation of eIF-2. The phosphorylation state of eIF-2α is elevated under a variety of physiological conditions such as interferon (IFN) treatment (Samuel, 1979a; 1979b; Pestka *et al.*, 1987) and haemin deficiency (Farrell, 1977; Ochoa, 1983). In such cases activity of eIF-2 is inhibited by phosphorylation of the α subunit. For example, low concentrations of dsRNA activate a factor in reticulocytes in the presence of

haeme, termed the dsRNA activated inhibitor (DAI). This factor has also been found in nucleated cells as an inactive precursor which is induced by treatment with interferon and the presence of dsRNA (Ochoa, 1983). Once induced, DAI phosphorylates the α subunit of the initiation factor eIF-2 thereby blocking initiation (Ochoa, 1983).

1.2.4 Interferon

Interferons (IFN) are a family of regulatory proteins affecting a number of biological processes in animal cells, including their ability to support infection with a range of RNA and DNA viruses, (Petska *et al.*, 1987). 3 classes of IFNs, alpha, beta and gamma have been identified with α and ß IFNs being of greatest importance in virus infection.

Two interferon induced dsRNA dependent enzymes have been identified and play an important role in the regulation of viral and cellular macromolecular synthesis and mRNA degradation (Pestka *et al.*, 1987):

1. Protein kinase
2. 2'-5' oligoadenylate synthetase.

1. P1/eIF-2α Protein kinase-Phosphoprotein phosphatase protein (DAI)

As described above the phosphorylation state of eIF-2α is a key control point of the initiation of translation. The phosphorylation state of two proteins, P1 and eIF-2α, which is modulated by a protein kinase and a phosphoprotein phosphatase, is very sensitive to IFN. Increases in the level of phosphorylation of these proteins in IFN-treated cell systems is dependent on virus infection (Rice *et al.*, 1985a; Samuel *et al.*, 1984) or the addition of dsRNA (Gupta, 1979).

The protein kinase (DAI) is activated by viral gene expression in the case of some viruses such as reovirus (Samuel *et al.*, 1984), vesicular stomatitis virus (VSV) (Sahni & Samuel, 1986) and encephalomyocarditis virus (EMCV) (Rice *et*

al., 1985a), thus providing a cellular defence against viral infection. However other viruses such as adenovirus (Anderson & Fennie, 1987) and vaccinia virus (Whitaker-Dowling & Younger, 1984) have adapted ways to overcome this host cell defence system, which will be discussed in section 1.3.

2. The (2'-5') oligoadenylate synthetase-nuclease system.

Another enzyme system induced in response to IFN and dsRNA is the (2'-5') oligoadenylate synthetase-nuclease system. This is also a latent enzyme system that is induced by IFN and dsRNA. There are 3 enzymes that play an important role in this system:

a. 2,5-$(A)_n$ synthetase

b. endoribonuclease

c. phophodiesterase.

The 2'5' oligoadenylate synthetase is induced by interferon and dsRNA hence polymerising ATP into a series of 2',5'-linked oligomers. These oligomers, termed 2-5A, bind and activate a latent cellular endoribonuclease which cleaves mRNA and rRNA. A 2'5' phosphodiesterase then degrades the 2-5A. This system is activated in EMCV- infected HeLa cells (Rice *et al.*, 1985a) and novel 2'5' oligoadenylates have also been found in IFN-treated vaccinia-virus infected cells (Rice *et al.*, 1985b).

1.2.5 Summary

Protein synthesis is a highly regulated series of events. When a virus enters the cell it utilises the cell's facilities for translating its own proteins. Viruses have adapted various ways of promoting their own protein synthesis over that of the host, in many cases resulting in the inhibition of host cell protein

synthesis and the eventual death of the cell. These mechanisms will be discussed in the following sections.

1.3 VIRUS INDUCED CONTROL OF HOST CELL PROTEIN SYNTHESIS.

The efficient replication of a virus depends on its ability to harness the host cell macromolecular machinery at a variety of levels. Many viruses have evolved mechanisms which enable them to dominate various components of the host cell machinery: for example, the host cell translational apparatus. A wide variety of such mechanisms have been described including (recently reviewed by Schneider & Shenk, 1987):

1. Degradation of host cell mRNA e.g Herpes simplex virus (HSV).
2. Inactivation of translation factors e.g Poliovirus.
3. Production of factors specifically inhibiting cellular translation e.g Adenovirus.
4. Viral mRNAs that out-compete cellular mRNAs e.g VSV.

1.3.1 Inhibition of host cell protein synthesis by poliovirus

Poliovirus is a member of the picornaviradae, a group of viruses whose genome is composed of positive sense single stranded RNA. The viral genomic RNA is translated in one open reading frame into a polyprotein precursor that on subsequent proteolysis releases the final viral proteins (Rueckert & Wimmer, 1984). The initial proteolysis of the polyprotein yields 3 precursors P1, P2 and P3 in a cleavage event mediated by two virus-encoded proteinases, polypeptides 3C and 2A. The primary proteolytic products then split into smaller protein products.

An important feature of picornaviral mRNA is that it lacks the blocked methylated cap structure found in most mRNAs (Nomoto *et al.*, 1976), and which under normal conditions is required for efficient translation (discussed in section 1). In place of the 5' cap structure picornaviruses have a pUp sequence (Hewlett, 1976).

Penham (1963) first reported that infection of cells with poliovirus causes a rapid and marked inhibition of cellular protein synthesis. It is also able to interfere with the translation of a variety of other viral mRNAs in co-infection experiments, for example, VSV (Ehrenfeld & Lund, 1977) and adenovirus (Bablanian & Russell, 1974), which in contrast to poliovirus have mRNA containing a 5' methylated cap structure similar to that of host cell mRNA. This observation led to the proposal that the difference between the 5' termini of polioviral and cellular mRNA could be a basis for the inhibition of host protein synthesis. This idea was further supported by Fernandez-Munz and Darnell (1976), who concluded that host mRNA was not destroyed and remained functional after poliovirus infection. The central postulate of this hypothesis was that a mechanism existed in infected cells to translate viral uncapped mRNA in preference to host capped mRNA.

To investigate this hypothesis further Rose *et al.* (1978) used VSV mRNA in extracts derived from poliovirus infected and uninfected HeLa cells to study the mechanism of inhibition. They found that addition of eIF-4B (purified from rabbit reticulocytes) to the assays overcame the poliovirus-associated inhibition of VSV translation mentioned above. This suggested that eIF4B, which is required for the translation of capped mRNA, may be lost or inactivated by poliovirus. When this activity of eIF-4B was first observed it was termed the 'restoring factor'. Trachsel *et al.*(1980) purified this 'factor' from poliovirus infected cells and found that the activity co-purified with a 24 kDa polypeptide that was identical to the cap binding protein (CBP1) originally described by Sonenberg *et*

al. (1978). This observation led to a new hypothesis in which poliovirus infection resulted in the inactivation of the cap-dependent recognition of mRNA that occurs during initiation. When the 24 kDa protein was purified it was nearly always found in association with eIF-3 (Lee & Sonenberg, 1982). To further investigate the alterations of eIF-3 and associated proteins (such as CBP1) following virus infection in HeLa cells, polyclonal antibodies against rabbit reticulocyte eIF-3 were used. A polypeptide of 220 kDa (p220) was found to disappear at 2.5 hours post infection (p220 is one of the components of the cap-binding complex eIF-4F (section 1)). Its disappearance was associated with the appearance of several smaller proteins (Etchison *et al.*, 1982). The cleavage of this protein correlated with viral infection: the loss of p220 occurred at the same time as cellular protein synthesis began to decrease. The findings of Etchison and collaborators suggested that a viral product must mediate the cleavage of p220. Lee *et al.* (1985) showed that this product was not the well-defined viral protease protein 3C.

In an attempt to elucidate the nature of the viral product cleaving p220, Bernstein *et al.* (1985) generated a range of single codon insertion mutants using a full length cDNA clone of the virus (Racaniello & Baltimore, 1981). One of these mutants contained an extra amino acid in the P2 segment of the virus coding for protein 2A. When this was transfected into a variety of cell lines there was no rapid inhibition of host cell protein synthesis associated with wildtype virus infection, nor were significant levels of p220 cleavage proteins evident in the mutant infected cells, thus implying that protein 2A may have a role in the cleavage of p220. Using an *in vitro* translation assay system to study p220 cleavage by protein 2A, Krausslich *et al.* (1987) found that synthetic mRNA transcripts encoding the 2A region of the viral genome completely cleaved p220. To investigate this cleavage *in vivo* Sun and Baltimore (1989) determined that protein 2A, produced from a cDNA clone, in HeLa cells inhibits

the translation of a wide range of reporter genes and it cleaved p220 into the products that were also found in poliovirus infected HeLa cells. The results of Kräusslich *et al.* (1987) and Sun and Baltimore (1989) led to a model proposing that protein 2A activates a cellular protease which cleaves p220. Wyckoff *et al.* (1990) studied the ability of protein 2A expressed in *E.Coli* to induce p220 cleavage and found that cleavage was incomplete, suggesting a cellular factor was also involved. HeLa cell extracts were fractionated by various methods and the capacity of each fraction to cleave p220 in the presence of protein 2A produced from *E.Coli* was analysed. eIF-3 was found to be required for protein 2A to completely cleave p220 (Wyckoff *et al.* 1990).

Thus poliovirus inhibits host protein synthesis by production of a protease, protein 2A, that cleaves protein p220, a component of the eIF-4F initiation complex, in the presence of initiation factor eIF-3. (Recently reviewed by Sonenberg, 1987).

Other viruses controlling host shut-off by cleavage of the cellular protein p220 include foot and mouth disease virus (Devaney *et al.*, 1988) and human rhinovirus 14. (Etchison & Fout, 1985).

1.3.2 Control of host cell macromolecular synthesis by VSV

VSV is a member of the rhabdoviridae, a group of viruses which contain negative sense single stranded RNA as their genome. The virion contains the enzymes necessary to translate the negative stranded RNA to positive stranded (messenger) RNA. The mRNA of this virus is synthesised by a virion associated transcriptase and mimics host cell RNA in having a 5' cap structure and 3' polyadenylated tail. The viral genome has a leader sequence of 45 nucleotides and is transcribed into 5 monocistronic mRNAs, each encoding a specific protein, N, NS, M, G and L respectively. The genome is transcribed: 3'-Leader-N-NS-M-G-L-5' (Ball & White, 1976).

Infection of a wide variety of animal cells with VSV results in inhibition of cellular DNA, RNA and protein synthesis and finally cell death. This discussion will focus on the control of cellular protein synthesis by the virus. VSV infection of mouse L-cells results in a visible reduction in the synthesis of cellular proteins within 5 hours of infection (M^{C}Allister & Wagner, 1976). This inhibition process requires transcription but not replication of the VSV genome as the virion itself is unable to shut off host transcription (Wek and Wagner 1979).

Initial studies on the inhibition mechanisms in this case suggested that it occurred at the level of initiation. Thus Nuss *et al.* (1975) proposed that VSV mRNA initiated protein synthesis with greater efficiency than cellular mRNA, in so doing out-competing the host mRNA for the available ribosomes. Using cell free translation systems Lodish and Porter (1980; 1981) provided experimental support for this proposal. However several other groups have reported that a viral specified function is required for host shut-off. Stanners *et al.* (1977) termed this virus-specified product the P function and isolated and partially characterised a P^{-} mutant having a lower capacity to inhibit host protein synthesis than the wild type virus. Using temperature sensitive (t.s.) mutants Marvalidi *et al.* (1977) proposed that the N and NS gene products may be involved in this shut-off. Successive viral mRNA can be inhibited with increasing doses of U.V irradiation, the furthest from the 3' end of the viral genome being inactivated first. By comparing the relative doses of U.V irradiation required to cause inhibition of host protein synthesis, Marvalidi *et al.*(1978) confirmed that the viral N gene and possibly the NS gene (i.e. the two smallest gene products) were required for the inhibition of cellular protein synthesis in mouse L-cells. Support that only the 3' end of the viral genome was required was provided by Schnitzlein *et al.* (1983) who reported that 5' Defective Interfering (DI) virus

particles had little effect on the capacity of the wild type virus to inhibit cellular protein synthesis.

In an attempt to dissect the molecular mechanisms of inhibition of protein synthesis by VSV Dunigan *et al.* (1983) used cell-free extracts from U.V irradiated virus. They found that the U.V inactivation curve consisted of two phases, suggesting that two viral transcription products were involved in inhibition of cellular protein synthesis. One of these products was 373 nucleotides and the other was 42 nucleotides. U.V inactivation of the larger transcription product resulted in a 40% decrease in the capacity of the virus to shut off host protein synthesis. The smaller product was the same size as the leader RNA which had previously been shown to inhibit cellular RNA synthesis (M^{c}Gowan & Wanger, 1981). To further study the role of the leader RNA in host shut-off, Dunigan *et al.* (1986) examined the relationship between the extent of shut-off and accumulation of leader mRNA in cells infected by wild type VSV and different mutants. These mutants were defective in either their ability to shut-off RNA and protein synthesis or in replication. From these studies Dunigan et al. (1986) confirmed that two VSV transcription products of the VSV genome were required to cause inhibition.

In summary, two VSV transcription products, possibly the leader RNA and the N protein, are required to inhibit host protein synthesis. This inhibition occurs at the level of initiation. However, to date there is no conclusive evidence to indicate at which stage this inhibition occurs (reviewed by Wagner, 1987).

1.3.3 Control of protein synthesis by adenovirus

Adenoviruses are a group of double stranded DNA containing viruses. Adenovirus mRNA has all the major characteristics of host mRNA and they have

been widely used as models for studying the splicing process in eukaryotes (Grabowski *et al.* 1984).

Host cell protein synthesis in adenovirus infected cells is depressed at late times in infection. Babich *et al.*(1983) showed that host mRNA from infected cells could still be translated in *in vitro* translating systems, suggesting that inhibition of protein synthesis may occur at the level of initiation of protein synthesis of pre-existing cellular mRNA.

Adenovirus infection results in the production of a range of small virion-associated RNAs of about 160 nucleotides in length (VA RNAI (VAI) and VA RNAII). VAI is synthesised in large amounts at late times in infection when it is the dominant RNA species in the cytoplasm. This occurs at a time comparable to the commencement of host shut-off. To investigate the functions of VAI, Thimmappaya *et al.*(1982) generated a range of mutations in the VAI region of the adenovirus 5 genome. One of these mutants, *d1331*, was defective in VAI. This mutant has been widely investigated and forms the current understanding of the control of protein synthesis by adenovirus. *d1331* does not grow well compared to the wild type virus, suggesting that VAI is required for efficient translation of viral mRNA at late times post infection. Thimmappaya *et al.* (1982) suggested that VAI could have a role in the initiation of translation of viral mRNAs by counteracting a cellular antiviral response at late times after infection.

Reichel *et al.*(1985) showed that translational activity could be restored to cell free extracts derived from *d1331* infected cells by the addition of purified eIF-2 and eIF-2B from reticulocytes lysates. This finding led to the proposal that the virus may cause inhibition by interfering with initiation at the level of eIF-2 recycling (discussed in section 1.2.3). The Pi/eIF-2α kinase (DAI) was found to be activated in *d1331* infected but not in wild type infected cells (Schneider *et al.*, 1985; Siekierka *et al.*, 1985). This led to the proposal that VAI directly

inhibits the expression of DAI. VAI has extensive double stranded character (Akusjärvi *et al.*, 1980) and it is conceivable that the action of VAI is such that it increases ds RNA concentrations in infected cells to levels that prevent DAI activation. In *d1331* infected cells such levels of RNA would not be present, therefore DAI would be activated by other ds RNAs produced during viral infection thus preventing the continuation of protein synthesis. Subsequently to this hypothesis Kitajewski *et al.* (1986) showed that this could not be the case as VAI could not activate the kinase and therefore did not act as a simple dsRNA. O'Malley *et al.* (1986) confirmed that the protein kinase responsible for phosphorylation of eIF-2α in *d1331* infected cells was DAI. From the results of their experiments they proposed that *in vivo* VAI counteracts the activation of DAI by dsRNA which is generated by late viral transcription. This proposal was further supported by Maran and Mathews (1988) who demonstrated that the dsRNA responsible for DAI activation in *d1331* infected cells was derived from transcription off both strands of the viral genome.

Katze *et al.* (1987) used monoclonal antibodies (Laurent *et al.*, 1985) to gain a greater insight into the role of DAI and VAI in the regulation of protein synthesis in adenovirus infected cells. Levels of DAI were found to be similar in *d1331* and wild-type infected cells and these in turn were the same as those found in uninfected cells. However during infection with the *d1331* the protein kinase became highly autophosphorylated and hence, highly inactivated. Katze *et al.* (1987) showed that VAI (absent in *d1331* infected cells) prevented this autophosphorylation *in vitro* by directly complexing with the eIF-2α protein kinase, DAI, thereby inactivating it and allowing the translation of late viral proteins. This hypothesis was further supported by Galabru *et al.* (1989) and by O'Malley *et al.* (1989) who demonstrated that adenovirus 2 did not inhibit host protein synthesis in cell lines deficient in DAI kinase activity.

In summary, adenovirus appears to control host cell protein synthesis by the synthesis of VAI, a small virion associated RNA, which complexes with DAI, an antiviral cellular response system, thereby inactivating the kinase and allowing the translation of late viral mRNA at the expense of cellular mRNA.

Other viruses that have evolved mechanisms of combating cellular antiviral defence systems by phosphorylation of eIF-2α include Epstein Barr virus (Clarke *et al.*, 1990), mengovirus (De Stefano, 1990), HIV (Edery *et al.* (1989); Gunnery *et al.*, 1990) and influenza virus (Katze *et al.*, 1986).

1.3.4 Control of host cell protein synthesis by influenza virus

Influenza virus is an enveloped RNA virus. The genome consists of eight discrete segments of negative single-stranded RNA. Each segment encodes a single protein except for the two smallest RNA segments, each of which encode two individual proteins. In contrast to other RNA viruses transcription of the virion RNA occurs in the nucleus and utilises capped RNA primers 10 -13 nucleotides long, which are cleaved from newly synthesised cellular transcripts to initiate viral RNA transcription (Herz *et al.*, 1981).

Influenza virus effectively inhibits host cell protein synthesis during infection (Lazarowitz, 1971; Skehel, 1972). This event most likely involves a mechanism blocking the translation of host mRNAs before and after initiation. Initial reports by Inglis (1982) suggested that inhibition was caused by virus-induced degradation of host cell mRNAs in the cytoplasm. By co-infecting cells with adeno and influenza virus, Katze and Krug (1984) determined that influenza viral mRNAs could overcome the blocks adenovirus exerted against the host cell mRNA. The viral mRNAs were also efficiently exported from the nucleus and translated. Katze and Krug (1984) concluded that influenza virus established its

own mechanism for transport and translation of viral mRNA by utilising influenza virus-specific nuclear transport and translation systems.

Katze and Krug (1984) demonstrated a reduction in the appearance of newly processed mRNA in the cytoplasm 1-2 hours post infection. This event was thought to be a consequence of cleavage of the 5' cap of the newly formed cellular transcripts by the influenza viral cap dependent endonuclease. However there was no significant degradation of cytoplasmic mRNA. The cellular mRNA present in the cell, when inhibition of host protein synthesis was observed, was functional in *in vitro* translation systems, further indicating a system for selective translation of viral mRNA rather that cellular mRNA degradation as the mechanism responsible for host shut-off.

When cells infected with the adenovirus mutant *d1331* (discussed in section 1.2.3) were superinfected with influenza, only influenza viral proteins were synthesised (Katze & Krug, 1984). Katze *et al.* (1986) showed that this selective translation resulted from a partial suppression by influenza virus of the DAI kinase activity, which is expressed after *d1331* infection. Thus the influenza virus gene product acts against dsRNA activation of the eIF-2α kinase. Katze *et al.* (1986) proposed that a viral gene product suppressed the dsRNA-dependent eIF-2α kinase (DAI) which was induced by IFN in response to viral infection. Further to these studies Lee *et al.* (1990) purified and partially characterised a DAI repressor factor from influenza infected cells. This inhibitor was found to be a cellular not a viral protein. Lee *et al.* (1990) propose that the repressor of DAI acts in a similar way to adenovirus VAI, by directly interacting with the kinase, thereby irreversibly inactivating it.

1.3.5 Inhibition of host protein synthesis by HSV

Another mechanism for controlling host protein synthesis is illustrated by HSV. When HSV infects a variety of tissue culture cells there is a marked

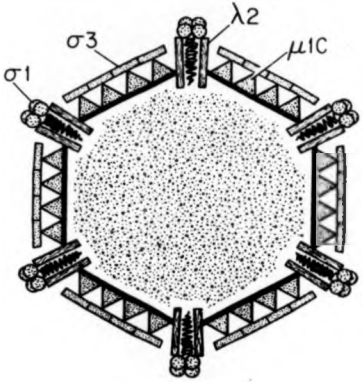
suppression of host protein synthesis (Roizman *et al.*, 1965). Nishioka and Silverstein (1977) determined that the mechanism of this suppression involved degradation of the cellular mRNA present in the cell prior to infection. Shut-off of host cell protein synthesis by HSV is mediated by a viral gene product termed the virion host shut-off factor (vhs) (Read & Frenkel, 1983). This product plays a role in the degradation of mRNA possibly by activating a pre-existing cellular RNAase that degrades cellular and early viral mRNAs thus allowing translation of the late viral mRNA (Strom & Frenkel, 1987). The host shut-off function may be part of a mechanism that the virus has evolved to regulate the expression of its own genes (Kwong & Frenkel, 1987; 1988; 1989).

Vaccinia virus also controls host shut-off by mRNA degradation (Rice & Roberts, 1983). However the mechanism of vaccinia virus host shut-off is thought to involve the interferon 2-5A system discussed in section 1.2.4 (Pacha & Condit, 1985; Whitaker-Dowling & Younger, 1984).

1.3.6 Summary

The preceding sections have outlined a variety of the mechanisms viruses have evolved to ensure their survival in the host cell. Some of these mechanisms are well understood, such as that of poliovirus. However, there is still a degree of controversy concerning the mechanisms by which other viruses such as influenza and VSV take control of the host cell translational machinery. Reovirus has also adapted ways to control host protein synthesis and the proposed mechanisms are discussed in section 1.5.

FIGURE 2: STRUCTURE OF THE REOVIRUS PARTICLE



(Illustrated by P. Battaglino, from Schiff & Fields, 1989)

1.4 REOVIRUSES

1.4.1 Structure and physicochemical properties of reovirus

Mammalian reoviruses are the type members of the orthoreovirus genus of the virus family reoviridae. The virus particle is approximately 75 nm in diameter. Unlike many other viruses they do not have a lipid envelope, but do possess a double capsid shell (figure 2). Each shell consists of subunits or capsomers arranged in icosohedral symmetry (Rhim *et al.*, 1961). The outer shell is composed of three proteins, $\sigma 3$, $\mu 1c$ and $\sigma 1$. It is readily removed by digestion with proteases such as chymotrypsin and trypsin resulting in single shelled viral cores (Joklik, 1972). The core has the unique structural feature of 12 prominent icosohedrally located projections or spikes which are thought to be pentameric aggregates of the lambda 2 protein (Ralph *et al.*, 1980). These spikes are hollow, providing a means of access to and exit from the interior of the particle (Luftig *et al.*, 1972). The virus core also contains the enzymes necessary to transcribe the 10 viral genes into capped mRNA. These enzymes include:

1. ds → ss RNA transcriptase (Shatkin & Sipe, 1968)
2. 5' Terminal guanyltransferase (Furuichi *et al.*, 1975)
3. 5' Terminal methylase (Furuichi & Shatkin, 1976)

In the 1960s Gomatos and collaborators demonstrated that the viral genome was composed of 10 segments of double stranded RNA falling into three size classes, large (L), medium (M) and small (S). The individual segments can be readily resolved by polyacrylamide gel electrophoresis (Shatkin *et al.*, 1968) which reveals that there are three L gene segments (L1-L3 molecular weight 2.3-2.5 x 10^6), three M gene segments (M1-M3 molecular weight 1.5-1.6 x 10^6)

and four S gene segments (S1-S4 molecular weight 0.6-0.9 x 10^6). The 10 segments are present in equimolar amounts in the virion.

mRNA is synthesised in the core and is extruded through the 12 projections or spikes located at the vertices of the core structure (Gillies *et al.*, 1971) into the cytoplasm where it enters the translation process. The mRNA encodes the viral proteins which also fall into 3 size classes: in order of decreasing size these are: lamda, mu and sigma.

1.4.2 Replication cycle of reovirus

The entry of reovirus into the host cell involves specific interaction of the σ1 protein on the outer capsid shell with receptor molecules on the cell surface (Lee *et al.*, 1981). Initially Co *et al.* (1985a; 1985b) proposed that the reovirus receptor closely resembled the ß-adrenergic receptor. However, this proposal was discredited by Choi *et al.* (1988), and Paul *et al.* (1989) provided evidence that sialic acid-bearing glycoproteins were a major receptor component on both mouse L-cells and human erythrocytes for reovirus binding, thus proposing that σ1 recognition of such receptors triggered a series of events leading to internalisation of the virion. Virions are taken into the cell by receptor mediated endocytosis forming phagocytic vacuoles (Silverstein & Dales, 1968) which migrate towards the centre of the cell and fuse with lysosomes.

Studies using ISVPs, generated by chymotrypsin digestion (Borsa *et al.*, 1973a; Borsa *et al.*, 1973b; Borsa *et al.*, 1979), as described in section 1.4.1, have shown that they directly penetrate L-cells without appearing in lysosomes (Borsa *et al.*, 1979). These particles are fully infectious once inside the cell (Borsa *et al.*, 1973a) suggesting that a second pathway for reovirus entry into target cells may exist.

Reoviruses have evolved a unique mechanism for uncoating of reovirus particles (Dales, 1973). This occurs within the lysosomes and involves proteolytic digestion of the outer capsid by lysosmal hydrolases (Chang & Zweerink, 1971; Silverstein *et al.*, 1972) resulting in the removal of σ3 and cleavage of μ1 to μ1c. This process requires a low pH and is an essential step in the infectious cycle (Canning & Fields, 1983; Sturzenbecker *et al.*, 1987). The genome is left intact within the inner capsid, termed the parental subviral particle (SVP) or core. These particles are released from the lysosome into the cytoplasm. The viral enzymes are only activated once the parental virion is uncoated (Silverstein *et al.*, 1972; Chang & Zweerink, 1971). Thus when the parental SVPs are released into the cytoplasm they are ready to transcribe the genome dsRNA into viral mRNA. Several studies suggest that a conformational change is important in producing transcriptionally active particles. This may involve a conformational change that prevents genomic RNA cross-linking with protein (Morgan & Zweerink, 1975). This event may be induced by large monovalent cations that facilitate full activation of the transcriptase following proteolytic digestion (Nagai *et al.*, 1984).

The transcriptase reaction is fully conservative. Both RNA strands of the parental dsRNA remain in the SVP and neither strand appears among the transcription products (Levin *et al.*, 1970; Schonberg *et al.*, 1971). The minus strand of each of the 10 parental RNA segments is transcribed into mRNA (Joklik, 1970; Skehel & Joklik, 1969) producing full length, complete copies of each gene segment. The viral enzymes guanyl transferase and methylase are activated at the same time as the transcriptase, adding a 5' cap structure to the early transcription products (Furuichi *et al.*, 1975a; 1975b). However these transcripts do not contain a modified 3' end, that is, they have no poly A tail.

'Early' transcription occurs within the cores and produces capped mRNA (Chang & Zweerink, 1971; Silverstein *et al.*, 1972). These serve as mRNA

Table 2: Transcription and translation frequency of Reovirus genes

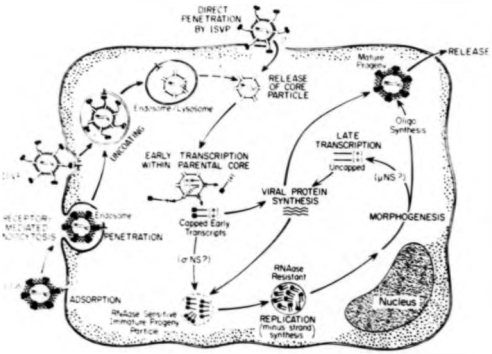
Gene	Protein	Relative transcription frequency	Relative translation frequency
L1	3	1	0.6
L2	2	1	3
L3	1	1	2
M1	μ2	3	0.6
M2	μ1	6	20
M3	μNS	10	10
S1	σ1	10	1
S2	σ2	10	4
S3	σNS	20	6
S4	σ3	20	14

Adapted from Joklik (1981).

templates for protein synthesis and as templates for minus strand synthesis (Furuichi *et al.*, 1975a). Transcription of early reovirus mRNAs appears to be regulated *in vivo*. The plus strand of the L1, M3, S3 and S4 genes appear before other early capped mRNAs (Nonoyama *et al.*, 1974) and have been referred to as pre-early mRNA (Zarbl & Millward, 1983). The remaining six dsRNA segments appear to require synthesis of viral proteins but not viral replication to be transcribed (Drayna & Fields, 1982; Spandidos & Graham, 1976). By 6 hours post infection all 10 dsRNA segments are transcribed (Nonoyama *et al.*, 1974). However, the number of copies varies (table 2, adapted from Joklik, 1981). These findings suggested that the viral transcriptase can only transcribe L1, M3, S3 and S4 until a viral protein product is synthesised in sufficient amounts to allow full transcription to occur. A second model for this regulation proposes that a host cell repressor protein interacts with the uncoated virus particle and in so doing prevents transcription of the six 'late' genes. A protein encoded by one or more of the 'early' genes may depress this factor thus allowing transcription of the late genes (Nonoyama *et al.*, 1974; Zarbl & Millward, 1983). Further evidence for this process was reported by Spandidos and Graham (1976), who showed that the 'early ' genes were the only ones transcribed by avian reoviruses in non-permissive L-cells indicating that the avain reovirus polypeptides were incapable of inactivating the L-cell repressor.

The processes described to date occur within the parental SVPs. Zweerink and collaborators (1972; 1974) proposed that dsRNA replicated within nascent progeny SVPs, which form when the 10 segments of plus sense ssRNA in the cytoplasm associate with viral proteins to form a virion-like particle. In these particles minus strand RNA is synthesised using capped plus strand RNA as a template (Acs *et al.*, 1971; Sakuma & Watanabe, 1972). Once the complementary minus strand is synthesised it remains associated with the plus strand (Sakuma & Watanabe, 1972). In this way 10 unique dsRNA segments

FIGURE 3: DIAGRAM OF THE REOVIRUS REPLICATION CYCLE



(Illustrated by P. Battaglino, from Schiff & Fields, 1989)

assemble in each nascent particle. However, the mechanism by which this occurs remains unknown. The progeny dsRNA are formed within the nascent SVP and remain there (Acs *et al.*, 1971). Free minus strand ssRNA and free dsRNA are never found in infected cells. Once dsRNA synthesis is complete the progeny SVPs undergo structural rearrangements that lead to the formation of particles resembling cores, and they become RNAase resistant and mRNAs encoding viral proteins (late mRNAs) are synthesised. These particles have latent mRNA capping enzymes, thus producing mRNA with uncapped 5' ends (Skup *et al.*, 1981) which synthesise further viral proteins. Mature virions are formed by the assembly of the structural polypeptides, $\sigma 1$, $\sigma 3$ and $\mu 1c$ to form the outer capsid shell. The mature virions also contain adenine-rich short oligonucleotides which constitute 15-20 % of the total RNA (Bellamy & Joklik, 1967). These are abortive transcripts formed after the inactivation of the transcriptase during the last stages of maturation (Zarbl *et al.* 1980a; 1980b). Finally, the mature virions are released by cell lysis via steps that to date remain undefined. The replication cycle is outlined in figure 3.

After viral infection commences there is a gradual decrease in host-cell protein synthesis and an increase in viral protein synthesis (Ensminger & Tamm, 1969; Gomatos & Tamm, 1963). The proposed mechanisms by which the virus controls host protein synthesis will be discussed in section 1.5.

1.4.3. Biochemical and biological characteristics of reovirus

Three serotypes of mammalian reoviruses have been defined using haemagglutination inhibition and antibody neutralisation tests (Fields, 1982). These serotypes are commonly termed Types 1, 2 and 3 with the prototype strains being Lang, Jones and Dearing respectively.

Serological studies have indicated that reoviruses are ubiquitous in their distribution among mammals. In man infection generally occurs in childhood and by the age of sixteen 50-80% of the population have antibodies to the virus (Sharpe & Fields, 1985). The importance of infection in man is difficult to ascertain due to its ubiquity and the common absence of symptoms associated with infection. In contrast to man, mice are highly susceptible to reovirus induced disease and have been widely used as models to study various aspects of viral pathogenesis.

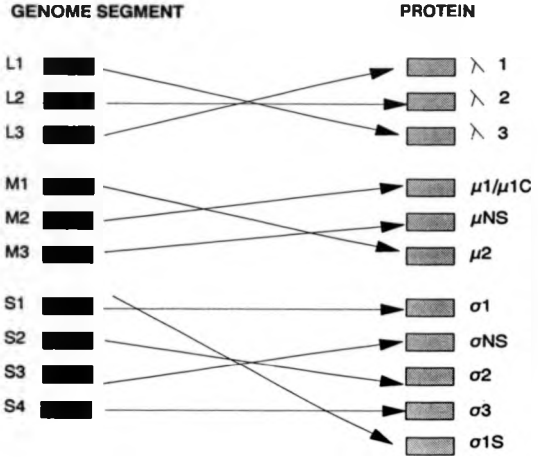
1.4.3.1 Gene-protein assignment

Mixed infections of reovirus in tissue culture cells result in reassortment of gene segments at high frequency to yield recombinant viruses of different genotypes which can be used in viral gene-function studies.

A range of t.s. mutants of the type 3 virus were initially generated by chemical mutagenesis (Fields & Joklik, 1969). Such mutants bearing a lesion in one of the genome segments were widely used in early studies of reovirus genetics. Genetic analysis of mixed infection between pairs of t.s. mutants revealed that certain mutant pairs yielded a high proportion of t.s. + recombinants, that is, they showed wild type activity, while others produced no detectable t.s. + mutants. This suggested that recombination was 'all or none', that is, recombination of the reovirus genome occurred through a mechanism of genome segment reassortment rather than true intermolecular recombination (Fields, 1971). However, proof that recombination occurred by reassortment required markers for the genome segments or gene products, so that the parental genes in the reassorted progeny virus could be identified.

These markers were identified when it was demonstrated that there was a

FIGURE 4: GENE-PROTEIN ASSIGNMENT



difference in electrophoretic migration among the genome segments of the three serotypes (Ramig *et al.*, 1977). For example the S1 gene of type 3 migrates less rapidly on PAGE than the S1 gene of type 1.

The parental origin of each genome segment in a recombinant could, in most cases, be easily determined by electrophoretic analysis of potential recombinants and this was used to demonstrate that the mechanism of recombination in reoviruses was in fact reassortment (Sharpe *et al.*, 1978).

Ramig *et al.* (1978) also used this type of recombinant analysis to map many of the t.s. lesions originally isolated by Fields and Joklik in the earlier genetic studies. Crosses were made between the t.s. mutants of serotype 3 and wildtype type 1. Screening of recombinants from such crosses identified the genome segment bearing the t.s. lesion since it was always replaced by the corresponding wild type gene segment. Similar experiments using wild type viruses showed that reassortment occurred at such a high frequency that it was not difficult to isolate monoreassortants (i.e. a progeny virus containing 9 segments from 1 serotype and 1 from the other). Such monoreassortants were used to assign specific polypeptides to the reassorted gene segment; this would be the only polypeptide having the same mobility as the parent contributing the single gene segment. This analysis was used to assign the polypeptides of the μ and σ classes to specific gene segments (Mustoe *et al.*, 1978). However there is little heterogeneity among the lamda polypeptides of the various serotypes so this method was unable to elucidate the protein assignment of these genes. Using a biochemical approach of *in vitro* translation of the denatured individual genome segments M^{C}Crae and Joklik (1978) made similar assignments for all 10 gene segments (figure 4). It has since been shown that the S1 gene is polycistronic, encoding proteins σ1 and p14 (Ernst & Shatkin, 1985; Jacobs & Samuel, 1985).

1.4.3.2 Reoviruses as models for studying pathogenesis

Mammalian reoviruses have been developed as a model for dissecting the molecular mechanisms of pathogenesis. Several unique properties of the virus have made this possible. These may be briefly summarized as follows:

a. Each serotype has unique pathogenic properties:

Although structurally quite similar, the three virus serotypes interact with their hosts to give very distinct patterns of disease (reviewed by Fields, 1982).

For example:

Intracerebral inoculation of less than 10 pfu of serotype 3 into neo-natal mice causes infection of the neurones (Margolis *et al.*, 1971) and results in a 100 % lethal encephalitis. If the mice are infected by the same route with serotype 1 an acute ependymitis occurs as the virus infects the ependymal cells (Kilham & Margolis, 1969). However when serotype 3 is given orally even at doses of 10^7 pfu of purified virus it is avirulent due to the apparent inability of the virus to escape from the intestinal tract. In contrast, reovirus serotype 1 multiplies in the intestine, later spreading to the brain where it produces a benign ependymitis, similar to that which develops after intracerebral injection (Rubin & Fields, 1980).

b. High frequency gene reassortment facilitates the isolation of virus recombinants with predetermined gene constellations:

Study of the biological properties of monoreassortants has proven to be a very useful technique for mapping the reovirus genome. Reoviruses have been widely used as a tool for studying the molecular mechanisms of pathogenesis by

assigning specific functions to specific genes. For example, in the pathogenic studies described above the difference in tissue tropism of the two serotypes was found to be a function of the S1 gene which encodes the σ1 protein. Type 3 σ1 was shown to specifically interact with neuronal receptors and type 1 σ1 with ependymal receptors (Weiner & Fields, 1977; Lee *et al.*, 1981). The ability of the virus to survive in the intestine was found to be a function of the M2 gene (Rubin & Fields, 1980; Hrdy *et al.* 1981; Drayna & Fields, 1982). This difference is due to the susceptibility of the proteins to chymotrypsin: type 1 μ1 is not cleaved by the enteric enzymes but type 3 μ1 is. Similarly type 1 and 3 σ1 proteins are differentially susceptible to trypsin and chymotrypsin (Yeung *et al.*, 1989), which may be related to the receptor activity of the virus in the intestinal tract.

The three serotypes of the virus also exhibit differences in their ability to control host cell macromolecular synthesis. Using monoreassortants, as discussed above, the genes responsible for the control of host cell DNA and protein synthesis have been identified (Sharpe & Fields, 1981, Sharpe & Fields, 1982). These will be discussed in the following section.

1.5 Control of host cell macromolecular synthesis by mammalian reovirus

Infection of monolayer cultures of mouse L-cells with reovirus type 3 results in a depression in host cell DNA and protein synthesis (Gomatos & Tamm, 1963). In contrast to these findings, infection of the same cell lines with type 1 reovirus has little or no effect on host DNA or protein synthesis (Sharpe & Fields, 1981; Munemitsu & Samuel, 1984).

1.5.1 Inhibition of host cell DNA synthesis

The inhibition of host DNA synthesis begins 8-10 hours post infection with reovirus type 3, prior to the onset of cytopathic effects (Cox & Shaw, 1974). This inhibition occurs without any detectable degradation of cellular DNA or alteration in the activity of either DNA polymerase or any of the other enzymes involved in DNA replication (Ensminger & Tamm, 1969a; 1969b). However, it does result in a blockade of multifocal initiation of new chain DNA synthesis by replication units, resulting in the accumulation of cells in the G1 phase (Hand *et al.*, 1971; 1974). The difference between types 1 and 3 in this property allowed Sharpe and Fields (1981) to use reassortants in mapping it to the S1 gene which encodes the σ1 and σ1S proteins (Ernst & Shatkin, 1985). Thus inhibition of DNA synthesis is dependent on the presence of the proteins derived from the S1 gene of the type 3 virus. The mechanism for this effect may include a direct receptor-mediated effect on a) DNA synthesis after virus binding or b) intracellular effects on σ1 or σ1S proteins, or c) S1 transcripts on synthetic machinery. Indeed, Gaulton and Greene (1989) concluded that the inhibition occurs through a direct receptor-triggered event that involves microaggregation but not intracellular processing of receptor molecules. The virus binds to a membrane protein and induces the inhibition of cellular DNA synthesis by either normal or aberrant signalling through this molecule. Further to these studies Fajardo and Shatkin (1990) studied the effect of the two type 3 S1 protein products in transfected mammalian cells on host DNA synthesis. They found expression of the proteins together or individually did not cause inhibition to the same extent as the whole virus. This suggested that other viral factors were required to interact with σ1 or σ1S to elicit the full inhibitory effect observed in type 3 infected cells.

1.5.2 Inhibition of host cell protein synthesis by mammalian reovirus

Initial studies on the effects of reovirus type 3 on host cell protein synthesis produced conflicting results. When monolayer cultures of L-cells were infected with the virus for up to 16 hours no inhibition of cellular protein synthesis was observed (Gomatas & Tamm, 1963). In contrast, inhibition was observed in suspension cultures at 10 hours post infection and beyond (Kudo & Graham, 1965). The difference in these observations was assigned to cell damage occurring in the suspension cultures (Ensminger & Tamm, 1969a), because at times when the inhibition was noticed the cells were no longer able to exclude trypan blue. However, in both of these studies total protein synthesis rather that host-specific protein synthesis was measured. Investigation of host-specific protein synthesis showed that inhibition occurred 3-6 hours earlier than inhibition of total protein synthesis in suspension cultures of L-cells, (Zweerink & Joklik, 1970), the time of onset of inhibition correlating with the start of viral mRNA synthesis. This led to an initial hypothesis for the mechanism of host shut-off which suggested that the host and viral mRNAs were involved in direct competition for some component of the protein synthesising machinery.

In recent years several alternative mechanisms for host shut-off by mammalian reovirus have been proposed.

1.5.2.1 mRNA competition

Throughout the infectious cycle the level of synthesis of individual viral polypeptides varies widely. Joklik (1974) proposed that this regulation partly occurred at the level of translation. *In vitro* translation studies revealed that the *in vitro* translation efficiency of a particular mRNA correlated closely with the

amount of the corresponding protein produced *in vivo* (Levin & Samuel, 1980). S4 and M2 mRNAs were found to be translated at the highest frequency and their proteins $\sigma 3$ and $\mu 1c$ were present in the largest amounts in the infected cell. By contrast other mRNAs, such as S1 and M1 mRNAs were poorly translated and their proteins $\sigma 1$ and $\mu 2$ were expressed in low amounts in the infected cell. The relative translational efficiencies of the viral mRNAs differ by as much as 100 fold (Gaillard & Joklik, 1985). The efficiency of expression of cDNAs of the reovirus S1 and S4 genes in animal cells was found to differ at the level of translation (Atwater *et al.* (1987). This discrepancy was resolved by Roner *et al.* (1989). Placing the 5' upstream sequence of the S4 gene in the same position in S1 resulted in a significant increase in the efficiency of S1 mRNA translation. Kozak (1981) determined that the nature of the sequence around the initiation sequence is important in determining the rate of translation. This suggested that the 5' upstream sequence of the S4 gene contains sequences enabling it to compete more efficiently for the translation apparatus.

Thach and colleagues suggested that such competition may play a role in regulating the translation of host and reovirus mRNA in infected cells (Walden *et al.*, 1981; Brendler *et al.*, 1981a; 1981b). Walden *et al.* (1981) concluded that major viral mRNAs such as S4, encoding $\sigma 3$, are poor initiators of translation relative to host mRNAs and this was possibly due to low affinity for an initiation component which discriminated between viral and host mRNA. Viral mRNA concentrations were found to be unusually high at maximal times of viral protein synthesis, suggesting that translation of viral mRNA was forced by mass action. On the basis of these results Thach and collaborators proposed a model whereby mRNA competes for a limiting message-discriminatory initiation factor in order to be translated. They also suggested that competition between mRNAs might be important in regulating the rate in initiation. This discriminatory factor was thought to be associated with the 40S ribosomal subunit (Ray *et al.*, 1983).

1.5.2.2 CAP independent translation

To investigate the mechanism by which the virus takes over host cell protein synthesis Skup and Millward (1980a; 1980b) studied changes in the methylation pattern of the viral mRNA during the process of viral infection. Using efficient cell-free protein synthesising systems derived from L-cells (Skup & Millward, 1977), comparisons were made between the ability of extracts prepared from uninfected and type 3 infected mouse L-cells to translate exogenously added reovirus mRNA *in vitro* (Skup & Millward, 1980a). Translation of uncapped viral mRNA was found to be highly efficient in extracts from infected cells. In contrast, capped reovirus mRNA or globin mRNA were efficiently translated in extracts from uninfected cells but not in infected cell extracts. From such observations Skup and Millward (1980a) proposed that the host cell translational machinery became modified as a result of viral infection such that it preferentially translated uncapped mRNA at late times in infection, possibly by a mechanism similar to that seen in poliovirus infected cells (Schneider & Shenk, 1987).

Further support for this hypothesis was obtained when Skup and Millward (1980b) studied the enzymatic activities associated with progeny SVPs isolated from L-cells at late times post infection when viral protein synthesis is at its maximum. They showed that the mRNA capping enzymes (guanyl transferase and RNA methylase) were not expressed in the progeny SVPs, that is, the capping enzymes were masked or latent. Thus the progeny SVPs synthesise uncapped mRNA. Analysis of the mRNA synthesised indicated that the 5' end was monophosphorylated having the structure pGpC. Skup *et al.* (1981) were also able to demonstrate that uncapped reovirus mRNA associated with polyribosomes at late times post infection. Therefore at late times in infection uncapped reovirus mRNA is apparently preferentially translated in comparison to

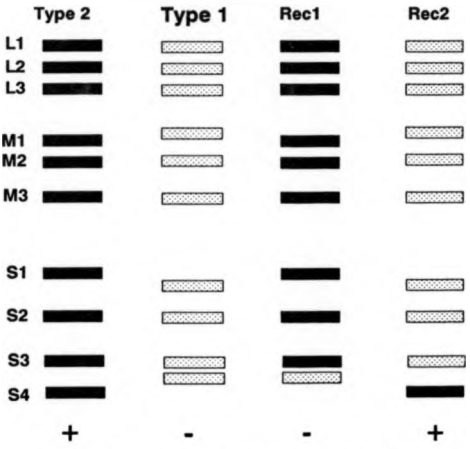
host capped mRNA, resulting in viral takeover and depression of host protein synthesis. In support of this hypothesis translation of reovirus uncapped mRNA synthesised *in vitro* in cell free extracts from infected L-cells was found to be insensitive to inhibition by a monoclonal antibody directed against the cap binding protein (CBP) (Sonenberg *et al.*, 1981).

However, other studies using the mouse cell line SC1 reported that during infection translation of reovirus type 3 mRNA remains cap dependent (Detjen *et al.*, 1982). Extracts made from uninfected and reovirus infected cell extracts were able to translate capped and uncapped globin mRNA with the same efficiency. These workers also obtained similar results in mouse L-cells with the exception that the shut-off of host translation was far greater in L-cells than in SC-1 cells. These results supported the work of Walden *et al.* (1981) suggesting that the regulation of protein synthesis in infected cells did not occur by a switch of cap dependent to cap independent translation.

To investigate the inconsistency of these results direct competition experiments between L-cell mRNA and late reovirus mRNA were carried out (Liemeux, 1984). The results indicated that late reovirus mRNAs did not compete with L-cell mRNA for a limiting component on the host translational apparatus in infected cell lysates, rather the virus induced an alteration in the cap dependent translational apparatus of L-cells. However the absence of the 5' cap structure could not be the only factor responsible for this switch because poliovirus mRNA was unable to be translated as efficiently in S-10 extracts prepared from reovirus infected cell extracts made at late times post infection as it was in uninfected cell extracts of L-cells.

The experiments by Liemeux *et al.* (1984) support the hypothesis that in extracts from infected cells certain initiation factors stimulating the translation of viral mRNA are more active, or are present in greater amounts, than are initiation factors required for the translation of host cell mRNA. Indeed,

FIGURE 5: GENE REASSORTMENT



Reassortants containing the S4 gene segment of type 2 and the other 9 gene segments of type 3 (Rec2) caused inhibition of host protein synthesis to the same extent as wild type type 2 (Sharpe and Fields, 1982).

Lemeux *et al.* (1984) showed that this stimulatory factor could be a viral product: a factor in infected L-cells specifically stimulated the translation of uncapped reovirus mRNA.

These results suggest that at late times in infection host cell protein synthesis is depressed as a result of the virus producing a factor to favour translation of its own mRNA.

1.5.3 Identification of the protein responsible for inhibition of protein synthesis

The mechanisms of reovirus control of host protein synthesis discussed above have involved study of type 3 reovirus. Other workers have investigated the ability of type 1 and type 2 to inhibit host protein synthesis.

Type 2 reovirus has also been reported to inhibit both DNA and protein synthesis in RA (human amnion) cells but had little effect on RNA synthesis (Loh & Soergel, 1965). Sharpe and Fields (1982) reported that reovirus type 2 inhibits protein synthesis in L-cell monolayers more rapidly and efficiently than type 3 virus. Using monoreassortants it was found that recombinant viruses required only the S4 gene to come from type 2 virus to be able to inhibit protein synthesis to the same extent as type 2 wild type. From these results they concluded that the S4 gene or at least its protein product, $\sigma 3$, is responsible for the shut-off of host cell protein synthesis (figure 5).

Further studies on the effects of the different serotypes were carried out by Munemitsu and Samuel (1984) who found that serotypes 1 and 3 differed significantly in their rates of multiplication and their effects on cellular protein synthesis. They found that at late times of infection there was a decrease in the overall rate of protein synthesis as noticed by others but not in the case of type 1 infected cells. Therefore there is a hierarchy of the ability of the virus to do this: type 2 > type 3 > type 1.

This property of reovirus has allowed it to be used as a model to study the molecular aspects of this important intracelluar pathogenic event in more detail.

1.5.3.1 Other properties of $\sigma 3$

Several other properties have been assigned to $\sigma 3$:

A. It has a role in establishing persistence.

B. It has a role in binding ds RNA.

C. It is a zinc binding protein.

A. Role in establishing persistence

Persistent infections of reovirus can be established by infecting L-cells with reovirus stocks that have been serially passaged at a high multiplicity of infection (Ahmed & Graham, 1977). Such viral stocks contain many types of mutants (deletion mutants, growth attenuated mutants and temperature sensitive mutants) (Ahmed & Fields, 1981). These stocks are referred to as defective interfering viral stocks (DI).

To investigate the mechanism of establishing persistence Ahmed and Fields (1982) co-infected cells with wild type 2 virus and DI stocks of type 3 which had established persistent infections. Analysis of the resulting persistently infected cell lines and the reassortant viruses showed they contained the S4 gene from the DI virus. As the S4 gene is responsible for inhibition of host cell protein synthesis a symbiotic cell-virus relationship leading to a persistent infection can only be established if the S4 gene of the type 2 virus is excluded from the lytic virus by selecting the S4 segment from the defective virus.

FIGURE 6: PROTEIN SEQUENCE OF TYPE 3 σ3 SHOWING AMINO ACID CHANGES IN TYPE 1 σ3

... = positions of amino acid identity

* = amino acid differences

B. Binding of double stranded RNA

Huisman and Joklik (1976) showed that σ3 binds strongly to ds RNA. This property of the protein could be involved in the control of host protein synthesis by binding to double stranded regions of rRNA, tRNA or mRNA (Sharpe & Field, 1982).

C. Zinc binding

σ3 has also been shown to be a zinc metalloprotein. (Schiff & Fields, 1988). This may influence the ability of σ3 to bind dsRNA and may be important for its proposed effects on regulating viral and host cell transcription and translation.

1.5.3.2 Sequence of the S4 gene

The S4 genes of type 3 (Giantini *et al.*, 1984) and type 1 (Atwater *et al.*, 1986) have been fully sequenced. The overall sequence homology between serotype 1 and 3 S4 gene is 94 % at the nucleotide level. The 2 genes are the same length (1,196 nucleotides) and the positions of the initiation AUG codon (nucleotides 33-35) and termination UAA codon (nucleotides 1128-1130) are conserved. There is a slightly greater homology in the 5' and 3' untranslated sequences showing 97 % homology at the nucleotide level. The predicted σ3 coding sequences, of 365 amino acids, display 96 % homology at the amino acid level. These differences are not clustered but are scattered throughout the gene (figure 6).

Despite the similarity of the S4 type 1 and S4 type 3 genes their ability to inhibit host cells protein synthesis varies:

Type 1 does not inhibit protein synthesis at late times in infection

Type 3 does inhibit protein synthesis at late times in infection.

1.6 Aims of the project

The preceding sections have discussed the hypothesis to account for the general mechanism by which reovirus exerts control on host cell protein synthesis. However, precise details of the molecular mechanism involved in the inhibition and the exact role of the S4 gene remain undefined.

The aims of this project were first to obtain full length cDNA clones of the S4 gene of serotypes 1 and 3. Then to use these cDNAs in developing an *in vitro* assay system to try and localise the region of the S4 gene responsible for the inhibition of translation of a eukaryotic protein. Thirdly, to develop an *in vivo* assay system again using the S4 cDNAs to gain a greater insight into the role of the S4 gene in causing the inhibition of host cell protein synthesis.

CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

The materials used in this study are listed in Table 3.

Table 3: Reagents used in this study.

REAGENT	SUPPLIER
1. Tissue culture	
All Tissue culture media	GIBCO/BRL
Amino acids	SIGMA
Antibiotics	SIGMA
Crystal violet	BDH
Foetal calf serum	GIBCO/BRL
$NaHCO_3$	BDH
New-Born calf serum	GIBCO/BRL
ß-Propiolactone	SIGMA
2. Gel Systems	
Acrylamide	FISONS
Agarose Type 2	SIGMA
Ammonium persulphate	BIORAD
Bromophenol blue	SIGMA
Ethidium Bromide	SIGMA
Fuji film	FUJI PHOTO LTD.

Gel tanks	NEW BRUNSWICK
Glycine	FISONS
LX24 X ray developer	EASTMAN KODAK COMPANY
FX40 X ray fixer	BIORAD
N'N-methylene bis acrylamide	BDH
PPO	BIORAD
Sodium dodecyl sulphate	BIORAD
Temed	BDH
Tris Base	BDH
Xylene cyanol FF	
3. DNA Techniques	
1kb DNA ladder	BRL
Agar	DIFCO LABS
AMV Reverse transcriptase	LIFE SCIENCES INC
Bacto-Tryptone	DIFCO LABS
Chloramphenicol	SIGMA
Calf Intestinal Phosphatase	BCL
Dithiothreitol	SIGMA
Klenow 1 DNA polymerase	PHARMACIA
EDTA	BDH
Electroporation cuvettes	BIORAD
Ethanol	HAYMAN LTD
Glycerol	BDH
IPTG	SIGMA

KCl	FISONS
LiCl	BDH
Lysozyme	SIGMA
$MgCl_2$	FISONS
Mouse liver t-RNA	Roger Jenkins
NaCl	FISONS
Nucleotide triphosphates	SIGMA
HYBOND Nylon filters	AMERSHAM
Phenol	FISONS
Restriction enzyme buffers	BRL
Restriction enzymes	BRL
RNA Ligase	BRL
RNAase A	BRL
Salmon Sperm DNA	SIGMA
Sodium Acetate	BDH
Sucrose	BDH
T4 DNA ligase	BCL
Triton	BDH
X-gal	NBL ENZYMES LTD
Yeast extract	OXOID
Yeast t-RNA	SIGMA

4. Radioactive chemicals

^{32}P-dGTP	AMERSHAM
^{32}P-rCTP	"
^{35}S-Methionine	"
^{3}H-Uridine	"

^{3}H-Uridine 5'Triphosphate	

5. *In vitro* Transcription

10x T7 transcription buffer	STRATAGENE
Ammonium Acetate	BDH
$m^{7}G(5')ppp(5')Gm$ (CAP)	PHARMACIA
RNA guard ^{TM}	PHARMACIA
RNAase free DNAase	BRL
T7 RNA polymerase	STRATAGENE

6. Protein techniques

BIORAD Protein assay kit	BIORAD
CAS amino acids	DIFCO
Creatine Phosphate	SIGMA
Creatine Phosphokinase	SIGMA
EGTA	FISONS
FITC-GAR	SIGMA
Haemin	BDH
Hepes	SIGMA
Mnase	PHARMACIA
NET-290 Acetyl co-enzyme A	NEN RESEARCH PRODUCTS
NP40	SIGMA
ONPG	SIGMA
Optiphase scintillation fluid	LKB
Protein A Sepharose	SIGMA
Sodium deoxycholate	BDH

ß-mercaptoethanol	BDH
Trichloroacetic acid	FSA
Toluene	RHONE POULENC

2.1.2 Cells and virus

Mouse L-cells were obtained from M.A.McCrae.

COS-1 and HeLa cells were obtained from K.Leppard.

Reovirus serotypes 1,2 and 3 were obtained from M.A.McCrae.

2.1.3. Bacterial strains

The bacterial strains MC1061 and JM103 used in this study were obtained from lab stocks from M.A.McCrae.

2.1.4: Media, Buffers and Solutions used in this study

The media , buffers and solutions used in this study are listed below.

1. Tissue culture:

Met-free G-MEM

2 x GMEM without Leu, Val, Met , $NaHCO_3$	500 ml
Leucine (200 mM)	10 ml
Valine (200 mM)	10 ml
Glutamine (200 mM)	10 ml
$NaHCO_3$ (5 % w/v)	20 ml

Antibiotics (100μg/ml)	1 ml
Distilled water to	1 litre

Phosphate-Buffered saline (PBS)

Potassium Chloride	0.2 g
Sodium Chloride	8.18 g
Potassium dihydrogen orthophosphate	0.2 g
di-sodium hydrogen orthophosphate	1.15 g
Distilled water	1 litre

Crystal violet

7.5 % crystal violet in ethanol

Formal saline

PBS	350 ml
formaldehyde	150 ml

Overlay agar

2 x GMEM	50 ml
FCS	2 ml
Noble agar (1.8 %)	50 ml

Resuspension Buffer

Tris-HCl pH 8	50 mM
NaCl	10 mM
ß-mercaptoethanol	1.5 mM

2. Gel systems

10 x T.B.E. Buffer pH 8	
Tris base	108 g/litre
EDTA	9.5 g/litre
Boric acid	55 g/litre

5 x Loading buffer	
Glycerol	50 %
Bromophenol blue	0.01 %
Xylene cyanol FF	0.01 %
T.B.E	1 x

10 x alkali gel buffer	
NaOH	0.3 M
EDTA	20 mM

10 x alkali loading buffer	
NaOH	0.3 M
EDTA	20 mM
Glycerol	50 %
Bromocresol green	0.01 %

10 x MOPS buffer pH 7	
MOPS	0.2 M
NaAc	50 mM
EDTA	10 mM

4 x separating buffer pH 8.8

Tris base	36.3 g
SDS	0.8 g
Distilled water to	200 ml

4 x stacking buffer pH 6.8

Tris Base	12.1 g
SDS	0.8 g
Distilled water to	200 ml

10 % SDS Polyacrylamide gel

	10 % Gel	5 % Stacker
Acrylamide-bis (30:0.8)	8.3 ml	2 ml
4 x separating buffer	6.25 ml	-
4 x stacking buffer	-	3 ml
Glycerol	2.5	-
100mg/ml APS	0.2 ml	3 ml
TEMED	12.5 µl	15 µl
Distilled water to	8 ml	4 ml

5 x Cracking buffer

SDS	10 %
ß-mercaptoethanol	2.5 %
glycerol	50 %
Gel stacking buffer	1 x
Bromophenol blue	0.01 %

10 x CIP buffer	
Tris-HCl pH 9	0.5 M
$MgCl_2$	10 mM
$ZnCl_2$	1 mM
Spermidine	10 mM

L-Broth	
Bactotryptone	10 g
NaCl	5 g
Yeast Extract	5 g
Distilled water to	1 litre

L-Agar	
Bactotryptone	10 g
NaCl	5 g
Yeast Extract	5 g
Agar	15 g
Distilled water to	1 litre

Ligation buffer	
2M Tris-HCl pH 7.5	6.7 µl
1M $MgCl_2$	1 µl
1M DTT	0.5µl
100 mM ATP	2 µl
H_20	9.3 µl

10 x Klenow Polymerase I buffer

Tris-HCl pH 7.5	0.5 M
$MgSO_4$	0.1 M
DTT	1 mM
BSA	100 μg/ml

T.N.E. Buffer

NaCl	0.2 M
Tris-HCl pH 7.5	50 mM
EDTA	1 mM

20 X SSC

NaCl	3 M
Sodium citrate	0.3 M

100 x Denhardts solution

BSA	2 g
Ficoll	2 g
PVPP	2 g
Distilled water to	1 litre

Prehybridisation mix

50 x Denhardts	4 ml
20 x SSC	6 ml
SDS	10 %
tRNA	
Spermidine	
Distilled water to	20 ml

5. *In vitro* Transcription

10 x T7 RNA polymerase buffer	
Tris-HCl pH 7.5	400 mM
$MgCl_2$	60 mM
Spermidine	20 mM
NaCl	100 mM

6. Protein Techniques

10 x Hepes buffered saline (HBS buffer)	
NaCl	4.09 g
Hepes	2.97 g
Na_2HPO_4	0.1 g
Distilled water to	50 ml

CAT lysis buffer	
Tris HCl pH 7.9	0.25 M
DTT	5 mM
Glycerol	10 %
NP40	0.2 %

2 x Immunoprecipitation buffer 3 (IP3)	
Tris-HCl pH 8	400 mM
NaCl	200 mM
EDTA	2 mM
NP40	2 %

Deoxycholate	2 %
SDS	0.2 %

Immunoprecipitation buffer 2: IP2

IP3 + 20 mg/ml BSA

Immunoprecipitation buffer 1: IP1

Tris-HCl pH 8	20 mM
NaCl	100 mM
EDTA	1 mM
NP40	1 %

2.2 METHODS

2.2. CELL CULTURE AND VIRUS PROPAGATION.

2.2.1. Cell culture:

Mouse L-cells were maintained in suspension culture in a sterile flat bottomed flask at 37°C in Jokliks modified minimal essential medium (MEMS) supplemented with 5 % foetal calf serum, glutamine and 100 μg/ml penicillin/streptomycin. The cell density was maintained at $5x10^5$ - $1x10^6$ cells/ml by splitting the culture 1:2 every 24 hours (M^{c}Crae 1985).

HeLa and COS-1 cells were maintained in 90 mm plastic tissue culture dishes in Dulbecco's modified Eagles medium (DMEM) supplemented with 10 % new-born calf serum and 10 % foetal calf serum respectively. Cells were split 1 : 10 every 4 days.

2.2.2. Preparation of virus inocula:

Confluent monolayers of L-cells were infected at a m.o.i of no greater than 0.1. Virus growth was allowed to proceed for 2-4 days until complete c.p.e. was observed. The contents of each flask were harvested and stored at -70°C until used.

2.2.3. Viral infectivity assay

Serial ten fold dilutions of the virus were prepared in PBS and were inoculated onto 12 or 24 well tissue culture dishes. Following one hour of

adsorption at 37°C the cells were overlaid with 1 x agar + 1 x Glasgow's modified minimal Eagles medium (GMEM) supplemented with 2 % FCS and incubated at 37°C for 4-5 days. The cells were fixed for 4 hours with formal saline, the overlay was removed and monolayers stained with crystal violet for 2-3 minutes. Excess stain was washed off and clear plaques counted.

2.2.4. Labelling of viral proteins

Confluent L-cell monolayers in 12 well dishes were infected at a m.o.i of 10. Infection was allowed to proceed for 12-15 hours before removing the medium, washing the cells in 1 x methionine-free (met-free) GMEMS and overlaying with met-free GMEMS and 2 % dialysed FCS for 1-2 hours. After this the medium was removed and replaced with 100 μl of ^{35}S-met (500 μCi/ml in PBS), and incubated for 20 minutes at 37°C. Cells were harvested in 300 μl of 10 mM Tris-HCl pH 8.5 by scraping the monolayers with rubber policemen.

2.2.5. Large scale growth of virus

Virus was routinely grown up in 4 litre batches of suspension L-cells. Prior to culture infection the cells were transferred from the 37°C water bath to 34°C as reovirus is cytotoxic to cells at 37°C. Cells were infected at an m.o.i. of 0.1 and virus growth allowed to proceed for 36 - 42 hours before transferring the culture to 4°C to allow the cells to settle out for at least 24 hours.

2.2.6. Virus purification

The settled viral and cellular debris were concentrated by centrifugation at 2000 rpm, 4°C, for 20 minutes in a bench centrifuge, resuspended in

FIGURE 7: CsCl GRADIENT PURIFICATION OF REOVIRUS

Reovirus was purified by isolation on CsCl gradients (ρ 1.2-1.4) as described in the text. The virus was harvested by side puncture and washed in 10mM Tris pH 8 to remove all traces of CsCl.

resuspension buffer and homogenised with 1/4 volume of Arcton 113 (trifluoroethane) 4-5 times using a Virtis 123 homogeniser. The aqueous phases were pooled together and finally the aqueous phase was back extracted with Arcton. Virus was concentrated by centrifugation at 100,000 g for 1 hour at 4°C in a M.S.E centrifuge (3 x 70 rotor). The pellet was resuspended in 50 mM Tris pH 8 and loaded onto a preformed $CsCl_2$ gradient of p1.2-1.4 which was centrifuged at 100,000 g at 4°C for 2 hours in a M.S.E. centrifuge (3 x 70 rotor). The p1.36 band was collected by side puncture, concentrated by centrifugation and resuspended in 50 mM Tris-HCl pH 8. (M^{c}Crae 1985). Figure 7 shows a typical virus gradient profile.

2.2.7. Extraction of genomic RNA from purified virus

The viral suspension (1 mg/ml) was made 1 % with respect to SDS and extracted 3 times with 50 mM Tris-HCl pH 8 saturated phenol. Residual phenol was removed by extracting 4 times with di-ethyl-ether and RNA collected by ethanol precipitation in the presence of 0.15 M LiCl. The concentration of the RNA was calculated on the basis of optical density at 260 nm.

2.3. GENERAL DNA TECHNIQUES

2.3.1. DNA precipitation

DNA was precipitated from aqueous solution on the addition of salt and ethanol. The salt routinely used was Lithium chloride. This was diluted from a 10 M stock to 0.15 M in the solution containing the DNA, prior to addition of 100 % ethanol and precipitation of the DNA (Sambrook *et al.*, 1989). The DNA

pellet was dried under vacuum and resuspended in T.E. buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

2.3.2. Phenol/Ether extraction

Proteins were removed from solutions containing DNA or RNA prior to ethanol precipitation with phenol saturated with 50 mM Tris-HCl pH 8. The DNA solution was made up to 100μl with water and an equal volume of phenol was added. After mixing, the phases were separated by brief microfugation. The upper aqueous layer was removed and extracted with an equal volume of di-ethyl-ether. The upper ether phase was removed and the lower aqueous phase was ethanol precipitated.

2.3.3. Restriction endonuclease digestion of DNA

Plasmid DNA (10 μg) was digested with the appropriate enzymes in a final volume of 20 μl containing 2 μl of the appropriate 10 x enzyme buffer and restriction enzyme. Generally 4 Units of enzyme was used per sample and the digestion carried out by incubating overnight at 37°C or the optimal temperature for enzyme activity as recommended by the supplier.

2.3.4: Large Scale Plasmid Preparation

A 10 ml culture containing the relevant plasmid DNA was grown overnight at 37°C. This was diluted into 1 litre of L-Broth and grown at 37°C until the OD 590 reached 0.6, chloramphenicol was added to a final concentration of 150 μg/ml and the plasmid DNA amplified by overnight incubation at 37°C. Cells were harvested, lysed and plasmid DNA isolated as described by Sambrook *et al.*, (1989). DNA was ethanol precipitated and the OD 260 used to calculate the amount of DNA recovered.

2.3.5: Agarose gel electrophoresis of DNA

Two types of agarose gel electrophoresis were employed depending on the nature of the DNA under investigation.

a. Neutral gels

b. Alkaline gels

a. Neutral gels:

1-1.5 g of agarose/100 ml of sterile Distilled water (SDW) was dissolved in a microwave and allowed to cool to 48°C. To this 10 mls of 10 x T.B.E. and 10 µl of ethidium bromide (10 mg/ml) were added.

The gels were cast, allowed to set and were placed in 1 x TBE. 20 µl of DNA sample was mixed with 5 µl of 5 x Loading buffer and loaded into the wells. Most gels were run at 250 mA for 1-3 hours or until the lower dye front reached the bottom of the gel.

1kb Ladder (BRL) was routinely used as a marker for agarose gels. Gels were photographed under ultraviolet light using Polaroid 667 positive film.

b. Alkaline agarose gels

These gels were typically run for examining the products of c-DNA synthesis.

To every 90 ml of molten agar 10 ml of 10 x alkali gel buffer was added. The gels were placed in 1 x alkali gel buffer and DNA samples were mixed with 10 x alkali gel loading buffer.

2.3.6. DNA isolation from agarose gels

DNA was isolated by two methods depending on the nature of the fragment.

a. Electroelution

This method was typically used for the isolation of radioactive c-DNA products as described by Sambrook *et al.*, (1989). The c-DNA band was located by autoradiography and the gel piece excised using a scalpel.

b. Direct elution of DNA from the gel

Non-radioactive DNA fragments to be isolated were visualised and a slit cut in front of the relevant band. A piece of 3 mm Whatman filter paper backed with dialysis tubing was placed in the slit. Most of the buffer was removed from the gel tank, such that buffer was only touching the top of the gel. The gel was run for 5 minutes at a maximum of 150 mA. The filter paper was transferred to a small eppendorf with a hole in the bottom and placed in a large eppendorf. The liquid was removed by microfuging for 10 minutes, followed by addition of 150 µl of T.N.E buffer to the filter paper and further microfugation to elute the DNA. The elutants were pooled and the process repeated 2-3 times, phenol/ether extracted and ethanol precipitated (Dretzen *et al.*, 1981).

2.3.7. Dephosphorylation of vector DNA

To remove the terminal 5' phosphate groups from linearised vector DNA, it was treated with calf intestinal phosphatase (CIP). The reaction mix consisted of <1 µg of digested vector DNA, 1 x CIP buffer and 1 Unit of CIP (Sambrook *et al.*, 1989)

2.3.8 Blunt ending of DNA

a. Klenow filling

5' overhangs of digested DNA (e.g. *BamHI*, *EcoRI*) were removed when required by Klenow filling the DNA. The reaction was allowed to proceed for 3

hours at 15°C and contained the relevant DNA, 20 mM dNTPs, 2 Units of Klenow DNA polymerase 1 and 1 x Klenow pol 1 buffer (Sambrook *et al.*, 1989)

b. S1 nuclease

3' underhangs of digested DNA (e.g. *PstI*) were removed when required by S1 nuclease as described by Sambrook *et al.*, (1989).

2.3.9. Ligation of DNA

DNA fragments were ligated with phosphatased vector DNA using a molar excess of insert to plasmid of 10:1. Ligation was allowed to proceed at 15°C overnight using 100 ng of vector DNA, and the relevant amount of insert DNA, 2 Units of DNA ligase and 1 Unit of RNA ligase in ligation buffer (Sambrook *et al.*, 1989).

2.3.10. Transformation

Ligated DNA samples were transformed into either competent *E.Coli* cells strain MC1061 ($recA^+$) or JM103 ($recA^-$), depending on the vector used.

Competent cells were generated by diluting an overnight culture 1 in 100 in L-Broth and incubating with agitation until the OD 590 reached 0.4-0.6.

Initially transformations were performed using the calcium chloride technique (Sambrook *et al.*, 1989). However, more recently they were performed by electroporation.

Each 20 ml of competent cells was washed twice in SDW. Cells were resuspended in half the initial volume of cold 10 % glycerol and respun at 2K for 10 minutes, 4°C in a bench centrifuge. They were then resuspended in 200 -

300 µl 10 % glycerol and either used directly or stored at -70°C until required. Cells were stored in such a way for up to 6 months.

For transformation, the ligation mix was ethanol precipitated to remove all traces of salt and the dried pellet resuspended in 10 µl of SDW. 40 µl of competent cells were added to the DNA and the mix transferred to an electroporation cuvette. The cells were pulsed in a BIORAD gene pulser at:

Capacitance	25µFD
Pulse controller	2000
Voltage	2.5K

A time pulse of 4.5 - 5 seconds resulted in a 'good pulse'.

The mix was transferred to 700 µl of L-broth and incubated at 37°C for 30 minutes before plating onto 1.5 % L-agar containing 100 µg/ml ampicillin or 10 µg/ml tetracycline depending on the plasmid under investigation. The plates were incubated overnight at 37°C. When JM103 / Bluescribe was the vector/bacteria combination used the transformation mixture was plated onto selective plates containing 100 µg/ml ampicillin, 40 µg/ml X-gal and 5 mM IPTG. Potential recombinants were identified by the white colony colour compared to the blue colour of vector only containing colonies.

2.3.11. Screening for potential recombinants

a. Minipreparation of plasmid DNA

20 - 30 colonies were normally picked from transformed plates per ligation. The transformed *E. coli* colonies were cultured in 10 mls L-broth overnight and plasmid DNA was prepared from these cultures by the alkaline lysis method as described by Sambrook *et al.*, (1989).

DNA inserts were detected by restriction enzyme digestion and analysed by agarose gel electrophoresis.

b. Screening for DNA by hybridisation techniques

In some cases it was necessary to screen large numbers of colonies for the insert DNA. In such cases 200 - 400 colonies were picked onto an L-agar plate containing the relevant antibiotic and incubated at 37°C overnight. The colony hybridisation method of Grunstein and Hogness (1975) was used. Colonies were overlaid with a nylon filter for 3 minutes instead of the traditional nitrocellulose filter. After lysing and fixing the colonies, the DNA was firmly bound to the filter by exposing to U.V illumination for 3 minutes. The filter was then placed in a plastic bag containing prehybridisation mix, sealed and incubated at 68°C for at least 1 hour.

The filter was then hybridised with a radioactive probe overnight at 68°C.

Non-hybridised radioactivity was removed by 4 washes in 2 x SSC and 0.5 % SDS for 20 - 30 minutes. The filter was dried and exposed to autoradiography. Colonies containing the insert could be detected and further checked for insert and orientation by mini-plasmid preparations, restriction enzyme digestion and agarose gel electrophoresis.

c. Preparation of radioactive probe

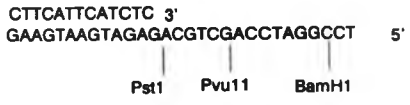
Probes were generally prepared by Nick translation using < 1 μg of the relevant insert DNA in a reaction mix of 1 x Poll buffer, containing 1 μl of DNAase (1 mg/ml diluted 1:10000), 1 Unit of Klenow I polymerase and 100 μCi of α^{32}P dGTP (Sambrook *et al.*, 1989). The reaction was incubated at 15°C for 3 hours and stopped by the addition of 30 μl of 2 x column buffer and 140 μl of 1 x column buffer.

Labelled DNA was separated from unincorporated radioactivity by running a Sephadex G50 column, equilibrated with 1 x column buffer. Twenty fractions (10 drops) were collected and counted by Cerenkov radiation in a LKB 1219 Rack-beta scintillation counter. The incorporated peak was pooled and the DNA

FIGURE 8: SEQUENCE OF THE PRIMERS USED FOR cDNA SYNTHESIS OF THE S4 GENE

5' end primer for the S4 gene

3' end primer for the S4 gene



ethanol precipitated, resuspended in a suitable volume (300 μl), boiled for 10 minutes and quench cooled prior to use in hybridisation.

2.3.12. Autoradiography

Filters or gels bearing $\alpha^{32}P$ radioactive isotope were exposed at room temperature or at -70°C using a Lightening-plus intensifying screen to Fuji X-ray film, for times varying between several hours and several weeks depending on the amount of isotope present.

2.4. c-DNA CLONING OF THE S4 GENE

2.4.1. Preparation of oligonucleotides

With the sequence available for the S4 gene of serotypes 1 and 3, oligonucleotides were specifically synthesised for the 5' and 3' ends of the gene (figure 8). These were used as primers for reverse transcription (Kurtz & Nicodemus, 1981) of the S4 gene. The important features of the oligos are discussed in section 4.2.

2.4.2. Purification of oligonucleotides

Oligonucleotides were resuspended in SDW and loaded onto a 20 % vertical acrylamide gel (20 % acrylamide, 42 % urea, 1 x T.B.E) and run in 1 x T.B.E. buffer. The oligos were excised from the gel and isolated by shaking overnight in 5 ml of elution buffer (10 mM MgAc, 500 mM NH_4Ac, 0.1 % SDS, 1mM EDTA) and run through a Sephadex G25 column overlaid with DEAE Sephacel anion exchanger. The column was equilibrated with 50 mM NH_4HCO_3 and the oligo eluted with 1 M NaCl/50 mM NH_4HCO_3. 10 drop fractions were collected, the OD 260 measured, the peak of absorbance pooled

and freeze dried. The oligo was resuspended in SDW and its concentration determined from the OD 260.

2.4.3. Preparation of the RNA template

10 μg of ds viral RNA was precipitated and resuspended in 30 μl of 1 mM EDTA. The solution was made 90 % with respect to DMSO and incubated at 45°C for 20 minutes, (thus lowering the t.m. of the RNA) followed by rapid precipitation of the RNA to denature the RNA.

2.4.4. c-DNA synthesis

c-DNA was synthesised following the basic method developed by M^{c}Crae and M^{c}Corquodale (1982).

As the primers were specific for the S4 gene, total single stranded viral RNA was used as the template for the reaction. The amount of oligo required for each reaction was calculated on the basis of using a 100 - 1000 x excess of the oligo over the calculated amount of S4 gene.

The RNA was diluted up to 40 μl in SDW and an equal volume of 5 mM Methyl mecuric hydroxide was added. The mixture was incubated at 20 °C for 3 minutes. To this, 120 μl of reverse transcription reaction mix was added.

The reaction mix consisted of the following:

Tris-HCl pH 8.3	75 mM
$MgCl_2$	180 mM
dATP	1.5 mM
dCTP	1.5 mM
dTTP	1.5 mM
dGTP	0.75 mM
DTT	15 mM

Oligos	c.a. 100μg/ml
α ^{32}P dGTP	50 μCi
Reverse transcriptase	30 Units
SDW to	120 μl

The reaction was allowed to proceed for 5 minutes at room temperature, 42°C for 60 minutes and finally 46°C for 10 minutes. Un-incorporated radioactivity was separated through a Sephadex G-50 column as described in section 2.3.11.

The overhanging end of the c-DNA, generated by the oligonucleotides, were filled using Klenow I polymerase and the c-DNA isolated on a denaturing alkaline gel followed by electroelution.

The cloning vector pAT153 was digested with *Pst I* and poly G tailed while the c-DNA was poly C tailed as described by M^{C}Crae and M^{C}Corquodale (1982), vector and cDNA were hybridised and transformed into competent MC1061 cells.

As the ampicillin resistant region of the plasmid was destroyed by *Pst I* digestion potential clones were initially screened for tetracycline resistance. Potential positives (i.e. those ampicillin sensitive) were further screened directly by mini-plasmid preparations or if there were large numbers of potential positives by Grunstein hybridisation, using a S4 cDNA probe, followed by mini-plasmid preparations and agarose gel electrophoresis of any potentially positive colonies.

2.5. *IN VITRO* TRANSCRIPTION

Using the techniques described in section 2.3 full length S4 c-DNA of types 1 and 3 (S4-1 and S4-3) were subcloned into the vector Bluescribe such

that the 5' end of the gene was adjacent to the T7 promoter, thus allowing *in vitro* transcription analysis using T7 RNA polymerase.

2.5.1. Linearisation of plasmid template

The T7 plasmid was linearised downstream of the coding sequence by digestion with *PstI* (S4-3) and *BamHI* (S4-1), to produce as authentic sized transcripts as possible. Generally 50 μg of DNA was digested and complete linearisation was confirmed by agarose gel electrophoresis. The linearised DNA was phenol extracted, precipitated and resuspended in SDW to a concentration of 1 mg/ml.

2.5.2. Preparation of radioactive *in vitro* transcripts

Transcripts containing α ^{32}P-r CTP were produced for size analysis of the T7 run off transcripts.

The reaction was made up at room temperature and incubated at 37°C for 1 hour. The final reaction mixture contained the following:

Reagent	Final conc
Plasmid DNA	2 μg
rUTP, rATP,r GTP	500 μM
RNAsin	1 U/μl
BSA	125 U/μl
DTT	10 mM
Transcription buffer	1 x
^{32}P rCTP	50 μCi
T7 RNA polymerase	10 U

The final volume routinely used was 50 μl.

2.5.3. Size analysis of T7 transcripts

T7 transcripts were analysed by electrophoresis on 1.5 % agarose gels containing 6 % formaldehyde in 1 x MOPS buffer pH 7. The T7 transcripts were denatured in 20 μl of denaturing solution (500 μl DIF, 100 μl 10 x MOPS buffer, 150 μl formaldehyde), at 60°C for 15 minutes and quench cooled on dry ice. The samples were mixed with 2 μl of loading buffer and gels were run at 250 mA for 3 hours, fixed in 10 % glycine, dried under vacuum below 60°C to avoid melting of the agarose, and then autoradiographed to detect the labelled transcripts.

2.5.4. Synthesis of unlabelled and capped transcripts

For *in vitro* translation analysis capped RNA transcripts were produced. To enable calculation of the amount of RNA produced 10 μCi of ^{3}H- rUTP was incorporated into the reaction. This was vacuum dried to remove ethanol and resuspended in 25 μl SDW. The final reaction mix contained the following:

Reagent	final conc
rATP, rUTP	1 mM
rCTP	
rGTP	0.5 mM
Transcription buffer	1 x
DTT	2 mM
RNAsin	0.4 U
DNA	5 μg

CAP	0.5 mM
T7 RNA Polymerase	0.5 U

The final reaction volume was routinely 50 μl.

The reaction was incubated at 37°C for 30 minutes prior to the addition of 1.5 μl 10 mM rGTP and incubation continued for 1 hour at 37°C.

In each *in vitro* transcription reaction the DNA template was removed by digestion with 2 Units of RNAase free DNAase and incubated for 15 minutes at 37°C prior to precipitation using 0.7 M NH_4Ac to separate the RNA transcripts from rNTPs. This RNA was directly used in *in vitro* translation reactions.

2.5.5. Calculation of the amount of T7 transcript produced

The dried mRNA pellet was resuspended in 50 μl of SDW. 2 μl of this was precipitated in 1 ml of 10 % TCA and incubated on ice for 15 minutes. The precipitate was collected on 2.5 cm Whatman No.1 filter discs by passing through a suction funnel, washed twice with 5 % TCA, twice with ethanol, allowed to dry and counted in 'OPTIPHASE' scintillation fluid on a LKB 1212 scintillation counter. The amount of mRNA produced per reaction could then be calculated.

For example:

The cpm produced from the *in vitro* transcription reactions ranged from 2 x 10^3 cpm - 3.5 x 10^3cpm. The amount of mRNA produced per reaction was calculated as follows:

1. The concentration of cold UTP added = 10 mM

In 50 μl have 25 nmoles of cold UTP.

2. Given that 1 μCi of ^{3}H labelled UTP = 2.54 x 10^6 cpm

The amount of UTP as TCA precipitatable RNA can be calculated:

$$\frac{\text{TCA ppt. counts}}{\text{No. of counts in 10 } \mu\text{Ci } ^3\text{H UTP}} \times \text{amount of cold UTP}$$

3. For example: If 2.8×10^3 cpm were precipitated then:

$$\frac{[2.8 \times 10^3 \times 25] \times 25}{2.54 \times 10^6} = \text{nmoles of UTP}$$

$= 0.63$ nmoles of UTP.

4. The average molecular weight of a ribonucleotide = 360

   The amount of RNA produced

   $= [0.68 \times 360 \times 4]$ ng of RNA

   $= 979$ ng

5. The efficiency of counting of the scintillation counter:

From 1 μCi of ^{3}H UTP expect 2.54×10^6 cpm

When 1 μCi was counted it gave 2.2×10^5 cpm.

The efficiency of counting = 9 %

Therefore the total amount of RNA produced was 10 μg.

2.6 *IN VITRO* TRANSLATION

2.6.1. The rabbit reticulocyte lysate system

2.6.1.1. Preparation of lysate

The rabbit reticulocyte lysate used in this study was prepared as described by Hunt *et al.* (1972) by M.A. M^{c}Crae. Aliquots of the lysate were removed from liquid N_2 prior to micrococcal nuclease (Mnase) treatment to remove endogenous mRNA (Pelham & Jackson, 1976).

To 2 ml of lysate were added:

Creatine Kinase (5 mg/ml)	20 µl
Haemin (1 mM)	80 µl
$CaCl_2$ (100 mM)	20 µl
Mnase (1mg/ml)	30 µl

The reaction was incubated at 20°C for 12.5 minutes followed by the addition of 10 µl of 0.5 M EGTA, to chelate the calcium ions. The treated lysate was aliquoted into suitable sizes and stored in liquid N_2 until required.

2.6.1.2. *In vitro* translation

m-RNA from *in vitro* transcription reactions was resuspended to a concentration of approximately 0.6 mg/ml. Generally 1 - 2 µl of mRNA was used per reaction.

The components of the reaction are listed below:

20 x Energy mix:	di-Potassium-ATP	20 mM
	Na-GTP	4 mM
	Tris pH 7.5	0.2 M

This was aliquoted and stored at -20°C. When required 145 mg of di-Tris creatine phosphate and 2 mg of creatine phosphokinase were added to each 2 mls of energy mix.

The reaction mix consisted of the following:

Reagent	Final conc.
Tris pH 7.5	0.625 mM
amino acids	100 μM
Energy mix	1 x
KCl	30 mM
Mg^{2+}	0.5 mM
tRNA	45 $\mu g/\mu l$

Lastly 12.5 μl of lysate and 50 μCi of ^{35}S-met were added and the reaction incubated at 30°C for 1 hour.

The reaction volume routinely used was 25 μl.

2.6.2. *In vitro* translation in S-10 cell extracts

2.6.2.1. Preparation of S-10 cell extracts

S-10 cell extracts were prepared from 1 litre of Type 1 infected, Type 3 infected and uninfected mouse L-cells. The cells were infected at 10 pfu/cell for 16 hours at 34°C and S-10 extracts were prepared as described by Skup and Millward (1977). 1 ml aliquots were stored in liquid N_2 until required.

Endogenous mRNA was removed by treating 1 ml aliquots with 75 Units Mnase and 1 mM $CaCl_2$ at room temperature for 10 minutes. The calcium ions were chelated by the addition of 1.2 mM 2'-deoxythymidine-3',5'-diphosphate. The Mnase treated extracts were aliquoted and stored in liquid N_2 until required.

2.6.2.2. *In vitro* translation

mRNA from *in vitro* transcriptions was resuspended to a concentration of 1 mg/ml. Generally 1 μg of mRNA was used per reaction. The components of the reaction mix are listed below:

5 x Master Mix:

Reagent	Final conc
Hepes	50 mM
KAc	600 mM
MgAc	2.5 mM
Spermidine	250 μM
DTT	5 mM
GTP	2.5 mM
amino acids	100 μM
CPKase	2 mg/ml

To each reaction were added: 10 μl Nuclease treated extract
50 μCi ^{35}S-met
4 μl 5 x master mix
6 μl mRNA + H_2O

The reaction was allowed to proceed at 30°C for 2 hours.

2.7. Measurement of incorporation of radioactivity

For *in vitro* translation analysis, 2.5 µl aliquots were applied to 2.5 cm Whatman No.1 filter discs at various times throughout the reaction, and precipitated in 5 % cold TCA for at least 10 minutes. The filter discs were boiled for 10 minutes in 5 % TCA / 3 % CAS amino acids, followed by 2 washes in ethanol and 2 washes in ether, air dried and the amount of ^{35}S-met incorporated into protein determined by scintillation counting in 'OPTIPHASE' scintillation fluid.

2.8. Analysis of protein products

Protein products were analysed on 10 % vertical polyacrylamide gels using the Laemmli discontinuous buffer system (Laemmli, 1970). Electrophoresis was continued at 40 mA for 3 hours and gels were treated with PPO (2,5 Diphenyloxozole) and DMSO to allow fluorography and dried onto Whatman No.1 filter paper under vacuum for 1 hour at 60 °C, prior to autoradiography.

2.9. TRANSFECTION OF DNA INTO MAMMALIAN CELLS

2.9.1. Electroporation

Mouse L-cells were taken from suspension culture, concentrated by centrifugation at 2 K, 4°C for 10 minutes in a bench centrifuge and resuspended to 10^7 cells/ml in cold PBS. 0.8 mls of cells were mixed with 1 - 10 µg of DNA and the mix electroporated at 0.5 Volts, resulting in a time constant of 0.7 - 0.8 seconds. The samples were incubated on ice for 10 minutes and added to 60 mm

tissue culture dishes containing 5 mls of medium and 10 % FCS. Cells were harvested in 1 ml of cold PBS 48 hours post transfection.

2.9.2. Calcium phosphate transfection

L-cells, HeLa and COS-1 cells were used in transfection assays.

5×10^5 cells were plated onto 60 mm tissue culture dishes and left to form a monolayer by incubating overnight at 37°C. 3 hours prior to transfection the cells were fed with fresh medium containing 10 % serum. For each transfection the following mix was made up:

Relevant DNA	10 μg
sssDNA 1mg/ml	1 μl
1.25 M $CaCl_2$	10 μl
T.E. buffer to	50 μl

50 μl of 2x HBS was added to this mixture and a DNA precipitate allowed to form by standing for 15 minutes at room temperature. 900 μl of media containing 10 % serum was then added. The cells were overlaid with the transfection mixture, incubated at 37°C for 3 hours, with occasional rocking to ensure the DNA was taken up by the cells, and then glycerol shocked for 1 minute with 15 % glycerol in 1 x HBS, washed twice in PBS and overlaid with media + 10 % serum. The cells were harvested in 1 ml of cold PBS 48 hours post transfection The cells were pelleted in a microfuge for 10 minutes and the pellets stored at -20°C until required.

2.10 ANALYSIS OF REPORTER GENE EXPRESSION

2.10.1. Preparation of cell lysates

The cell pellets (from section 2.7) were resuspended in 150 μl of cold CAT lysis buffer incubated on ice for 10 minutes and sonicated in a sonication bath for 4 x 15 second bursts. The nuclei were removed by microfugation for 10 minutes at 4°C.

2.10.2. Determination of the amount of protein in the lysate

The amount of protein used in each reaction was standardised using the BIORAD protein assay kit. 1 μl of the cell lysate was diluted into 1 ml of a 1:5 dilution of the BIORAD assay kit. The amount of protein in each sample was determined by the OD 595 and a standard amount of protein was used per set of assays.

2.10.3. Chloramphenicol acetyl transferase (CAT) assays

CAT assays were performed by the two fluor diffusion assay as described by Neuman *et al.* (1987).

The cell extracts were made up to 50 μl with 100 mM Tris-HCl buffer pH 7.5 and heated at 70°C for 15 minutes to destroy background activity. The reaction mix consisted of:

10 mM Chloramphenicol	50 μl
1 M Tris-HCl buffer pH 7.5	25 μl
^{3}H-acetyl co-enzyme A	0.1 μCi
Distilled H_2O	124 μl

The reaction mix was mixed with the heat treated cell extract, placed in a 5 ml scintillation vial and overlaid with non aqueous scintillation fluid (Toluene + 0.5 % PPO). The reactions were incubated at room temperature (Eastman,

1987) in a scintillation counter with the samples being counted every 15 - 30 minutes for 1 minute, depending on the number of samples under investigation.

2.10.4. ß-galactosidase assays

ß-galactosidase assays were performed as described by Sambrook *et al.* (1989).

Cell extracts containing equal amounts of protein were combined with the following reagents.

100 x Magnesium buffer	3 µl
ONPG	66 µl
Sodium Phosphate buffer to	
a final volume of	300 µl

The reaction was incubated at 37°C for 15 minutes until a yellow colour appeared and the reaction stopped by the addition of 0.5 ml 1 M Na_2CO_3. The amount of enzyme activity was determined spectrophotometrically at 410 nm.

2.11. PRODUCTION OF POLYCLONAL ANTISERUM AGAINST *e*3

2.11.1 Inactivation of reovirus

Antiserum was raised against reovirus type 3 in a New Zealand white rabbit. However, reovirus is highly virulent to mice and to avoid infection of mice in the animal house it had to be inactivated prior to use. To do this 1 mg of virus was made up to 1 ml in 50 mM Tris-HCl pH 7.5. To this 10 µl of a 1:10 dilution of ß-propriolactone was added.. The mixture was left at 4 °C overnight. The following day it was dialysed against several changes of prechilled (4°C)

PBS. The virus was checked for inactivity by plaque assay prior to infection of the animal.

2.11.2 Production of polyclonal antisera

The animal was subcutaneously injected with 0.5 mg CsCl gradient purified, ß-propiolactone inactivated virus, emulsified in complete Freund's adjuvant. Two subsequent boosts , 0.3 mg each, were given in incomplete Freund's adjuvant and the animal was bled 10 days post injection. The activity of the antiserum was checked by plaque reduction assay as described by Offit *et al.* (1984).

2.12 DETECTION OF ø3 IN TRANSFECTED CELLS

2.12.1. Immunoprecipitation

2.12.1.1. Labelling of cells with ^{35}S-met

60 mm tissue culture dishes of transfected cells were labelled for 3 hours at 48 hours post transfection with 100 µCi of ^{35}S-met per dish in met-free GMEMS. The cells were harvested in 300 µl of 10 mM Tris-HCl pH 8.5 and subjected to 3 x freeze thawing followed by 4 x 20 second bursts of sonication in a sonication bath. The cell debris was removed by microfugation for 10 minutes at 4°C and the amount of labelled protein in each sample was determined by TCA precipitation and equal amounts were used for immunoprecipitation.

2.12.1.2. Immunoprecipitation

Samples were made up to 100 µl with 2 x immunoprecipitation buffer 3 (IP3), 10 µl of neat serum was added and the mix incubated for 1 hour at 4°C

with gentle agitation. The sample was then added to pre-swollen protein A sepharose (5mg in 100 μl of IP3) and incubated for 1 hour as above. The supernatant was removed by microfugation for 1 minute and non specifically bound molecules were removed from the beads by sequential washes in IP2, IP2 + 1M NaCl, twice in 1 x IP3 and finally IP1.

The sample was made up to 30 μl with 50 mM Tris-HCl buffer pH 8 and 10 μl of cracking buffer was added prior to boiling the samples for 3 minutes. The immunoprecipitated products were analysed on a 10 % polyacrylamide gel as described in section 2.8.

2.12.2. Immunofluorescence

For immunofluorescence, transfections were performed in 12 well tissue culture dishes. 1 coverslip was placed in each well prior to plating out the cells. It was important to keep the cells as subconfluent as possible. 48 hours post transfection the cells were formaldehyde fixed. This involved washing the monolayers 4 x in PBS, followed by incubating in 3.7 % formaldehyde at room temperature for 5 minutes. The cells were then incubated in 3.7 % formaldehyde + 0.1 % Triton X 100 for 5 minutes and finally in 1 % BSA in PBS for 10 minutes. The fixed monolayers were washed twice with PBS prior to incubation with 20 μl of antisera diluted 1:50 for 1 hour at 37°C. The monolayers were washed twice with PBS and were overlaid with 20 μl of fluorescent conjugate (FITCGAR) diluted 1:40, followed by incubation at 37°C for a further hour in the dark. The monolayers were thoroughly rinsed in PBS to remove any non bound conjugate, mounted onto slides in 10 % glycerol in PBS and viewed under the immunofluorescent microscope.

As a control for immunofluorescence cells were infected with reovirus type 3 at 0.1 m.o.i for 16 hours prior to fixing the cells as described above.

CHAPTER 3

INHIBITION OF HOST CELL PROTEIN SYNTHESIS BY MAMMALIAN REOVIRUS TYPES 1 AND 3

3.1 Introduction

As mentioned in section 1.6 the overall aim of this project was to gain a greater insight into the mechanism underlying the differential effects that types 1 and 3 reovirus have on the inhibition of host cell protein synthesis. These effects were first reported by Sharpe and Fields (1982), who showed that the different abilities of type 2 and type 3 to inhibit host cell protein synthesis was a property of the S4 gene. Subsequently Munemitsu and Samuel (1984) demonstrated that type 1 and type 3 also inhibit host cell protein synthesis to differing extents. The type 1 phenotype does not inhibit protein synthesis at late times in infection whereas type 3 does. Before starting work on the role of the S4 gene in this shut-off it seemed necessary to confirm that the results published by others held true under the conditions employed in our laboratory. That is, could it be demonstrated that reovirus type 3 inhibits host cell protein synthesis to a greater degree than does type 1?

3.2 Rationale

Mouse L-cells were seeded into 12 well tissue culture dishes ($5x10^5$ cells/dish). The cells were infected at 10 pfu/cell with type 1, type 2 or type 3 reovirus. Mock-infected cells were used as a control. 5, 10, 20, 25 and 30 hours post infection, cells were pulse labelled with ^{35}S-met for 30 minutes as described in materials and methods, and harvested by lysing the cells on the plates as described by Munemitsu and Samuel (1984). The recovery of cells was estimated by measuring the total amount of protein present in each sample using the Biorad protein assay kit. The amount of ^{35}S-met incorporated into a standard number of cell equivalents was determined by TCA precipitation. Equivalent amounts of labelled protein were also analysed on 10 % acrylamide gels.

3.3 Results

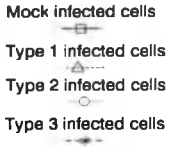
Figure 9 shows that in type 1 infected cells total protein synthesis continues at approximately the same rate as mock infected cells even at late times in infection. However, in type 3 and type 2 infected cells inhibition of protein synthesis commences 20 hours post infection. When equal amounts of labelled protein were analysed on a 10% polyacrylamide gel it was evident that the rates of viral protein synthesis differed between the serotypes (figure 10). As can be seen in figure 10 viral protein synthesis commences between 5 and 15 hours post infection by which time all the viral proteins are being synthesised (tracks A - D 15 hours). In type 3 infected cells viral protein synthesis reaches maximal levels at 15 hours post infection (track D 15 hours), by contrast in type 1 infected cells the maximal level of viral protein synthesis is not reached until at least 25 hours post infection (track B 25 hours). At late times in infection (20 hours +) inhibition of host protein synthesis is evident in type 2 and type 3 infected tracks (tracks C and D, 20 and 25 hours post infection). For example, the intensity of the actin band in type 2 and type 3 infected cells clearly decreases from 20 hours onwards when compared to that of type 1 and un-infected tracks (A - D, 20 and 25 hours).

3.4 Conclusion

The results from this experiment confirm the findings of Munemitsu and Samuel (1984), namely that type 3 reovirus inhibits host protein synthesis to a greater degree than type 1 reovirus. The results also show that the level of type 3 reovirus synthesis reaches a maximum earlier in infection that does type 1.

FIGURE 9: PROTEIN SYNTHESIS IN TYPE 1, 2 AND 3 INFECTED AND MOCK INFECTED L-CELLS.

Mouse L-cells were infected at 10 pfu/cell of type 1, type 2 and type 3 reovirus and mock infected cells were used as a control. At 5, 10, 15, 20, 25 and 30 hours post infection the cells were pulse labelled with ^{35}S-met as described in Methods. The amount of ^{35}S-met incorporated into a standard number of cell equivalents was determined by TCA precipitation as described in section 3.2.



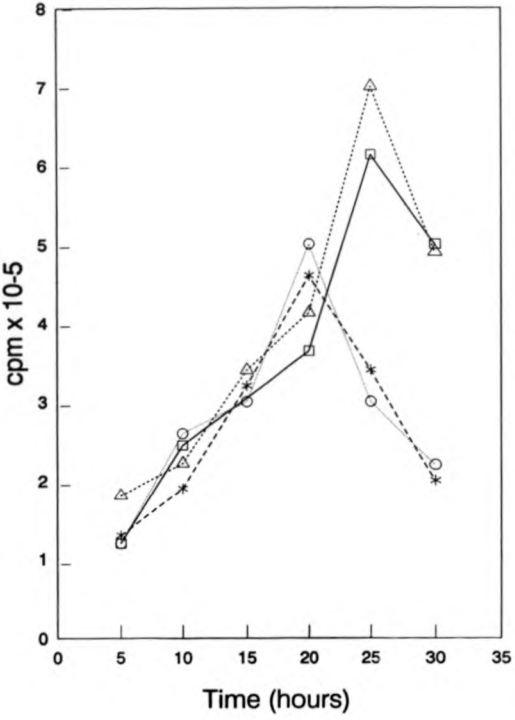

cpm x 10-5
Time (hours)
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30
35

FIGURE 10: PROFILE OF PROTEIN SYNTHESIS IN REOVIRUS TYPE 1, 2 AND 3 INFECTED L-CELLS AT VARIOUS TIMES POST INFECTION.

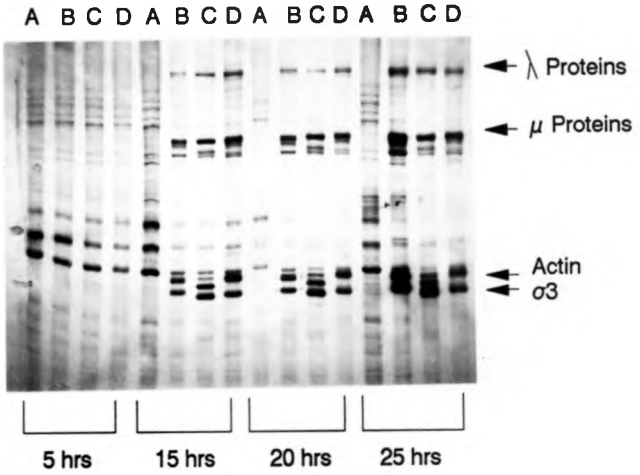
A = CONTROL

B = TYPE 1 INFECTED

C = TYPE 2 INFECTED

D = TYPE 3 INFECTED

Equal amounts of labelled protein from 5, 15, 20 and 25 hour time points were analysed on a 10 % polyacrylamide gel which was fluorographed as described in Materials and Methods.


A B C D A B C D A B C D A B C D
λ Proteins
μ Proteins
Actin
σ3
5 hrs
15 hrs
20 hrs
25 hrs

CHAPTER 4

DEVELOPMENT OF REAGENTS

4.1 Introduction

To investigate the role of the S4 gene in the shut-off of host protein synthesis by mammalian reovirus it was first necessary to obtain full length c-DNA clones of the S4 gene from both type 1 and 3 virus (S4-1 and S4-3). The sequence of the two S4 genes was available (Atwater *et al.*, 1986) and allowed for the construction of oligonucleotides specific to the 5' and 3' ends of the S4 genes (figure 8, Materials and Methods). These were used to specifically prime S4 c-DNA synthesis using total viral dsRNA as a template.

The oligonucleotides contain unique restriction enzyme sites overhanging the ends of the gene, the sites used being chosen on the basis that they did not occur within the gene, but were present in the M13 polylinker. This was done in order that the full length cDNA generated would have a set of unique restriction enzyme sites at either end to allow for easy manipulation of the cloned gene into other vectors. The restriction enzyme sites *Eco47III* and *PstI* were constructed to allow precise cutting at the 5' and 3' ends of the genes, such that an exact sized S4 c-DNA could be isolated if necessary. S4-1 cDNA contains an internal *PstI* site, however *PstI* was the only restriction enzyme sequence that could be constructed to cut precisely at the 3' end of the gene. Due to the high homology at the 5' and 3' ends of the S4-1 and S4-3 genes the same 5' and 3' oligonucleotides were used in cDNA synthesis (figure 8).

4.2 c-DNA cloning of the S4 gene

The cloning strategy initially used is outlined in figure 11 and the methods employed are described in Materials and Methods.

The cDNA was cloned into the *PstI* site of pAT153. pAT153 is resistant to ampicillin and tetracycline. *PstI* lies within the ampicillin resistance region of the

FIGURE 11: CLONING STRATEGY

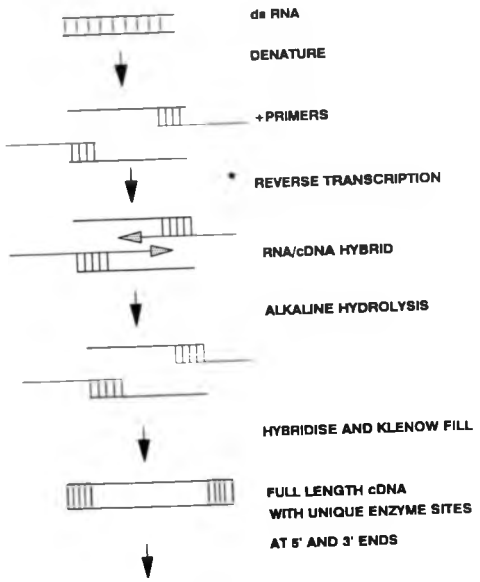
Full length cDNA was synthesised according to the basic reaction described by McCrae and McCorquodale (1982) and described in Methods.

dsRNA was denatured and the resulting ssRNA hybridised with the primers illustrated in figure 8.

Two RNA/cDNA hybrids were produced by reverse transcription and the RNA removed by alkaline hydrolysis. The complementary cDNA strands were hybridised and the overhanging ends produced by the primers filled in by Klenow I polymerase as described in Methods.

The full length cDNA was isolated from a 1.5 % alkaline agarose gel. It was C-tailed and the cloning vector pAT153 was digested with *Pst*I, and G-tailed. cDNA and vector DNA were hybridised together and transformed into the *E. coli* strain MC1061. Full length inserts were identified by antibiotic screening, mini-plasmid preparations and restriction enzyme analysis (figure 12).

* Point of PCR where the cDNA was amplified by McCrae (personnel communication, 1987).


ds RNA
DENATURE
+PRIMERS
REVERSE TRANSCRIPTION
RNA/cDNA HYBRID
ALKALINE HYDROLYSIS
HYBRIDISE AND KLENOW FILL
FULL LENGTH cDNA
WITH UNIQUE ENZYME SITES
AT 5' AND 3' ENDS
C-TAIL cDNA AND HYBRIDISE WITH
G-TAILED pAT153 LINEARISED WITH PSTI

plasmid, therefore potential cDNA clones were identified by screening for ampicillin sensitivity. Any ampicillin sensitive colonies were screened for the S4 gene by mini-plasmid preparations and restriction enzyme analysis. However initial attempts using the strategy outlined in figure 11 failed to isolate any full length cDNA clones. One possibility for this was that insufficient cDNA was being made from the dsRNA template as only faint cDNA bands were isolated from alkaline agarose gels. The polymerase chain reaction (PCR) was used to directly amplify the initial cDNA product. This procedure increased the amount of cDNA produced at least 50 fold. The cDNA was amplified and successfully cloned into the *PstI* site of the cloning vector pAT153 by McCrae (personnel communication, 1987) using the strategy outlined in figure 11. The resulting colonies were screened for the S4 gene by digestion with *Hind*III and *BamH*I (present in the oligonucleotides at the 5' and 3' ends of the gene respectively) which released a 1.2 kb insert of S4-1 and S4-3 genes (figure 12). Further enzyme digests also agreed with the predicted enzyme analysis obtained from the sequence of the genes. Single digestion with *HindIII* (in the 5' oligo) and *BamHI* (in the 3' oligo) also orientated the S4 inserts.

4.3 Initial subcloning of the S4 gene

In order to use the S4 genes for investigations they had to be transferred from the pAT153 vector to a more versatile vector suitable for producing mRNA transcripts *in vitro*, and for manipulation to other vectors for *in vivo* experiments. The Bluescribe vector which contains the T3 and T7 RNA polymerase promoters in the coding region of the *LacZ* gene, at either end of the M13 polylinker, met these requirements. Recombinants were readily selected in the host strain, JM103, on L-amp plates containing IPTG and X-gal as colonies carrying a viral insert were white compared to the blue colonies obtained when only the vector plasmid was present.

FIGURE 12: IDENTIFICATION OF FULL LENGTH cDNA CLONES

M1 = pBr322 digested with *Hinfl*

A = S4-1 digested with *PstI*

B = S4-1 digested with *HindIII/BamHI*

C = S4-3 digested with *PstI*

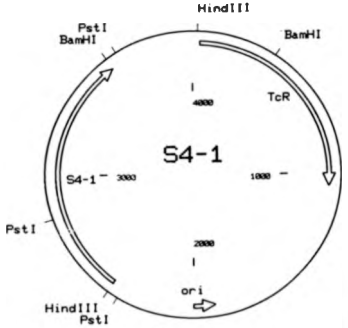
D = S4-3 digested with *HindIII/BamHI*

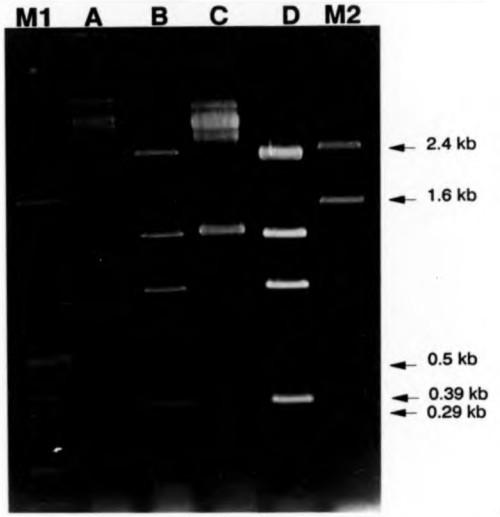
M2 = pBr322 digested with *AccI*

Full length cDNA inserts in pAT153 were identified by *HindIII/BamHI* digestion, releasing a 1.2 Kb insert (tracks B and D). The digest shown in track A is only a partial digest, however the relevant bands can be clearly seen. The bands released by *PstI* digestion are slightly larger than those predicted for S4-1 and S4-3 due to the presence of the G and C tails added to the cDNA and vector during the cloning procedure. *PstI* digestion of S4-3 released full length S4-3 (1.2 Kb), track C, however S4-1 contains an internal *PstI* at postion 489 resulting in two bands, 700 bp and 489 bp, track A. This site was used to distinguish S4-1 and S4-3.

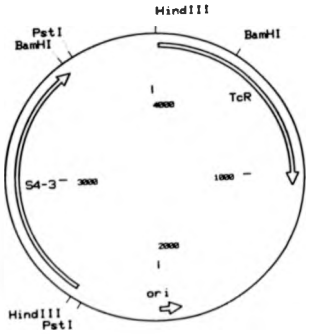
The plasmids are also represented and the position of the restriction enzymes used identified.

A: S4-1 in pAT153





B: S4-3 in pAT153



The two S4 genes were subcloned into the Bluescribe polylinker in such a way that the 5' end of the gene was adjacent to the promoter for T7 RNA polymerase (T7 promoter), to allow for synthesis of S4 mRNA *in vitro* using T7 RNA polymerase. The subcloning procedure is outlined in figure 13. The ligated DNA was transformed into JM103 and grown on L-amp plates containing X-gal and IPTG. Potential positives i.e white colonies, were picked and screened for S4 inserts by mini-plasmid preparations and restriction enzyme analysis. Full length S4 inserts were identified and orientated by *HindIII* digestion (figure 14).

4.4 Analysis of reagents for *in vitro* experiments

In vitro experiments aimed at determining the role of the S4 gene in inhibiting the translation of a eukaryotic protein will be discussed in the following chapters. For this work a eukaryotic gene, of approximately the same size as the S4 gene was required as a control in the transcription and translation studies. The ß-Actin gene fitted these requirements and a clone of it (T7ß-ACT) (obtained from K.Leppard, 1988), in Bluescribe was used in this work.

4.4.1 Synthesis of T7 transcripts

To synthesise full length mRNA transcripts of the S4 and ß-actin genes *in vitro* using T7 RNA polymerase, the genes were first linearised at the 3' end by digestion with *PstI* (S4-3), *BamHI* (S4-1) and *HindIII* (ß-actin).

The size of the mRNAs produced were analysed by the synthesis of high specific activity mRNA transcripts, which allowed detection of the transcription products by autoradiography, as described in Materials and Methods. Figure 15(a) shows that the S4-1 and S4-3 mRNA produced were full length (1.2 Kb) and the T7ß-ACT produced ß-actin mRNA (1.8 Kb) of the size predicted for a full length transcript (figure 15(b)).

FIGURE 13: SUBCLONING OF THE S4 GENE(S) INTO BLUESCRIBE.

The Bluescribe vector was prepared by first digesting with *EcoRI*. The liberated ends were blunted using Klenow DNA polymerase I. The blunt ended DNA was digested with *BamHI* and the 5' phosphate groups removed using Calf Intestinal Phosphatase (CIP) as described in Methods.

The S4 insert(s) was released from pAT153 by *NruI/BamHI* digestion. These enzyme sites are present in the oligos (figure 8) at the 5' and 3' ends of the gene respectively. The insert was isolated on and excised from a 1 % agarose gel as described in Methods. The vector and insert DNA mixture were ligated, transformed into JM103 and grown on L-amp plates containing X-gal and IPTG. Potential positives i.e. white colonies, were picked and screened for S4 inserts by mini-plasmid preparations and restriction enzyme analysis (figure 14).

The position of the T7 promoter (T7) is indicated in the diagram. The S4 gene was inserted down stream of this to allow for the synthesis of S4 mRNA using T7 RNA polymerase.

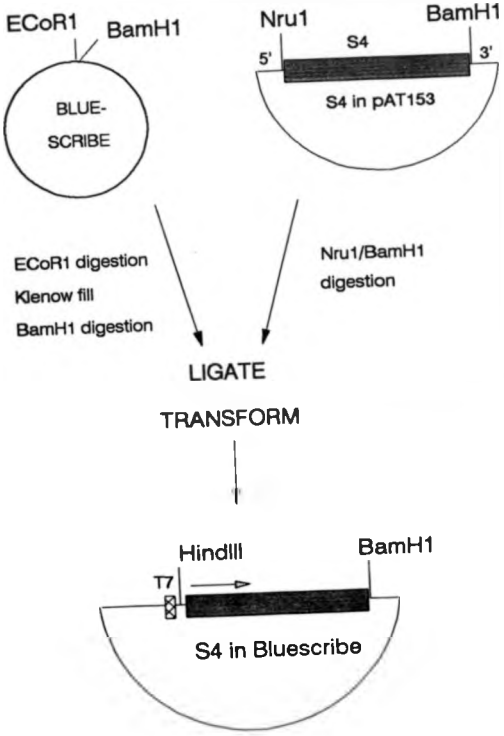

ECoR1
BamH1
BLUE-
SCRIBE
Nru1
BamH1
S4
5'
3'
S4 in pAT153
ECoR1 digestion
Klenow fill
BamH1 digestion
Nru1/BamH1
digestion
LIGATE
TRANSFORM
HindIII
BamH1
T7
S4 in Bluescribe

FIGURE 14: IDENTIFICATION OF THE S4 GENE(S) IN BLUESCRIBE.

A = S4-1

B = S4-3

M = BRL 1Kb ladder

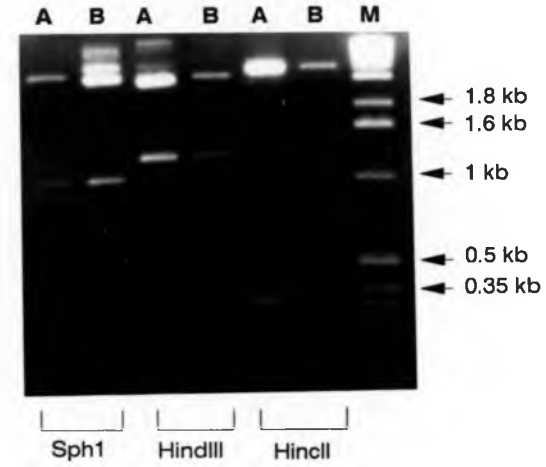
S4 inserts in Bluescribe were identified by *HindIII*,*SphI* and *HincII* digestion and were size analysed on a 1 % agarose gel.

HindIII and *SphI* were used to orientate the inserts. *SphI* cuts at position 184 in both S4-1 and S4-3 and in the Bluescribe polylinker at the 3' end of the gene, releasing a 1 Kb insert. *HindIII* cuts at the 5' and 3' ends of the gene releasing a 1.2 Kb full length S4-1 and S4-3 insert.

HincII was used to identify the two genes, as it cuts S4-1 once at position 857 and S4-3 twice at position 437 and 857 and in the polylinker at the 3' end of the gene resulting in one band of 350 bp for S4-1 and two bands of 350bp and 400 bp for S4-3.

The diagram beneath the photograph represents the bands released by the digests. The enzymes used did not cut elsewhere in the vector.

T7 = T7 promoter


A
B
A
B
A
B
M
1.8 kb
1.6 kb
1 kb
0.5 kb
0.35 kb
Sph1
HindIII
HincII

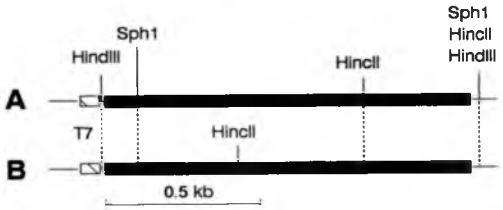

Sph1
HincII
HindIII
HindIII
Sph1
HincII
A
T7
HincII
B
0.5 kb

FIGURE 15: ANALYSIS OF mRNA TRANSCRIPTS

A. SIZE ANALYSIS OF S4-1 AND S4-3 mRNA

A = S4-1 mRNA transcript 1.2 kb

B = S4-3 mRNA transcript 1.2 kb

C = Reovirus M2 mRNA transcript 2.2 kb

D = Reovirus S1 mRNA transcript 1.4 kb

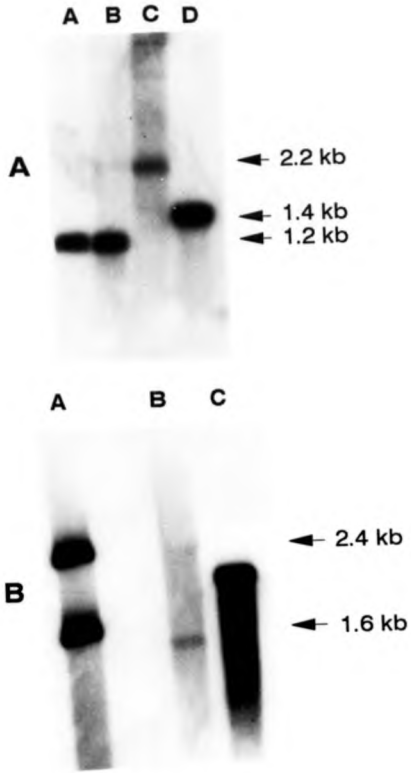
B. SIZE ANALYSIS OF ß-ACTIN mRNA

A = Radioactively labelled pBr322 digested with *AccI*

B = S4-3 mRNA transcript 1.2 kb

C = ß-actin mRNA transcript 1.8 kb

mRNA was produced by *in vitro* transcription using T7 RNA polymerase as described in Methods. The transcripts were size analysed on 1.5 % agarose gels containing 6 % formaldehyde in 1 x MOPS buffer pH 7 as described in Methods. The radioactively labelled DNA marker was prepared by incorporating 50 μCi of α^{32}P dGTP into a Klenow I DNA polymerase reaction as described in Methods.


A B C D
A
2.2 kb
1.4 kb
1.2 kb
A B C
B
2.4 kb
1.6 kb

Stocks of 'unlabelled' capped mRNA transcripts were prepared for *in vitro* translation analysis as described in Materials and Methods.

4.5 Summary

Full length cDNA clones of S4-1 and S4-3 in the vector pAT153 were made available for this work. These were transferred to the more versatile Bluescribe vector such that mRNA sense transcripts could be produced *in vitro* using T7 RNA polymerase. ß-actin was chosen as a control eukaryotic cell gene for the *in vitro* translation studies to be discussed in the following chapters. The ß-actin gene was also available in a clone from which mRNA transcripts could be produced *in vitro* using T7 RNA polymerase. The transcripts produced *in vitro* were all of the size predicted for full length mRNAs (S4-1 and S4-3 1.2 Kb and ß-actin 1.8 Kb). 10 μg of 5' capped mRNA was routinely produced for *in vitro* translation assay systems. The systems in which this mRNA was used were:

1. The rabbit reticulocyte lysate system
2. S-10 cell free *in vitro* translation system

The development of these systems and the experiments carried out will be discussed in the following chapters.

THE EFFECT OF σ3 ON THE TRANSLATION OF EUKARYOTIC mRNA

Introduction

The mRNA produced in Chapter 4 was used in several *in vitro* translation systems. The effect of σ3 produced *in vitro* on the translation of eukaryotic proteins was investigated, in an attempt to gain a greater insight into the underlying differences the two serotypes have on the inhibition of host protein synthesis. The use of the rabbit reticulocyte lysate system will be discussed in chapter 5 and the use of the translation capacity of cell free extracts prepared from reovirus infected and uninfected L-cells in chapter 6. However there are many limitations to *in vitro* experiments and to ascertain if the effects observed in the *in vitro* systems hold true for events that occur in infected cells the development of an *in vivo* assay system also using S4 cDNA will be discussed in chapters 7, 8 and 9.

CHAPTER 5

THE RABBIT RETICULOCYTE LYSATE SYSTEM

5.1 Introduction

The rabbit reticulocyte lysate *in vitro* translation system is a very efficient eukaryotic cell free protein synthesising system. Endogenous mRNA can be destroyed by pre-treatment of the lysate with micrococcal nuclease (Mnase) allowing the specific translation of exogenously added mRNA (Pelham & Jackson, 1976). This system was used to study the effect $\sigma 3$, synthesised *in vitro* from T7 mRNA transcripts, had on the *in vitro* translation of ß-actin mRNA.

5.2 Optimisation of the *in vitro* translation system

Endogenous mRNA was removed by treatment of the lysate with Mnase and *in vitro* translation reactions were performed as outlined in methods. The activity of the reticulocyte lysate system is sensitive to Mg^{2+}, K^+ and mRNA concentrations. To optimise these parameters S4 mRNA was translated at varying concentrations of K^+ while keeping the Mg^{2+} concentration constant. The optimum level of K^+ ions was found to be 90 mM (Figure 16(a)). To optimise the Mg^{2+} concentration, the K^+ concentration was kept constant at 90 mM and Mg^{2+} concentration varied. Figure 16(b) shows that optimum incorporation of ^{35}S-met into protein was achieved at 1 mM Mg^{2+}.

Using these optimal salt conditions the ability of the system to translate various concentrations of mRNA was investigated. Figure 17(a) shows that optimal translation was achieved at 40 ng/μl after which the system becomes saturated for mRNA. Finally the ability of the system to translate equivalent amounts of capped and uncapped mRNA was studied. Figure 17(b) shows that capped mRNA is translated slightly more efficiently than uncapped mRNA, as reported for the reticulocyte system (Dasso & Jackson, 1989).

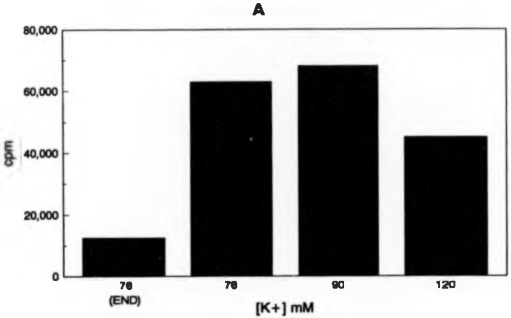
FIGURE 16a: OPTIMISATION OF RETICULOCYTE LYSATE FOR [K^+]

In vitro translations were carried out at Mg^{2+} and S4 mRNA concentrations of 1 mM and 40 ng/μl respectively. The K^+ concentration was varied over the range 76 mM to 120 mM. The results are expressed as the amount of ^{35}S-met incorporated into protein at the 60 minute time point of the reaction, determined by TCA precipitation as described in Methods.

END = endogenous

FIGURE 16b: OPTIMISATION OF RETICULOCYTE LYSATE FOR [Mg^{2+}]

The K^+ and S4 mRNA concentrations were maintained at 90 mM and 40 ng/μl respectively. The Mg^{2+} concentration was varied over the range 0.5 mM to 10 mM.


A
80,000
60,000
40,000
20,000
0
cpm
76
(END)
76
90
120
[K+] mM

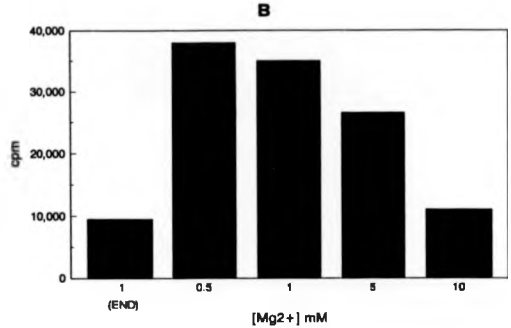

B
40,000
30,000
20,000
10,000
0
cpm
1
(END)
0.5
1
5
10
[Mg2+] mM

FIGURE 17a: OPTIMISATION OF mRNA CONCENTRATION

The Mg^{2+} and K^{+} concentrations were maintained at 1 mM and 90 mM respectively. The S4 mRNA concentration was varied in the range of 20 ng/μl to 80 ng/μl.

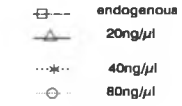
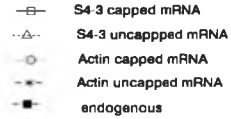


FIGURE 17b: TRANSLATION OF CAPPED AND UNCAPPED mRNA

Capped and uncapped S4 and actin mRNA were translated at the optimal conditions described in figure 16.



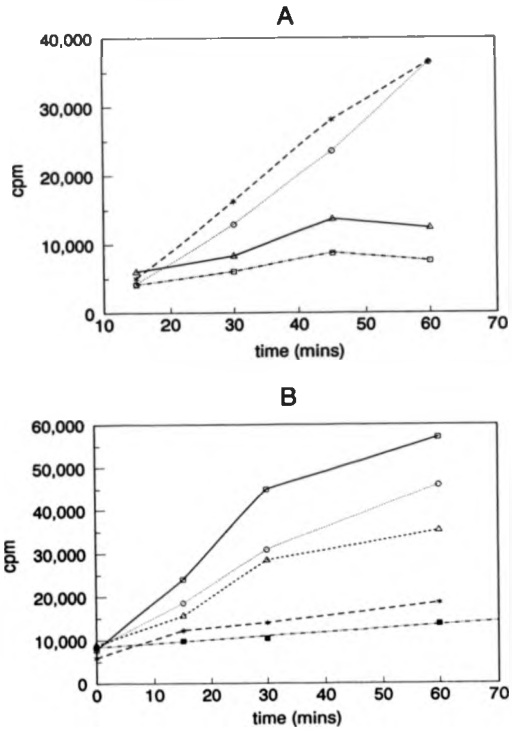

A
40,000
30,000
20,000
10,000
0
cpm
10
20
30
40
50
60
70
time (mins)
B
60,000
50,000
40,000
30,000
20,000
10,000
0
cpm
0
10
20
30
40
50
60
70
time (mins)

FIGURE 18: SIZE ANALYSIS OF *IN VITRO* TRANSLATION PRODUCTS.

Act = ß-actin

σ3.3 A = type 3 σ3 from S4-3 BS

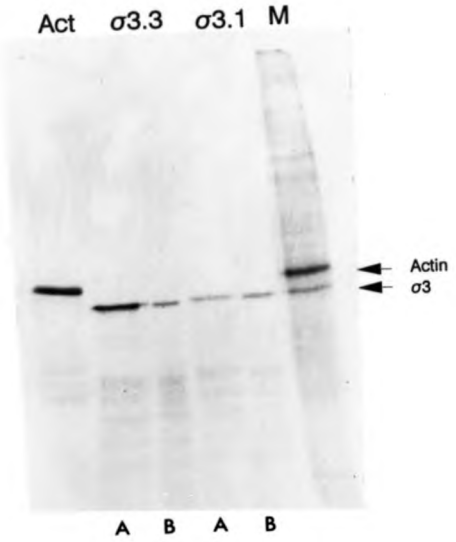
σ3.3 B = type 3 σ3 from HPA-3 (see figure 32)

σ3.1 A = type 1 σ3 from S4-1 BS

σ3.1 B = type 1 σ3 from HPA-1 (see figure 32)

M = Type 3 infected cell lysate

S4-1, S4-3 and ß-actin mRNAs were translated in reticulocyte lysates as described in Methods. The translation products were fractionated on a 10 % polyacrylamide gel run at 40 mA for 3 hours and fluorographed as described in Methods.
The translation efficiencies of both S4-1 and S4-3 mRNA were the same (data not shown).


Act
σ3.3
σ3.1
M
Actin
σ3
A
B
A
B

Thus the optimal conditions determined for the reticulocyte lysate and those used in the following experiments were 90 mM for K^+, 1 mM for Mg^{2+}, and 40 ng/μl for mRNA .

5.3 Size analysis of *in vitro* translation products

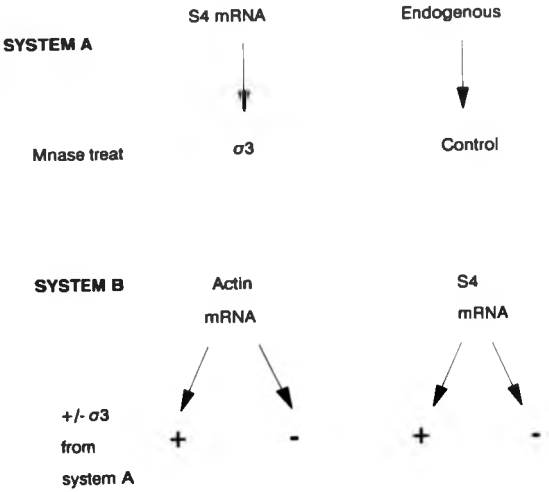
The size of the translation products produced from the *in vitro* synthesised S4 mRNA and ß-actin mRNA were analysed on 10 % polyacrylamide gels. Figure 18 shows that *in vitro* transcription produces functional mRNA which is translated to authentic sized σ3 and ß-actin proteins in the rabbit reticulocyte lysate system.

5.4 The effect of type 3 σ3 on the translation of a eukaryotic protein.

5.4.1 Rationale

To investigate the effect of type 3 σ3 on the translation of a eukaryotic protein the experimental rationale outlined in figure 19 was developed. It involves the use of two *in vitro* translation systems: A and B. The strategy was to investigate the effect of the translation products synthesised in A on the *in vitro* translation of mRNA in B. In A a comparatively large amount of type 3 σ3 was synthesised. As a negative control *in vitro* translation was carried out without the addition of mRNA to the system (i.e. endogenous). At the end of the reaction the remaining mRNA and tRNA in the system were removed by Mnase treatment so they would not interfere with the *in vitro* translation capacity of system B. Increasing amounts of σ3 or endogenous from system A were added to system B containing ß-actin or S4 mRNA. The activity of each reaction in system B was determined by TCA precipitation of 5 μl aliquots of each reaction at 15 minute time intervals. Equal volumes of each of the reaction mixes were analysed on 10 % polyacrylamide gels.

FIGURE 19: RATIONALE FOR IN VITRO ASSAY SYSTEM



ANALYSIS OF PROTEIN PRODUCTS

5.4.2 Results

Figure 20 shows that there was a gradual decrease in the extent of ^{35}S-met incorporation with addition of increasing amounts of σ3 or endogenous from A to the translation of ß-actin or S4-3 in *in vitro* translation B. There was little difference in the amount of stimulation of S4-3 translation between the addition of equal volumes of σ3 or endogenous samples from A. However a slight decrease in the translation of ß-actin was observed on the addition of σ3 to B compared to the addition of endogenous, suggesting that σ3 may have a slight inhibitory effect on ß-Actin translation.

To further analyse the effects of σ3 on ß-actin translation equal volumes of the samples from B were fractionated on 10 % polyacrylamide gels. Figure 21 shows that σ3 from A was being carried through to the second reaction. However, there was no obvious difference in the intensity of the ß-actin bands produced in those where σ3 was added when compared to those in which negative control was added. This suggested that in this system σ3 has no direct effect on ß-actin translation.

One possible explanation for the apparent inhibition evident in figure 20, was that addition of increasing amounts of A to B might lead to changes in salt concentration. To investigate this possibility, following Mnase treatment of A it was dialysed overnight against 10 mM Tris pH 7.5 before being added to the second translation reactions. Unfortunately both σ3 and endogenous reactions derived dialysates prevented the synthesis of both σ3 and ß-actin in the second translation assay (data not shown).

5.5 Summary

The rabbit reticulocyte lysate system was used to examine the effect of type 3 σ3 on the translation of cellular and viral mRNA. Full length σ3 and ß-actin

FIGURE 20: THE EFFECT OF TYPE 3 σ3 ON THE TRANSLATION OF ACTIN AND S4-3 mRNA.

A = Actin mRNA + σ3 from system A

B = Actin mRNA + endogenous from system A

C = S4-3 mRNA + σ3 from system A

D = S4-3 mRNA + endogenous from system A

The effect of type 3 σ3 produced in system A on the translation of actin or S4 mRNA in system B was examined as described in section 5.1.4.1. *In vitro* translations were carried out as described in Methods.

The results are expressed as the fold increase in stimulation of ^{35}S-met incorporation between 0 and 60 minutes of translation.

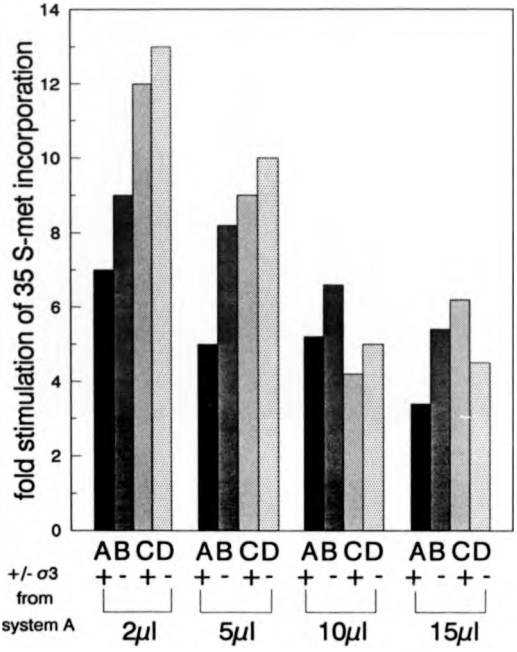
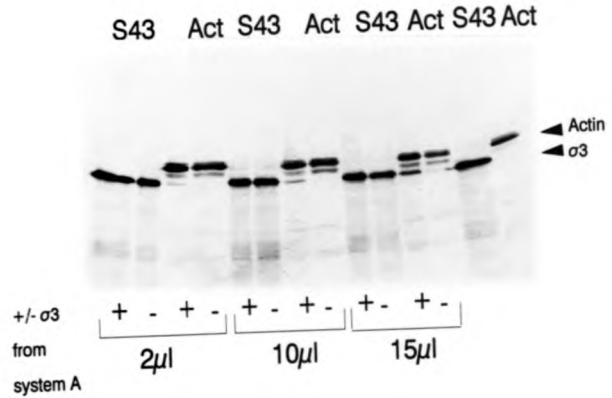

fold stimulation of 35 S-met incorporation
14
12
10
8
6
4
2
0
AB CD AB CD AB CD AB CD
+/- σ3 from system A
+ - + - + - + - + - + - + - + -
2μl
5μl
10μl
15μl

FIGURE 21: THE EFFECT OF TYPE 3 σ3 ON THE TRANSLATION OF ACTIN AND S4 mRNA.

Equal volumes of the reactions from system B were analysed on 10 % polyacrylamide gels, run at 40 mA for 3 hours and fluorographed as described in Methods.


S43 Act S43 Act S43 Act S43 Act
Actin
σ3
+/- σ3
+ - + - + - + - + - + -
from
system A
2μl
10μl
15μl

proteins were produced by the system. However, the *in vitro* translation assay system developed to investigate the effect of type 3 σ3 on translation of ß-actin provided no further insight into how this protein may act in inhibition of host cell protein synthesis in infected cells.

CHAPTER 6

TRANSLATION OF S4 AND β-ACTIN mRNA IN UNINFECTED AND INFECTED S-10 L-CELL EXTRACTS.

6.1 Introduction

Cell-free extracts prepared from mock infected and viral infected cells have been widely used to study the factors involved in viral control of host protein synthesis. For example, Jen and Thach (1982) used lysates prepared from encephalomyocarditis virus-infected (EMCV) L-cells to investigate the ability to translate capped mRNAs. Many of the experiments used to determine the mechanism by which poliovirus inhibits host cell protein synthesis were determined by comparison of cellular and viral mRNA translation in extracts prepared from poliovirus-infected and uninfected HeLa cells. Skup and Millward (1977) reported that mRNA could be efficiently translated in cell-free extracts prepared from mock-infected or reovirus infected L-cells. We developed this system to examine the ability of cell free extracts prepared from type 1 infected , type 3 infected and uninfected L-cells to translate viral and ß-actin mRNA prepared by *in vitro* transcription.

The objective of these experiments was to determine if the different extracts varied in their translational potential, that is, do type 3 infected cell extracts translate ß-actin mRNA with the same efficiency as those from type 1 infected and uninfected cells?

6.2 Optimisation of *in vitro* translation derived from infected and uninfected L-cells

6.2.1 Mnase treatment

The amount of endogenous mRNA removed by Mnase treatment and the ability of exogenous mRNA to restore the translational ability of the extract was examined at Mnase treatments of 5 and 10 minutes.

FIGURE 22: TIME COURSE FOR Mnase TREATMENT OF S-10 CELL EXTRACTS.

A = Non Mnase treated extract

B = 10 minute Mnase treated extract + 50 ng/μl S4 mRNA

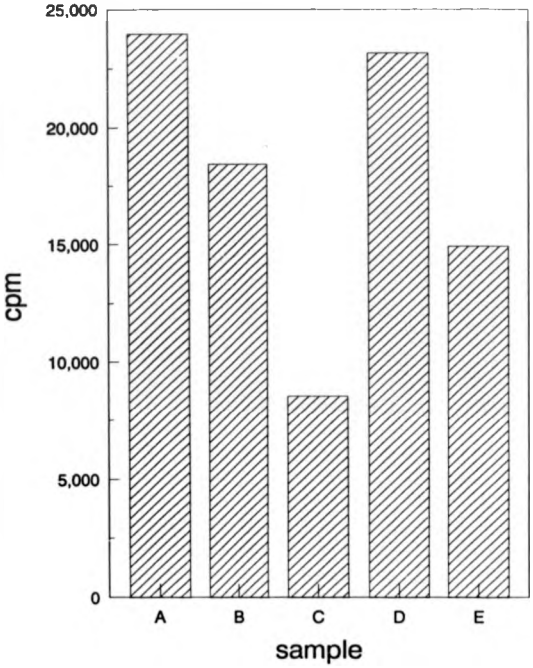
C = 10 minute Mnase treated extract endogenous.

D = 5 minute Mnase treated extract + 50 ng/μl S4 mRNA

E = 5 minute Mnase treated extract endogenous

The S-10 cell extracts were treated with Mnase for 5 or 10 minutes as described in Methods. *In vitro* translations were carried out using 50 ng/μl of S4 mRNA, 1 mM Mg^{2+} and 10 mM K^{+} as described in Methods.

The results are expressed as the total amount of ^{35}S-met incorporated into each sample at the 90 minute time point of the reaction.


25,000
20,000
15,000
10,000
5,000
0
cpm
A
B
C
D
E
sample

The results are shown in figure 22. As expected highest incorporation was seen in the non Mnase treated samples. Treatment of the extract for 5 minutes with Mnase reduced the endogenous activity to approximately one half its original activity and Mnase treatment for 10 minutes to one quarter. Despite the fact that the full activity of the extract was restored on addition of 50 ng/μl of S4 mRNA to the 5 minute Mnase treated extract, the actual fold stimulation seen compared to the endogenous control was only 1.5. The level of stimulation was slightly greater in the extract that had been treated for 10 minutes, even though the original activity given by the extract was not fully restored As a result of this experiment 10 minute treatment of Mnase was chosen for further studies.

6.2.1.2 K^+ and Mg^{2+} optimisation

The system was optimised for Mg^{2+} and K^+ concentrations (figure 23). The optimum concentration of Mg^{2+} was found to be 0.5 mM and for K^+ 10 mM.

6.2.1.3 mRNA optimisation

The capacity of the system to translate varying amounts of S4 mRNA was investigated using final concentrations of 50 ng/μl, 100 ng/μl, 200 ng/μl and 300 ng/μl. Analysis on 10 % polyacrylamide gels of the σ3 produced at these different levels of mRNA showed that there was no change in the amount synthesised (figure 24).

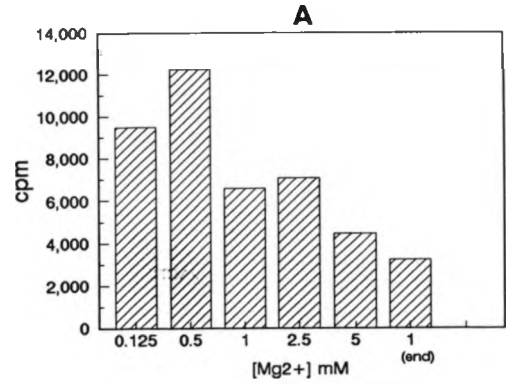
Therefore the optimum conditions for translation in S-10 extracts were: Mnase treatment of 10 minutes, 0.5 mM Mg^{2+}, 10 mM K^+ and 50 ng/μl mRNA. These conditions were used in subsequent experiments.

FIGURE 23a: OPTIMISATION OF S-10 EXTRACTS FOR Mg^{2+}

K^+ and mRNA concentrations were maintained at 10 mM and 50 ng/μl respectively. Mg^{2+} titration was over the range from 0.125 to 5 mM.

FIGURE 23b: OPTIMISATION OF S-10 EXTRACTS FOR K^+

Mg^{2+} and mRNA concentrations were maintained at 0.5 mM and 50 ng/μl respectively. K^+ titration was over the range 5 mM to 20 mM.


A
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
cpm
0.125
0.5
1
2.5
5
1
(end)
[Mg2+] mM

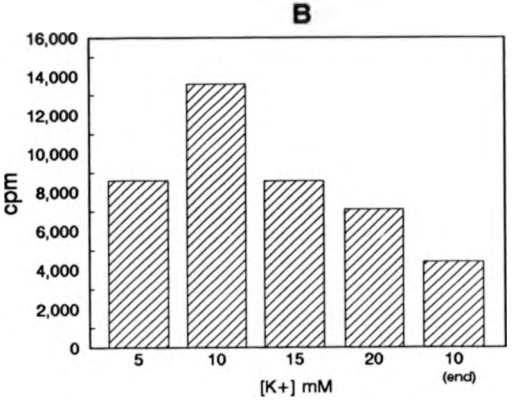
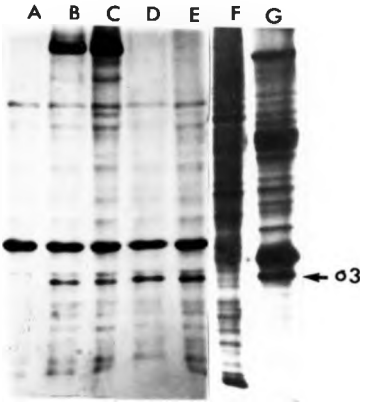

B
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
cpm
5
10
15
20
10
(end)
[K+] mM

FIGURE 24: mRNA OPTIMISATION OF S-10 CELL EXTRACTS



A = Endogenous control

B = 50 ng/μl of S4-3 mRNA

C = 100 ng/μl of S4-3 mRNA

D = 200 ng/μl of S4-1 mRNA

E = 300 ng/μl of S4-1 mRNA

F = Mock-infected cell lysate

G = Type 3 infected cell lysate

Increasing amounts of S4 mRNA were translated as described in Methods. The amount of σ3 produced was analysed on a 10 % polyacrylamide gel as described in Materials and Methods.

6.3 Capacity of each of the extracts to translate viral and cellular mRNA

The ability of the S-10 extracts prepared from uninfected, type 1 and type 3 infected L-cells to translate mRNA of viral and cellular origin (S4 and ß-actin respectively) was investigated. The effect of type 1 and 3 σ3 on the translation of endogenous mRNA remaining in the extracts after Mnase treatment was also examined. *In vitro* translations were carried out as described in Materials and Methods. The activity of each extract was qualitatively analysed on a 10 % polyacrylamide gel (figure 25). As can be seen from figure 25 cellular and viral mRNAs were translated in extracts prior to Mnase treatment (tracks A-C end - Mnase) compared to the endogenous proteins translated in Mnase treated extracts (tracks A-B + Mnase). It is evident that the total translation activity is less in the type 3 extracts (track B end -Mnase) than in type 1 infected and uninfected extracts (tracks A and C end -Mnase). In fact, the type 3 extract did not even translate exogenously added S4 mRNA efficiently (tracks B S4-1 and S4-3). ß-actin mRNA was not efficiently translated in any of the extracts (tracks A-C ACT). However, type 1 and 3 σ3 of the predicted size were synthesised in both uninfected and type 1 infected extracts (tracks A and C S4-1 and S4-3). Expression of type 3 σ3 in either of these extracts did not have any effect on the expression of the remaining endogenous proteins when compared to the endogenous tracks or those expressing type 1 σ3.

The presence of the high molecular weight protein products in extracts translating S4-3 mRNA was further investigated (figure 25 tracks A-C S4-3). The stock of S4-3 DNA which had been used in the previous experiments had been linearised with *PstI* prior to *in vitro* transcription. *PstI* digestion produces a 3' protruding end, which may cause non-specific initiation of RNA transcripts of the DNA template resulting in the synthesis of high molecular weight proteins. When

FIGURE 25: TRANSLATION PRODUCTS SYNTHESISED IN S-10 CELL EXTRACTS

A = Mock infected extract

B = Type 3 infected extract

C = Type 1 infected extract

S-10 *In vitro* translations were carried out as described in Methods and equal volumes of each sample were analysed on a 10 % polyacrylamide gel.

For an explanation of the high molecular weight protein present in the S4-3 tracks see figure 26.

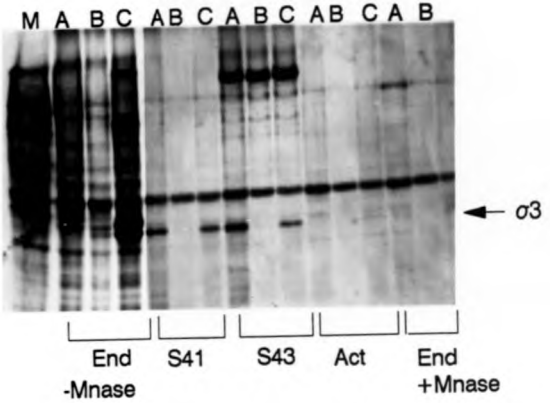
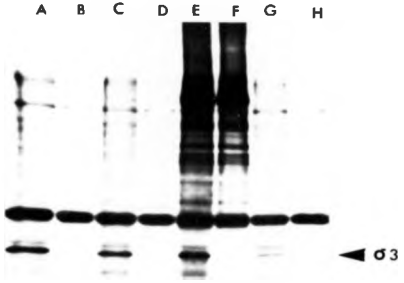

M A B C AB C A B C AB C A B
σ3
End -Mnase
S41
S43
Act
End +Mnase

FIGURE 26: IDENTIFICATION OF THE REASON FOR MULTIPLE BANDING OF S4-3.



A = S4-1/*BamHI* Type 1 extract

B = S4-1/*BamHI* Type 3 extract

C = S4-3/*BamHI* Type 1 extract

D = S4-3/*BamHI* Type 3 extract

E = S4-3/*PstI* Type 1 extract

F = S4-3/*PstI* Type 3 extract

G = S4-3/*PstI* (S1 nuclease treated) Type 1 extract

H = S4-3/*PstI* (S1 nuclease treated) Type 3 extract

S4-3 DNA was linearised with *PstI*, *BamHI* and the 3' protruding end produced by *PstI* digestion was removed by S1 nuclease as described in Materials and Methods. S4-3 mRNA was produced from each of the above DNA templates and σ3 expression analysed in S-10 cell extracts as previously described.

S4-3 was linearised with *BamHI* or the 3' *PstI* end was removed by S1 nuclease digestion this problem of multiple banding disappeared (figure 26).

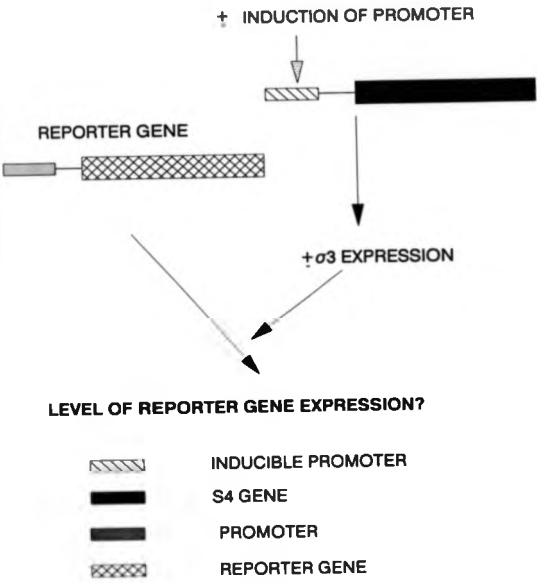
6.4 Summary

S-10 extracts prepared from type 1 infected and uninfected L-cells translated viral and cellular mRNA with the same capacity. However, extracts prepared from type 3 infected cells did not translate viral or cellular mRNA efficiently. Type 3 σ3 did not have any effect on the ability of type 1 infected or uninfected extracts to translate endogenous mRNA that had not been removed by Mnase treatment. These results further suggested that in vitro σ3 has no effect on the translation of eukaryotic mRNAs. The results presented in this chapter did not provide any further insight into the different abilities of reovirus types 1 and 3 to inhibit host protein synthesis.

CHAPTER 7

DEVELOPMENT OF AN *IN VIVO* ASSAY SYSTEM

FIGURE 27: RATIONALE OF IN VIVO EXPERIMENTS



7.1 Rationale

The rationale of the experiments discussed in the following chapters is outlined in figure 27.

To investigate the effect of σ3 on host cell protein synthesis *in vivo*, a system involving an easily assayable reporter gene as a marker for eukaryotic protein synthesis was developed. The rationale of these experiments was to have σ3 under the control of an inducible promoter, to allow for the analysis of reporter gene expression in the presence or absence of σ3 expression.

That is: does type 3 σ3 inhibit reporter gene expression more than type 1 σ3?

7.2 Introduction

The development of an *in vivo* assay system for monitoring the effect of σ3 on host protein synthesis required an easily assayable reporter gene to act as a marker for host protein synthesis. Several genes have been used as reporters including chloramphenicol acetyltransferase (CAT) (Gorman *et al.*, 1982a; 1982b), bacterial ß-galactosidase (ß-gal) (An *et al.*, 1982), and secreted alkaline phosphatase (SEAP) (Berger et al., 1988; Yoon et al., 1988). In each case the enzyme activity of the reporter can be easily assayed in cell lysates and at least in the case of CAT and ß-gal background levels are low in eukaryotic cells. The use of an inducible promoter to control the level of S4 transcription would allow reporter gene expression to be monitored in the presence or absence of σ3 expression. To ensure that every cell received equal amounts of both genes the S4 gene was initially linked onto the plasmid containing the reporter gene.

Several inducible promoter systems are available in eukaryotes including the mouse mammary tumour virus promoter which is regulated by steroid hormones (eg Lee *et al.*, 1984; Beato, 1989), and the HIV-LTR which is *trans*-activated by the

HIV tat protein. Sun and Baltimore (1989) used this latter system to investigate the role of poliovirus protein 2A in the inhibition of host protein synthesis. They found that in co-transfection experiments performed in HeLa cells, CAT reporter gene expression was inhibited when protein 2A was activated by tat induction of the HIV-LTR, thus suggesting protein 2A has a role in regulating the inhibition of host protein synthesis.

7.2.1 Regulation of the HIV-LTR

The HIV-LTR operates as a promoter in a variety of animal and human cell lines. This has been largely determined by using the CAT gene linked to the HIV-LTR, as a reporter gene for HIV driven gene expression (eg Sodoroski *et al.*, 1984). Initiation of transcription from the LTR relies on certain binding sites for cellular and viral transcription factors in the LTR. Transcription of the LTR is stimulated following transactivation by the tat protein. In HIV-infected cells the HIV-LTR initially produces basal rates of early mRNA, due to the binding of host transcription factors to the LTR. However, one of the early mRNAs produced is that for tat, and as levels of this protein increase, transcription from the LTR is stimulated by a *trans*-activation mechanism. In cells transfected with genes driven by the HIV-LTR this *trans*-activation can be mediated by co-transfection with a plasmid containing the tat gene under the control of a constitutive eukaryotic promoter (e.g Rosen *et al.*, 1986a). The tat protein is a 86 amino acid protein encoded by a mRNA produced by double splicing of the full length RNA (Muesing *et al.*, 1985). It acts by binding at the Transactivation Region or TAR sequence, located at the transcription start site, (bases + 1 - + 79). The TAR sequence forms a stem loop structure essential for tat induction of the HIV-LTR (Muesing, 1987).

The inducible transcription system described above met the requirements of the proposed experiments and was developed as an *in vivo* assay system to study the effect $\sigma 3$ has on cellular protein synthesis.

7.3 Construction of reporter plasmids containing the S4 gene

The S4 gene(s) was first subcloned into a plasmid containing the HIV-LTR. The plasmid used, $HXBH_2$, was obtained from C.Chapman (Chester Beatty Labs, London). It contained the HIV-LTR (550 bp) including the TAR sequence cloned into the polylinker of pUC18. The S4 gene was readily inserted downstream of the promoter as illustrated in figure 28. Clones containing the S4 inserts were identified by mini-plasmid preparation and restriction enzyme analysis and were termed HX-1 and HX-3 (figure 29).

Reovirus mRNA is not normally polyadenylated. However, to ensure correct processing and transport of mRNA within the transfected cells a poly A signal was added to the 3' end of the S4 gene(s). For this a vector (pSPPA4) (obtained from K.Leppard, 1990) containing the SV40 small T antigen splice signals upstream of the adenovirus E1b poly A signal (size 350 bp), present in the polylinker of the cloning vector Bluescribe (figure 30), was used. To do this the HIV-S4 insert(s) in the HX plasmid(s) was isolated and inserted upstream of the poly A signal in pSPPA4 as outlined in figure 30. Full length clones were detected by ***KpnI/HincII*** digestion and referred to as HPA-1 for S4-1 inserts and HPA-3 for S4-3 inserts (figure 31). The presence of a T7 promoter in the final plasmid upstream of the S4 gene allowed *in vitro* transcription-translation analysis to be used to determine that full length $\sigma 3$ was still produced, despite the addition of the promoter and poly A tail. Figure 32 shows that the final plasmids, HPA-1 and HPA-3, generate mRNA that encodes full length $\sigma 3$.

FIGURE 28: CONSTRUCTION OF HIV-LTR-S4 CONTAINING SUBCLONES:- HX-1 AND HX-3.

The plasmid HXBH2 containing the HIV-LTR (550bp) in the polylinker of pUC18, was digested with *HindIII/BamHI* and the 5' phosphate group was removed as described in Methods. The S4 gene(s) was removed from the S4BS clones (section 4) by *HindIII/BamHI* digestion and the fragment(s) isolated from a 1 % agarose gel as described in Methods. The vector and insert were ligated and transformed into JM103. Plasmids containing the S4 gene(s) downstream of the HIV-LTR were screened for by mini-plasmid preparations and restriction enzyme analysis (figure 29).

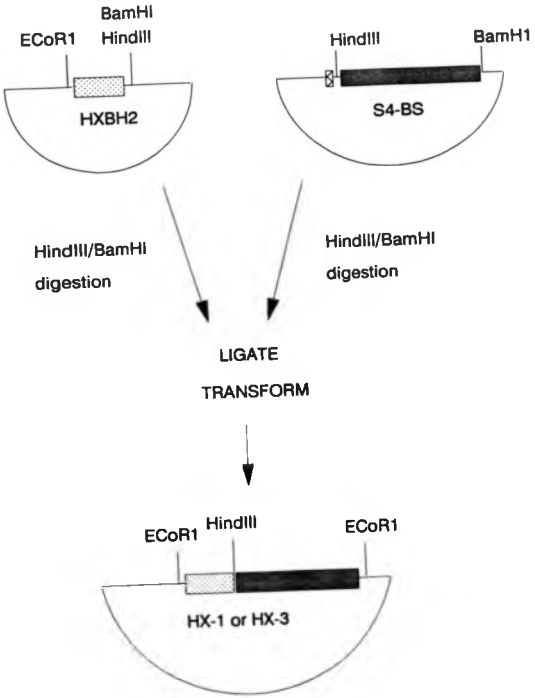

BamHI
ECoR1
HindIII
HXBH2
HindIII
BamH1
S4-BS
HindIII/BamHI
digestion
HindIII/BamHI
digestion
LIGATE
TRANSFORM
ECoR1
HindIII
ECoR1
HX-1 or HX-3

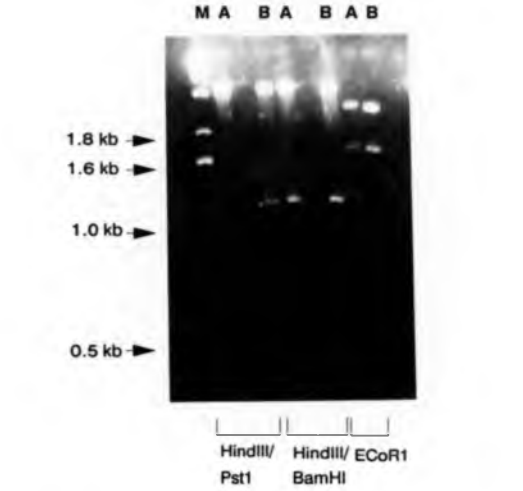
FIGURE 29: IDENTIFICATION OF HX-1 AND HX-3 SUBCLONES.

M = BRL 1 KB LADDER

A = HX-1

B = HX-3

Plasmids HX-1 and HX-3 were analysed on 1 % agarose gels. Full length HX-1 and HX-3 of 1.75 Kb were identified by *ECoRI* digestion. *HindIII/BamHI* digestion released full length S4 genes and *HindIII/PstI* digestion distinguished between HX-1 and HX-3, releasing 0.48 Kb and 0.7 Kb pieces from HX-1 and a full length 1.2 Kb insert from HX-3.


M A B A B A B
1.8 kb
1.6 kb
1.0 kb
0.5 kb
HindIII/ Pst1
HindIII/ BamHI
ECoR1

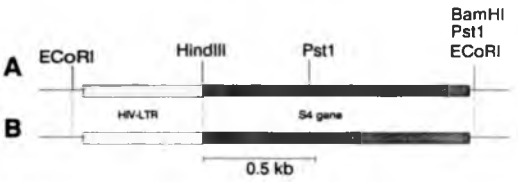
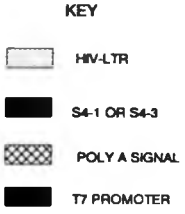

BamHI
Pst1
ECoRI
A
ECoRI
HindIII
Pst1
B
HIV-LTR
S4 gene
0.5 kb

FIGURE 30: CONSTRUCTION OF HIV-LTR-S4--POLY A CONTAINING SUBCLONES: HPA-1 AND HPA-3.

The plasmid pSPPA4 was digested with *EcoRI/SmaI* and the 5' phosphate group was removed as described in Methods. Full length (1.75 Kb) inserts of HX-1 and HX-3 were isolated on 1 % agarose gels following *EcoRI/SmaI* digestion. The vector and insert were ligated and transformed into JM103 as described in Methods. Plasmids containing the S4 gene(s) upstream of the poly A signal were identified by mini-plasmid preparations and restriction enzyme analysis (figure 31).



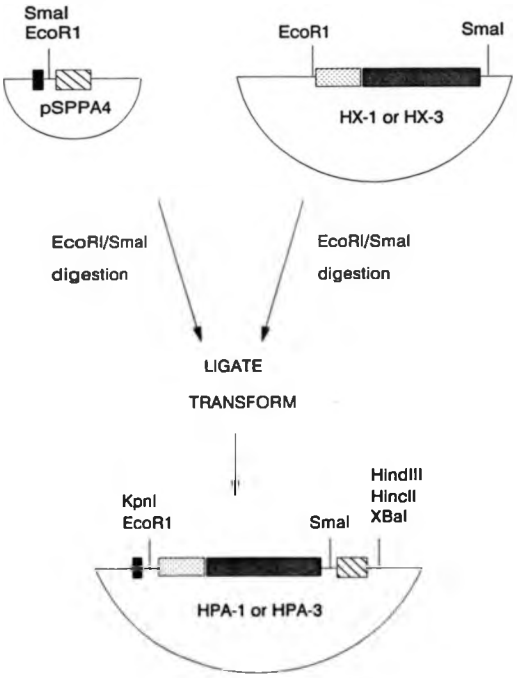

SmaI
EcoR1
pSPPA4
EcoR1
SmaI
HX-1 or HX-3
EcoRI/SmaI
digestion
EcoRI/SmaI
digestion
LIGATE
TRANSFORM
KpnI
EcoR1
SmaI
HindIII
HincII
XbaI
HPA-1 or HPA-3

FIGURE 31: IDENTIFICATION OF HPA-1 AND HPA-3

M = BRL 1KB LADDER

A1 = HPA-3 (undigested)

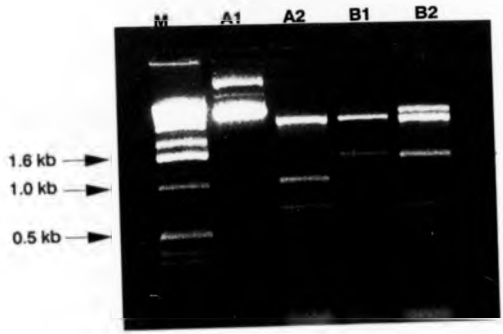
A2 = HPA-3

B1 = HPA-1

B2 = HPA-1

HPA-1 and HPA-3 inserts were analysed on 1 % agarose gels. Full length inserts were identified by *KpnI/HincII* digestion, which also distinguished between the two genes.

For HPA-1 two insert containing bands were released, one of 1.43 Kb and one of 0.7 Kb. For HPA-3, three insert containing bands of 0.98 Kb, 0.4 Kb and 0.7 Kb were released.


M
A1
A2
B1
B2
1.6 kb
1.0 kb
0.5 kb

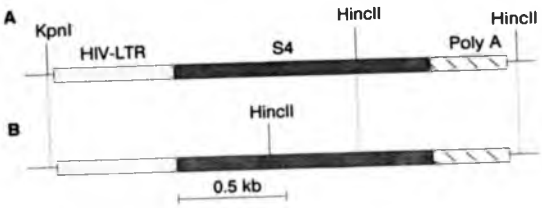

A
KpnI
HIV-LTR
S4
HincII
Poly A
HincII
B
HincII
0.5 kb

FIGURE 32: ANALYSIS OF PROTEIN PRODUCTS PRODUCED FROM HPA-1 AND HPA-3 .

A = ß-actin from T7-ß-ACT (section 4.4)

B = σ3 type 1 from original S4-1BS (section 4.3)

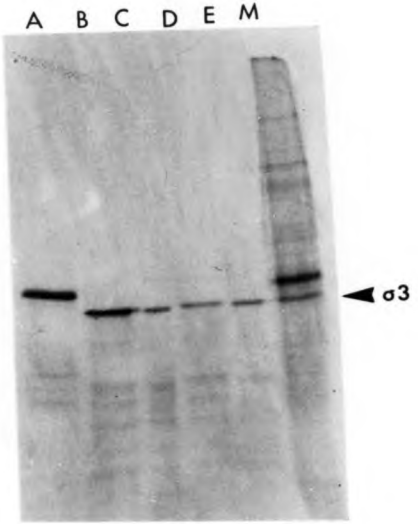
C = σ3 type 1 from HPA-1

D = σ3 type 3 from original S4-3BS (section 4.3)

E = σ3 type 3 from HPA-3

F = Marker: pulse labelled reovirus type 3 infected cell lysate

The relevant DNA was linearised by either *BamHI* (S4-1 and S4-3 BS clones), *XBal* (HPA-1 and HPA-3) or *HindIII* (T7-ACT) digestion. mRNA was synthesised *in vitro* using T7 RNA polymerase as described in Methods and in section 4.4. *In vitro* translation analysis, in reticulocyte lysates, was carried out as described in Methods. The protein products were analysed on a 10 % polyacrylamide gel, run for 3 hours at 40 mA. The position of full length σ3 is indicated on the right side of the figure.


A B C D E M
σ3

7.3.1 Insertion of HPA-1 and HPA-3 into a plasmid containing the CAT reporter gene

A plasmid with the CAT gene under the control of the cytomegalovirus immediate early promoter (CMV) was used to link the S4 gene(s) and CAT gene onto the same plasmid. CMV-CAT is based on the pSV_2-CAT plasmid range developed by Gorman *et al.* (1982). The ideal restriction enzyme site for insertion of the HPA gene(s) into pCMV-CAT had to meet several requirements:

1. It had to be unique in pCMV-CAT
2. It had not to interfere with CAT gene expression
3. It had not to interfere with the plasmid origin of replication
4. It had not to interfere with the plasmid ampicillin resistance.

The only restriction enzyme site which met these requirements was *BamHI*, located within the SV40 splicing signal region. The HPA gene(s) were ligated into the plasmid as outlined in figure 33. 200 colonies of the resulting transformation were picked and were screened for S4 DNA by hybridisation using a nick translated S4-DNA probe, prepared as described in Methods. Figure 34 shows the results of a typical screening procedure to identify S4-3 containing colonies. Several of the colonies hybridising with the S4 probe were picked and further screened for inserts by mini-plasmid preparations and restriction enzyme analysis. Figure 35 shows the restriction enzyme analysis used to identify S4-1 and S4-3 inserts. These plasmids were termed CMV-1 and CMV-3 respectively.

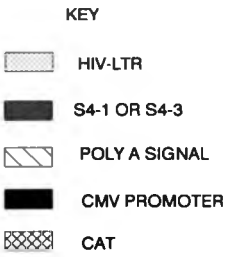
7.4 Analysis of CAT activity from reporter gene constructs

To do this, plasmid DNA was first transfected into cells using either electroporation or calcium phosphate transfection. Following incubation at 37°C for 48 hours cells were extracted and reporter gene expression assayed as described in

FIGURE 33: CONSTRUCTION OF PLASMIDS CONTAINING THE CAT REPORTER GENE LINKED TO THE S4 GENE.

pCMV-CAT was digested with *BamHI*, and treated with Klenow 1 polymerase to produce blunt ends prior to removal of the 5' phosphate group as described in Methods.

The HPA insert(s) (2.1 Kb) was isolated on a 1 % agarose gel following *ECoRI/XBal* digestion and treatment with Klenow 1 polymerase to produce blunt ends. Vector and insert were ligated and transformed into MC1061 and colonies containing CAT and S4 genes were identified by Grunstein hybridisation followed by mini-plasmid preparations and restriction enzyme analysis (figures 34 and 35).



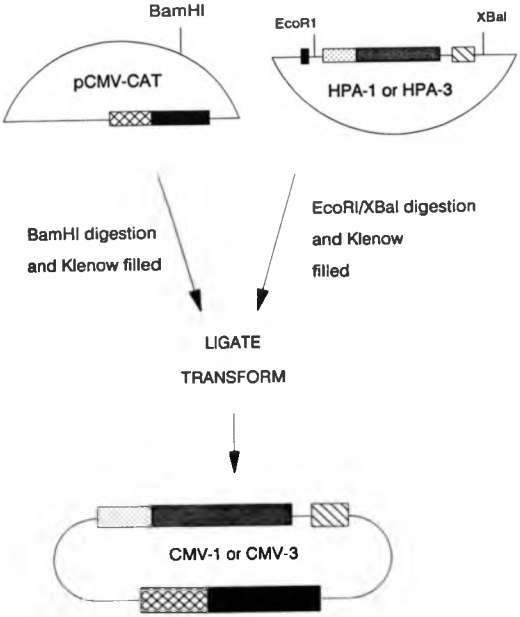

BamHI
pCMV-CAT
EcoR1
XBal
HPA-1 or HPA-3
BamHI digestion
and Klenow filled
EcoRI/XBal digestion
and Klenow
filled
LIGATE
TRANSFORM
CMV-1 or CMV-3

FIGURE 34: GRUNSTEIN HYBRIDISATION TO IDENTIFY S4 CONTAINING COLONIES.

200 colonies were picked from the transformed plates (figure 33) were screened for the S4 gene using a nick translated S4 DNA probe prepared as described in Methods. The colonies were hybridised as described in Methods. HPA-3 was used for a positive control for the hybridisation (bottom row of photograph). 15 potential positive colonies were picked and further screened for the S4 insert by mini-plasmid preparations and restriction enzyme analysis (figure 35).

FIGURE 35: IDENTIFICATION OF S4 INSERTS IN CMV-CAT: CMV-1 AND CMV-3

A = CMV-1

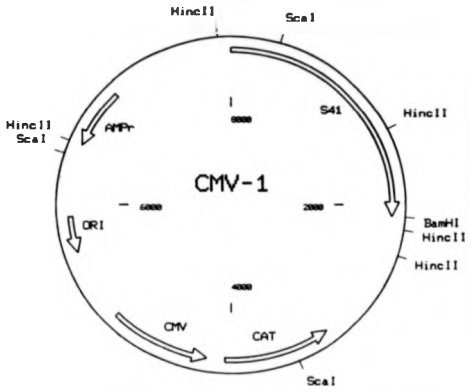
B = CMV-3

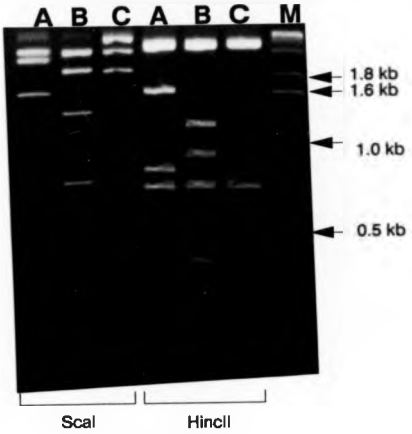
C = CMV-CAT

M = 1 KB BRL LADDER

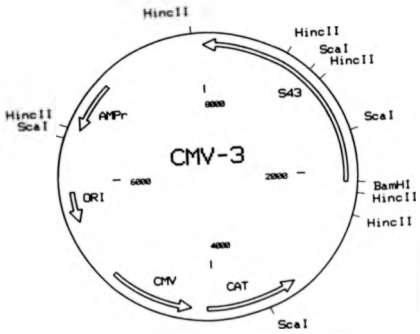
Potential CMV-1 and CMV-3 clones were analysed on a 1 % agarose gel. S4 inserts were identified by *ScaI* and *HincII* digestion, which determined the orientation of the inserts and distinguished between the two genes. The final orientations of the clones were different as is shown in figures A and B.

A. Orientation of CMV-1





B: Orientation of CMV-3



Methods. A plasmid containing the HIV-LTR driving CAT gene expression was used as a positive control for induction of the HIV-LTR by tat. In this control transfection tat was expressed under the control of the SV40 early promoter (termed TAT). Reactions were carried out in triplicate as described in Methods.

7.4.1 Optimisation of electroporation voltage

To determine the optimum voltage for electroporation, L-cells were electroporated with 10 μg of CMV-CAT DNA at 250, 500, 750 or 1000 Volts. The survival of the cells was determined by observing cell monolayers over the incubation period. When the cells were electroporated at 500 Volts there was approximately 35 % cell mortality. When the voltage was increased to more than 750 Volts the cell mortality was 70 % or greater. The relative CAT activity at each voltage value was assayed and maximum CAT activity was found in cells electroporated at 500 Volts (data not shown).

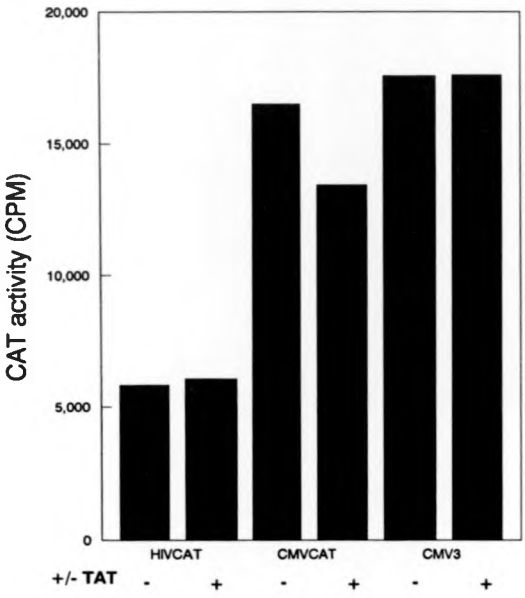
7.4.2. CAT activity in L-cells

To analyse CAT activity from reporter gene constructs the cells were electroporated at 500 Volts with a total of 20 μg of DNA (10 μg of CAT-S4 containing DNA and 10 μg of TAT or Bluescribe to keep the DNA input constant). Figure 36 shows that there is no difference in the relative CAT activities in the clones containing S4-3 in the presence or absence of TAT. This indicated that the insertion of S4 DNA into CMV-CAT did not disrupt CAT gene expression. In the control experiment, using co-transfection of HIV-CAT and TAT DNA, the expected increase in CAT activity was not seen. This suggested that the promoter might be non-functional in L-cells.

Rosen *et al.* (1986b) and Newstein *et al.* (1990) reported that the HIV-LTR system was non-functional in certain cell lines, particularly those of non-primate

FIGURE 36: CAT ACTIVITY FROM CMV-CAT-S4 CONTAINING PLASMIDS IN L-CELLS.

L-cells were transfected with 10 μg of CMV-CAT or CMV-3 and 10 μg of TAT or Bluescribe DNA, as a negative control for TAT activity. The plasmid, HIV-CAT, was used as a positive control for TAT induction of the HIV-LTR. CAT activity is expressed in cpm taken at the 120 minute time point of the reaction.


20,000
15,000
10,000
5,000
0
CAT activity (CPM)
HIVCAT
CMVCAT
CMV3
+/- TAT
-
+
-
+
-
+

origin. To investigate this in our system, a comparative study of HIV-LTR/TAT activity in L-cells and HeLa cells, a cell line where the HIV promoter is known to function (Cullen, 1986; Sun & Baltimore, 1989), was undertaken. For these experiments and all following experiments, cells were transfected using the calcium phosphate technique as it was found to produce more consistent results than electroporation. Figure 37 shows that TAT induction of HIV promoter activity was 20 fold in HeLa cells, whereas there was again no significant induction of CAT activity in L-cells, suggesting that they lack factors, possibly transcription factors, necessary for the HIV promoter to function.

7.4.3 Optimisation of TAT induction in HeLa cells

Several workers (Hauber and Cullen, 1989; Fenrick *et al.*, 1989) have reported that the level of TAT induction of the HIV-LTR is affected by the ratios of HIV-LTR DNA : TAT DNA used for transfection. To investigate this, HeLa cells were transfected with 10 μg HIV-CAT and increasing amounts of TAT DNA from 0.5 μg to 15 μg. Figure 38 shows that TAT induction reaches an optimum level at 10 μg of HIV-CAT and 4 μg of TAT DNA, resulting in greater than 50 fold stimulation of CAT activity.

7.4.4 The effect of σ3 on CAT gene expression in HeLa cells

To analyse the effect of σ3 on CAT gene expression. HeLa cells were co-transfected at the optimal HIV-LTR : TAT ratios obtained in the previous section. The CAT activity from CMV-1 and CMV-3 in the presence and absence of TAT was studied. Figure 39 shows that there was no significant difference in the expression from various CAT constructs suggesting σ3 had no effect on CAT gene expression or alternatively that σ3 was not being efficiently expressed.

FIGURE 37: COMPARISON OF TAT INDUCTION OF THE HIV-LTR IN HeLa CELLS AND L-CELLS

A = L-cells

B = HeLa cells

L-cells and HeLa cells were transfected with 10 μg of HIV-CAT and 10 μg of TAT DNA. The results show the progressive increase in CAT activity (cpm) throughout the reaction. There was no significant stimulation of CAT activity in L-cells. By contrast, in HeLa cells there was a 20 fold increase in CAT activity in the presence of TAT.

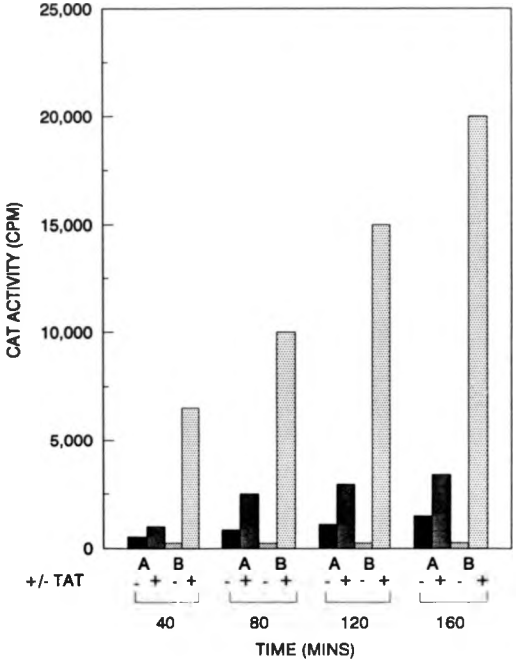

25,000
20,000
15,000
10,000
5,000
0
CAT ACTIVITY (CPM)
A B A B A B A B
- + - + - + - + - + - + - + - +
+/- TAT
40
80
120
160
TIME (MINS)

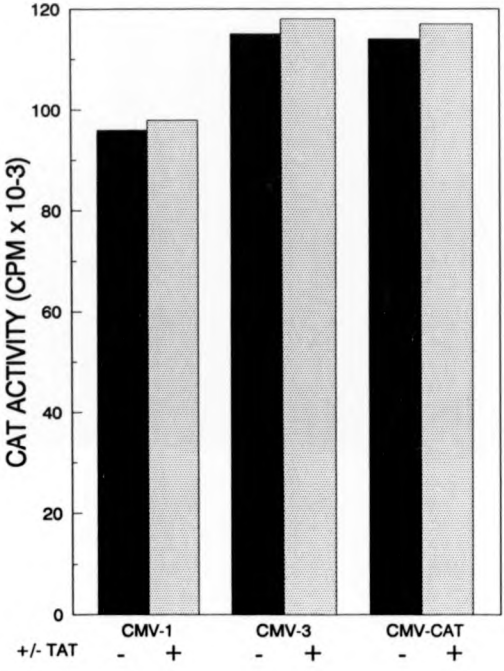
FIGURE 38: OPTIMISATION OF TAT INDUCTION IN HeLa CELLS.

HeLa cells were co-transfected with 10 μg of HIV-CAT and 0 - 10 μg of TAT DNA as described in Methods. The cells were harvested and extracts prepared for assaying CAT activity as described in Methods. The results are expressed as the fold stimulation of CAT activity over samples where no TAT was added.

FOLD STIMULATION CAT ACTIVITY
60
50
40
30
20
10
0
0
2
4
6
8
10
12
[TAT] μg

FIGURE 39: CAT ACTIVITY FROM CMV-CAT-S4 CONTAINING PLASMIDS IN HeLa CELLS.

HeLa cells were co-transfected with 10 μg CMV-1, CMV-3 or CMV-CAT and 4 μg of TAT DNA. The cells were harvested and extracts prepared to assay CAT activity as described in Methods. The results were expressed as the CAT activity (cpm) at 120 minutes into the reaction.


CAT ACTIVITY (CPM x 10-3)
120
100
80
60
40
20
0
CMV-1
CMV-3
CMV-CAT
+/- TAT
-
+
-
+
-
+

7.5 Detection of σ3 in transfected cells

To confirm if σ3 was being expressed in the experiments described in section 7.3, polyclonal antibody against reovirus type 3 was raised in a New Zealand white rabbit as described in Methods. This would allow for detection of σ3 in transfected cells by immunoprecipitation or immunofluorescence.

Before the serum was used to detect σ3 expression in transfected cells its activity against type 3 and type 1 infected cells was investigated. Figure 40 shows that the serum was active against type 3 and type 1 virus.

To confirm that σ3 was being produced during the reactions discussed in section 7.3.5, plates of CMV-1 and CMV-3 (section 7.2.2) transfected cells were labelled with ^{35}S-met 48 hours post transfection and extracts were immunoprecipitated as described in Methods. However no σ3 was detected in these samples, suggesting that σ3 was only being made at very low levels by the CMV-1 and CMV-3 CAT reporter plasmids.

The original HPA-1 and HPA-3 clones (section 7.2.1) from which the CMV constructs were derived had been shown to translate σ3 *in vitro* (figure 32). These clones were transfected into HeLa cells in the presence or absence of optimal TAT concentrations and their ability to synthesise σ3 *in vivo* was studied by immunoprecipitation and immunofluorescence. Again no σ3 was detected. Two questions therefore arose:

1. Was the S4 gene functional *in vivo*?
2. Was the HIV promoter functional?

FIGURE 40: DETECTION OF REOVIRUS TYPE 3 INFECTED CELLS BY IMMUNOFLUORESCENCE.

A = Reovirus type 1 infected cells + hyperimmune serum (phase/fluorescence)

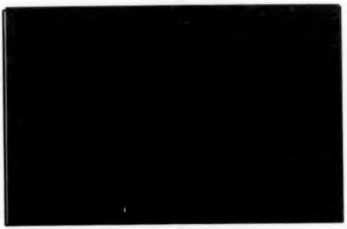
B = Reovirus type 3 infected cells + hyperimmune serum (phase/fluorescence)

C = Reovirus type 3 infected cells + hyperimmune serum (fluorescence viewed under high magnification)

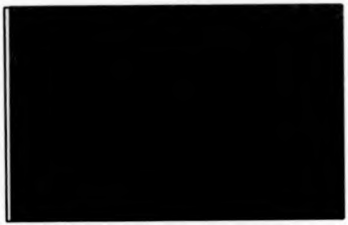
Monolayers of L-cells were infected with 0.1 pfu/cell or 0.5 pfu/cell of reovirus type 3 or reovirus type 1. Control cells were mock-infected. 18 hours post infection the cells were fixed and treated with polyclonal antibody to reovirus type 3 as described in Methods.

It is also clearly evident from these photographs that reovirus grows solely in the cytoplasm of the cells.

A



B



C

7.5.1 Development of a σ3 expressing system

One possible reason for the lack of detection of σ3 expression in the HPA-1, HPA-3 and CMV-1 and CMV-3 plasmids was that not enough σ3 was being expressed to be detected or to have any effect on CAT gene expression.

COS-1 cells are a transformed monkey cell line expressing the SV40 T antigen, and they are therefore able to replicate introduced plasmids containing the SV40 origin of replication (Gluzman, 1981). Banjeri (1981) observed that the expression of ß-globin in transfected cells was enhanced in the presence of the SV40 enhancer element.

To ascertain if the S4 gene was functionally expressed *in vivo* it was inserted into the polylinker region of pEXP6 (obtained from K.Leppard, 1990). This vector has the SV40 origin, promoter and SV40 poly A tail flanking either end of the standard polylinker. The S4 gene from the initial S4-pAT153 clone (figure 12, chapter 4) was inserted downstream of the SV40 promoter (figure 41), generating two individual subclones which were termed SV40-1 (containing S4-1) and SV40-3 (containing S4-3) (figure 42). These constructs were transfected into COS-1 cells, to allow for replication of the plasmid and σ3 synthesis was analysed by immunoprecipitation (figure 43). Figure 43 shows that both S4-1 and S4-3 genes were functional *in vivo* when expressed from the SV40 promoter. A strong σ3 band was precipitated from SV40-3 transfected cells but only a very faint signal was produced from SV40-1 transfected cells.

To investigate if the reason for lack of detection of expression from the HIV-S4 containing plasmids was due to insufficient σ3 being expressed, the SV40 origin was inserted into the HIV-S4 containing plasmids. The SV40 origin was available in a plasmid termed pSVE1 (obtained from K.Leppard, 1990) and could be isolated as a 350 bp piece. There was no suitable enzyme site available in CMV-1 or CMV-3 for insertion of the SV40 origin. Therefore it was inserted downstream of the poly A signal in the HPA clones at the *Sall* site, a unique site located in the polylinker

FIGURE 41: CONSTRUCTION OF PLASMIDS CONTAINING THE SV40 PROMOTER TO DRIVE S4 GENE EXPRESSION.

The plasmid pEXP6 contained the SV40 origin, early promoter and polyadenylation signals flanking either end of the M13 polylinker. This was digested with *HindIII* (immediately downstream of the promoter) and *BamHI*. The 5' phosphate group was removed as described in Methods. The S4 gene was released from pAT153 by *HindIII/BamHI* (figure 12) and the insert isolated from a 1 % agarose gel as described in Methods. The vector and insert mixture were ligated and transformed into MC1061. Colonies containing the plasmid were screened for by mini-plasmid preparations and restriction enzyme analysis (figure 42).

KEY

SV40 PROMOTER

POLY A SIGNAL

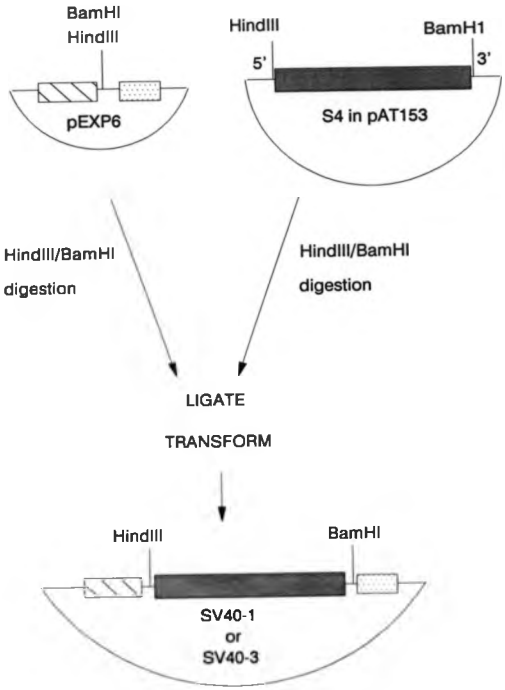

BamHI
HindIII
pEXP6
HindIII
BamH1
5'
3'
S4 in pAT153
HindIII/BamHI
digestion
HindIII/BamHI
digestion
LIGATE
TRANSFORM
HindIII
BamHI
SV40-1
or
SV40-3

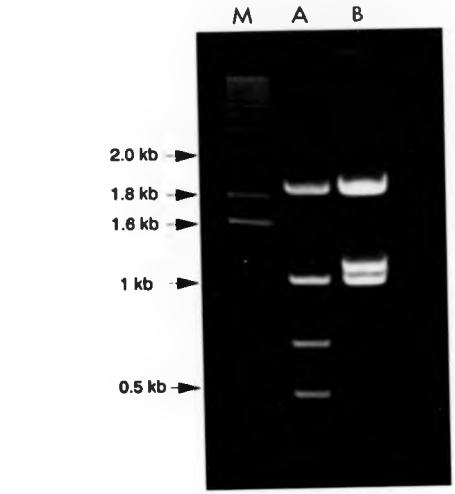
FIGURE 42: IDENTIFICATION OF SV40-S4 CONTAINING PLASMIDS.

M = 1 Kb BRL ladder

A = SV40-1

B = SV40-3

SV40-1 and SV40-3 subclones were analysed on 1 % agarose gels. Full length inserts were identified by *HindIII/PstI* digestion as described in Methods. SV40-1 results in 2 bands released from the S4 insert, one of 0.48 Kb and another of 0.7 Kb. SV40-3 results in the release of full length S4 gene.


M
A
B
2.0 kb
1.8 kb
1.6 kb
1 kb
0.5 kb

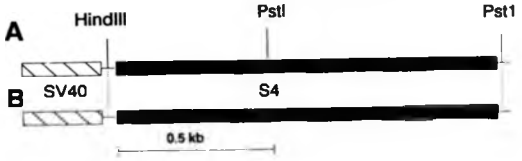

A
HindIII
PstI
Pst1
B
SV40
S4
0.5 kb

FIGURE 43: DETECTION OF $\sigma 3$ IN TRANSFECTED COS-1 CELLS BY IMMUNOPRECIPITATION.

A = SV40-1 + Hyperimmune serum

B = SV40-1 + Preimmune serum

C = SV40-3 + Preimmune serum

D = SV40-3 + Hyperimmune serum

E = HPA-1 + TAT + Hyperimmune serum

F = HPA-1 + TAT + Preimmune serum

G = HPA-3 + TAT + Hyperimmune serum

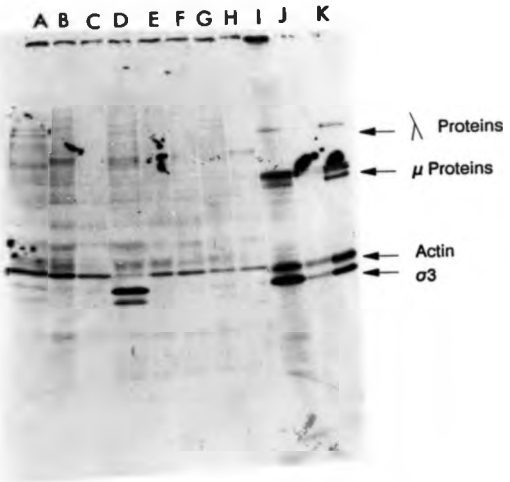
H = HPA-3 + TAT + Preimmune serum

I = Pulse labelled reovirus type 3 infected cell lysate + Hyperimmune serum

J = Pulse labelled reovirus type 3 infected cell lysate + Preimmune serum

K = Pulse labelled reovirus type 3 infected cell lysate

COS-1 cells were transfected with 10 μg of the relevant DNA and labelled 48 hours post transfection with ^{35}S-met, harvested and immunoprecipitated as described in Methods. As a positive control pulse labelled reovirus type 3 infected cells were immunoprecipitated. The precipitated proteins were fractionated on a 10 % polyacrylamide gel, electrophoresed for 3 hours at 40 mA and fluorographed.


A B C D E F G H I J K
λ Proteins
μ Proteins
Actin
σ3

(figure 44). These subclones were identified by mini-plasmid preparations and restriction enzyme analysis and were termed HIV-1 and HIV-3 (figure 45).

To obtain maximum expression from these constructs they were expressed in COS-1 cells. COS-1 cells were first optimised for TAT induction of the HIV-LTR, in case the optimal level differed from that found for HeLa cells. Figure 46(a) shows that optimal activity was a 10:1 ratio of HIV:TAT DNA and that induction of the HIV-LTR in COS-1 cells was greater than in HeLa cells (compare with figure 38). The relative CAT activity produced was also greater in COS-1 cells than in HeLa cells (figure 46(b)).

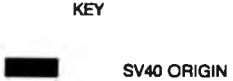
The HIV constructs and optimal TAT concentrations were co-transfected into COS-1 cells and σ3 expression analysed 48 hours post transfection by immunofluorescence as described in Methods. Figure 47 shows that type 3 σ3 was detected in cells co-transfected with HIV-3 and TAT and to a lesser extent in cells transfected with HIV-3 in the absence of TAT. By contrast type 1 σ3 was not detected by immunofluorescence.

7.5.2 Summary

Expression of σ3 was detected in transfected cells by inserting the SV40 origin of replication into the initial HPA plasmids produced in section 7.2. The SV40 origin increases the copy number of the plasmid in the cell hence producing more protein. However σ3 was also detected in cells transfected in the absence of TAT. A recent report by Proudfoot *et al.* (1990) has shown that transcription from the HIV-LTR in plasmids containing the SV40 origin of replication occurs without TAT induction, suggesting that expression from the HIV-LTR can be activated by plasmid replication. This may be the reason for the observations obtained in these experiments.

FIGURE 44: ADDITION OF THE SV40 ORIGIN OF REPLICATION TO HIV-S4-POLY A CONSTRUCTS.

The SV40 origin (350 bp) was isolated from plasmid pSVE1 by *EcoRI/HindIII* digestion and treated with Klenow I polymerase to produce blunt ends. The vector HPA-1 or HPA-3 was digested with *SalI*, treated with Klenow I polymerase and the 5' phosphate group removed as described in Methods. The SV40 origin insert was isolated from a 1.5 % agarose gel as described in Methods. Vector and insert were ligated and transformed into MC1061. Colonies containing the insert were analysed by mini-plasmid preparations and restriction enzyme analysis (figure 45). These were termed HIV-1 and HIV-3.



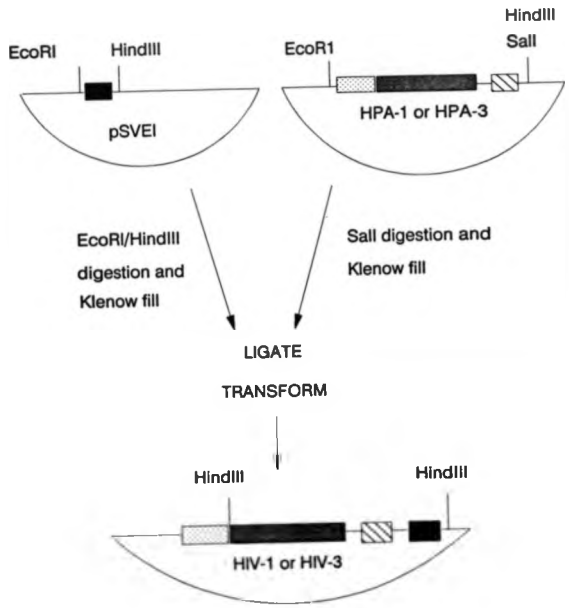

EcoRI
HindIII
pSVEI
EcoR1
HindIII
SalI
HPA-1 or HPA-3
EcoRI/HindIII
digestion and
Klenow fill
SalI digestion and
Klenow fill
LIGATE
TRANSFORM
HindIII
HindIII
HIV-1 or HIV-3

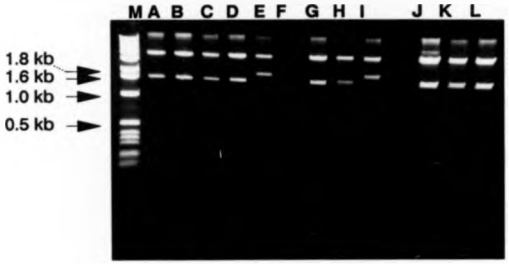
FIGURE 45: IDENTIFICATION OF HIV-1 AND HIV-3 SUBCLONES

M = 1KB BRL LADDER

A-F = POTENTIAL HIV-3

G-L = POTENTIAL HIV-1

HIV-1 and HIV-3 subclones were analysed on 1.5 % agarose gels. Full length subclones were identified by *HindIII* digestion. Those samples containing the insert released a fragment of 1.85 Kb (tracks E and I) and those with no insert released a fragment of 1.55 Kb.


M A B C D E F G H I J K L
1.8 kb
1.6 kb
1.0 kb
0.5 kb

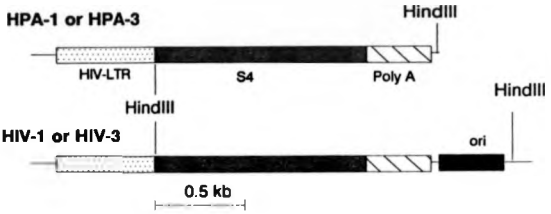

HPA-1 or HPA-3
HindIII
HIV-LTR
S4
Poly A
HindIII
HindIII
HIV-1 or HIV-3
ori
0.5 kb

FIGURE 46a: OPTIMISATION OF TAT INDUCTION OF THE HIV-LTR IN COS-1 CELLS.

COS-1 cells were transfected with 10 μg of HIV-CAT and 0 - 10 μg of TAT. The cells were harvested and CAT activity monitored as described in Methods. The results were expressed as the relative increase in CAT activity over HIV-CAT activity in the absence of TAT, which is taken as 1.

FIGURE 46b: COMPARISON OF EXPRESSION FROM THE HIV-LTR IN HeLa AND COS-1 CELLS.

The activity from the HIV-LTR expressed in COS-1 cells and HeLa cells was compared in parallel reactions.

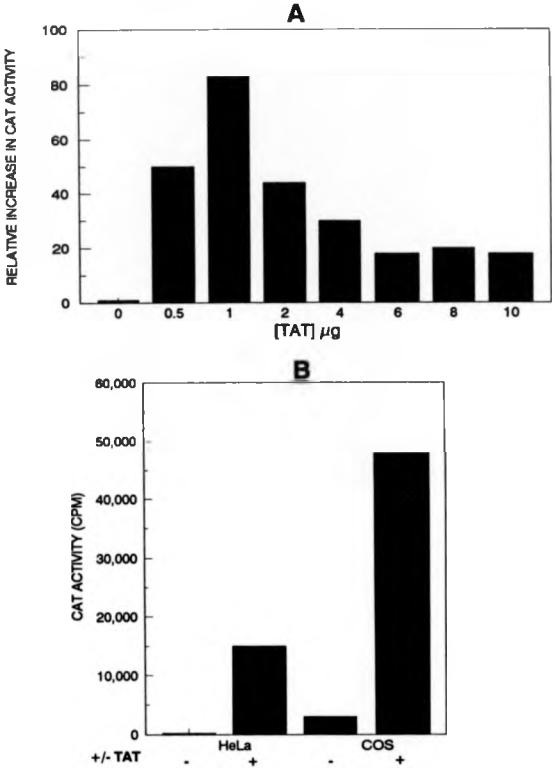

A
RELATIVE INCREASE IN CAT ACTIVITY
100
80
60
40
20
0
0
0.5
1
2
4
6
8
10
[TAT] μg
B
CAT ACTIVITY (CPM)
60,000
50,000
40,000
30,000
20,000
10,000
0
HeLa
COS
+/- TAT
-
+
-
+

FIGURE 47: DETECTION OF σ3 EXPRESSION IN TRANSFECTED CELLS BY IMMUNOFLUORESCENCE

A = HIV-3 + TAT (Phase/fluorescence)

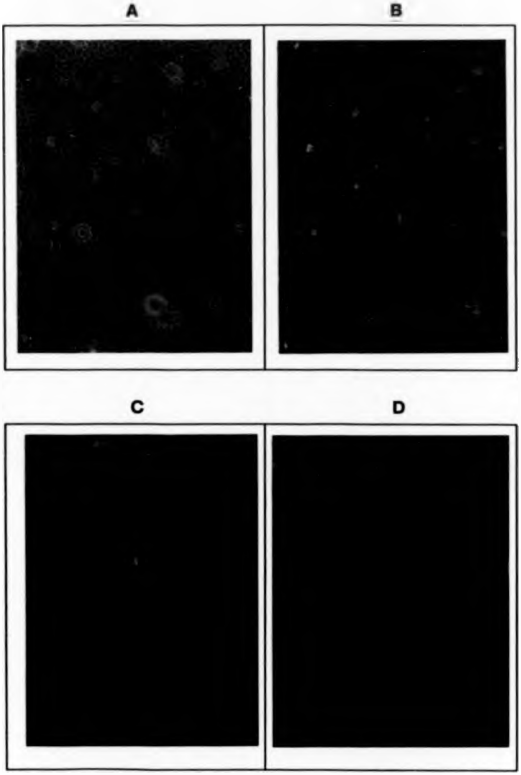
B = HIV-3 + TAT (Fluorescence)

C = HIV-3 - TAT (Fluorescence)

D = HIV-1 + TAT (Fluorescence)

COS-1 cells were transfected with the relevant DNA and the cells were fixed and prepared for immunofluorescence as described in Methods. σ3 type 3 was detected by immunofluorescence in cells transfected with HIV-3 in the presence or absence of TAT. It was also detected in cells transfected with SV40-3. However σ3 type 1 was not detected from the HIV-1 or SV40-1 constructs.

Magnification x 100


A
B
C
D

The fact that σ3 type 1 was not detected suggested that either it is not as efficiently expressed as σ3 type 3 or that the reovirus type 3 polyclonal antibody used in these experiments was not active against this protein. In the following experiments the latter was assumed to be true.

7.6 The effect of σ3 on CAT gene expression in COS-1 cells

COS-1 cells were co-transfected with HIV-1 or HIV-3 DNA and CMV-CAT in the presence or absence of TAT DNA. To ensure that all the cells expressing CAT also expressed σ3, a DNA ratio of 10 μg of S4 DNA to 1 μg of reporter gene DNA was used. To analyse σ3 expression, cells were also prepared for immunofluorescence. Figure 48 shows that there was no significant difference in CAT activity in any of the samples despite σ3 type 3 being detected by immunofluorescence.

7.6.1 Summary

Despite σ3 from type 3 virus being expressed in COS-1 cells, the protein had no effect on CAT gene expression in this system. There could be several reasons for this. First σ3 may not inhibit host protein synthesis on its own but may require some other viral factors. Another possibility is that insufficient σ3 was being produced to have any effect on CAT gene expression.

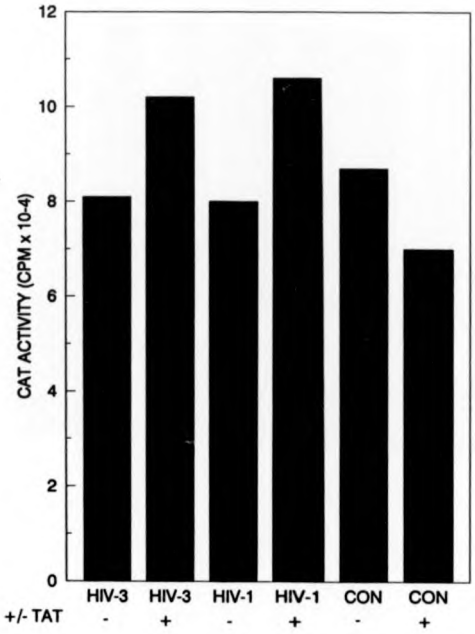
7.7. CMV-CAT gene expression in reovirus infected cells

To ascertain if the reason for the lack of inhibition of CAT gene expression produced by σ3 was due to it requiring other viral factors, the effect of type 1, type 2 and type 3 virus on CAT gene expression was studied.

FIGURE 48: THE EFFECT OF σ3 ON CAT GENE EXPRESSION IN COS-1 CELLS

COS-1 cells were co-transfected with 10 μg of HIV-S4 containing DNA, 1 μg of CMV-CAT and 1 μg of TAT. HPA-3 was used as a negative control. The cells were harvested and cell extracts analysed for CAT gene expression as described in Methods.

The results are expressed as the CAT activity at the 120 minute time point of the reaction.


CAT ACTIVITY (CPM x 10-4)
12
10
8
6
4
2
0
HIV-3
HIV-3
HIV-1
HIV-1
CON
CON
+/- TAT
-
+
-
+
-
+

These experiments involved infecting the cells with 5 pfu/cell of type 1, type 2 or type 3 virus. Control cells were mock-infected. The cells were infected 8 hours prior to transfection of CMV-CAT DNA to allow viral protein synthesis to begin. One set of samples was pulse labelled to ensure viral protein synthesis was occurring. The transfected cells were harvested and CAT activity was analysed as previously described. The results are shown in figure 49. Under the conditions of the experiment infection of L-cells with reovirus type 1, 2 or 3 did not cause inhibition of CAT protein synthesis. Viral protein synthesis was occurring in each of the sets of transfected cells as is shown in figure 50.

7.7.1 Summary

The results from this experiment suggest that the CAT gene may not, after all, be a suitable reporter plasmid for studying the mechanism of host shut-off by reovirus.

FIGURE 49: THE EFFECT OF REOVIRUS TYPE 1, TYPE 2 AND TYPE 3 ON CAT GENE EXPRESSION.

L-cells were infected with 5 pfu/cell of reovirus type 1, 2 or 3 eight hours prior to transfection with 10 μg of CMV-CAT. Mock infected cells were used as a control. The cells were harvested at 24 hours post transfection and CAT activity assayed as described in Methods. The results were expressed as the total increase in CAT activity from time point 0 to the end of the reaction.

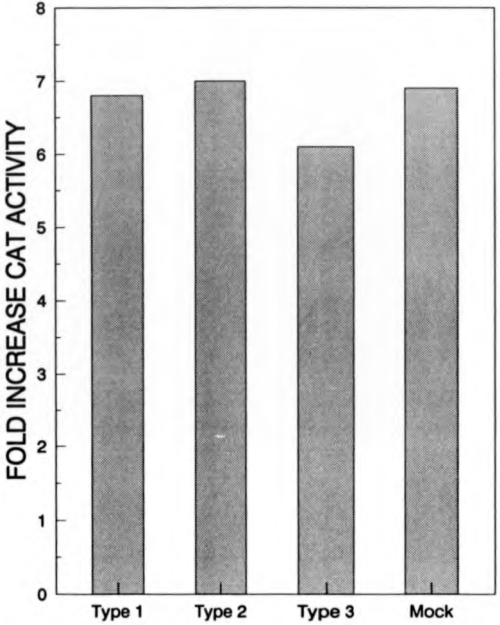
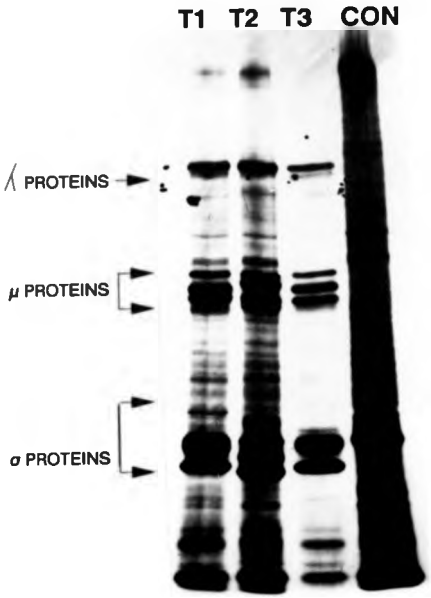

FOLD INCREASE CAT ACTIVITY
0
1
2
3
4
5
6
7
8
Type 1
Type 2
Type 3
Mock

FIGURE 50: VIRAL PROTEIN SYNTHESIS IN CELLS TRANSFECTED WITH CMV-CAT

In a parallel set of reactions to those described in figure 49 the cells were pulse labelled with ^{35}S-met as described in Methods. The proteins were fractionated on a 5-11 % gradient polyacrylamide gel to allow for complete resolution of the viral proteins.


T1
T2
T3
CON
λ PROTEINS
μ PROTEINS
σ PROTEINS

CHAPTER 8

THE EFFECT OF σ3 ON OTHER REPORTER GENES

8.1 Introduction

The results obtained in the previous chapter suggested that σ3 had no effect on CAT gene expression. To investigate if this phenomenon was true for other reporter genes the effect of σ3 on the *E.Coli LacZ* reporter gene system, which encodes the enzyme ß-galactosidase (ß-gal), was examined. This gene system was initially used in studies of bacterial gene regulation (Casadaban *et al.*, 1980) as enzyme activity can be simply measured by a spectrophotometric assay. Hall *et al.* (1983) produced a system in which the *LacZ* gene was fused to the early promoter of SV40, which facilitated its use in transient expression systems to measure ß-gal activity in transfected mammalian cells. This system has been successfully used as a reporter gene in a variety of mammalian systems, for example; it was used as a marker in the study of rous sarcoma virus (RSV) expression and replication (Norton and Coffin, 1985).

8.2 The effect of σ3 on ß-gal gene expression

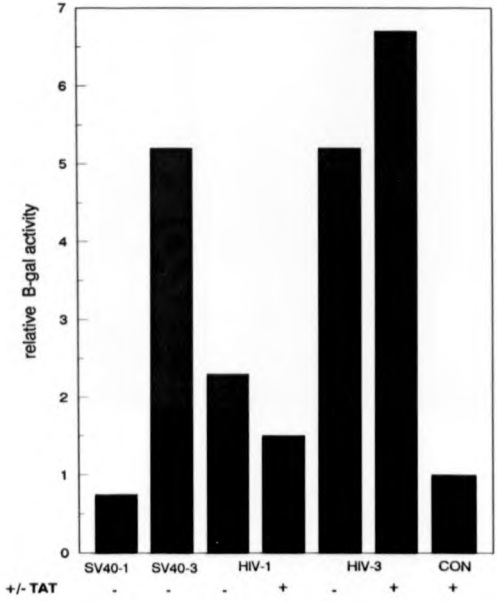
The basic rationale of the experiments looking at the effect of σ3 on ß-gal activity follows that used in assaying CAT activity, that is, a DNA ratio of 10 μg of σ3 expressing DNA: 1 μg of reporter gene DNA : 1 μg of TAT DNA was used. The control for the level of TAT induction of the HIV-LTR was a plasmid containing the *LacZ* gene under the control of the HIV promoter. The reporter plasmid, pCH110 (obtained from A.Easton, 1990), containing the *LacZ* gene under the control of the early SV40 promoter was used as a positive control.

8.2.1 Results

Figure 51 shows that in cells expressing type 3 σ3 a 5-7 fold increase in ß-gal activity was obtained, rather than the decrease expected if ß-gal was acting as a reporter gene for the inhibition of host cell protein synthesis by type 3 σ3. In

FIGURE 51: THE EFFECT OF σ3 ON ß-GALACTOSIDASE ACTIVITY.

COS-1 cells were co-transfected with either 10 μg of SV40-S4 constructs (illustrated in figure 41, chapter 7) or 10 μg of HIV-1 or HIV-3 (illustrated in figure 44, chapter 7) and 1 μg of TAT or Bluescribe. In all cases 1 μg of pCH110 *LacZ* reporter gene was co-transfected as described in Methods. The cells were harvested 48 hours post transfection and extracts were assayed for ß-gal activity as described in Methods. The results are expressed as the stimulation of ß-gal activity over the activity found in cells co-transfected with HPA-3 (containing the HIV-LTR, S4 and Poly A tail) / pCH110 control which was taken as 1.


relative B-gal activity
0
1
2
3
4
5
6
7
SV40-1
SV40-3
HIV-1
HIV-3
CON
+/- TAT
-
-
-
+
-
+
+

contrast there was no alteration in ß-gal expression in cells co-transfected with DNA able to express type 1 σ3.

8.3 The effect of σ3 on CAT gene expression from different CAT reporter genes

8.3.1 Introduction

It was surprising to find that σ3 had different effects on the two reporter gene systems. A major difference between the two reporter systems was that expression of the *Lac*Z gene was under the control of the SV40 promoter whereas CAT gene expression was under the control of the immediate early promoter of CMV. To investigate whether the effect of σ3 on reporter gene expression was promoter dependent, a variety of plasmids with the CAT gene under the control of the following constitutive promoters were examined:

1. Thymidine Kinase (TK) promoter of HSV
2. CMV promoter
3. Rous sarcoma virus (RSV) promoter
4. SV40 early promoter

The control plasmid in this experiment was the original pEXP6 plasmid from which the SV40-1 and SV40-3 plasmids had been derived (figure 42). COS-1 cells were co-transfected with 10 μg SV40-1, SV40-3 or pEXP6 and 1 μg of the relevant CAT plasmid.

8.3.2 Results

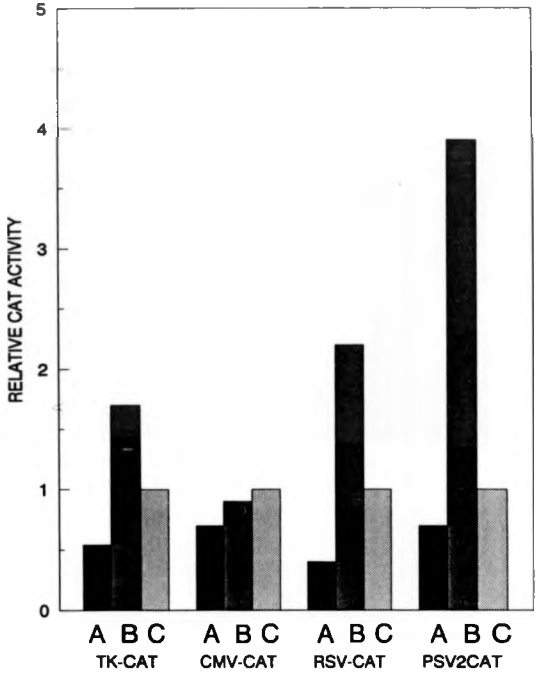
The results from this experiment are shown in figure 52. For each of the CAT reporter plasmids used there is little difference in the CAT activity produced from cells transfected with control DNA and SV40-1 DNA. However, for cells transfected with SV40-3, there is a marked stimulation in CAT activity

FIGURE 52: THE EFFECT OF σ3 ON REPORTER GENE EXPRESSION.

COS-1 cells were co-transfected with 10 μg of SV40-1 (column A), SV40-3 (column B) or pEXP6 (column C) and 1 μg of the various CAT plasmids in which CAT expression was under the control of the following promoters:

TK-CAT: Thymidine kinase promoter from HSV
CMV-CAT: Immediate early promoter from cytomegalovirus
RSV-CAT: Long terminal repeat of rous sarcoma virus
pSV_2CAT: SV40 early promoter

CAT activity from the cell extracts was analysed as described in Methods. The results are expressed as the difference in CAT activity compared to that found in cells transfected with pEXP6 and the relevant CAT plasmid as a control, which is taken as 1.


RELATIVE CAT ACTIVITY
5
4
3
2
1
0
A B C
TK-CAT
A B C
CMV-CAT
A B C
RSV-CAT
A B C
PSV2CAT

in all but CMV-CAT transfected cells. The increase is greatest in pSV_2CAT transfected cells (4 fold). Comparison of the relative CAT activity from each promoter is shown in figure 53. The relative CAT activity from the CMV-CAT promoter is at least 10 times greater than that from the other promoters.

When the effect of $\sigma 3$ on SV40-*LacZ* (pCH110) and SV40-CAT (pSV_2CAT) was compared the amount of stimulation of expression of both reporter genes produced by type 3 $\sigma 3$ was comparable (figure 54).

8.4 Summary

The results presented in this chapter show that type 3 $\sigma 3$ stimulated reporter gene expression but type 1 $\sigma 3$ had no effect on either ß-gal or CAT gene expression. This stimulation appears to be promoter specific. Type 3 $\sigma 3$ had no effect on CAT gene expression when it was under the control of the CMV promoter. However, when CAT gene expression was driven by SV40 or RSV promoters type 3 $\sigma 3$ caused a significant stimulation of expression. When the relative CAT activity from the various promoters was compared, the activity from the CMV promoter was found to be at least 10 times greater than that of the other promoters. One possible explanation for the failure of $\sigma 3$ to stimulate CMV-CAT gene expression is that the CAT activity produced from the CMV promoter was too great for $\sigma 3$ synthesised from either SV40 or HIV promoters to have any effect on it.

FIGURE 53: RELATIVE CAT ACTIVITY FROM VARIOUS CAT PROMOTERS

The CAT activity (cpm) of the different promoters described in figure 52 were compared. The results are expressed as the relative CAT activity at the 90 minute time point of the reaction.

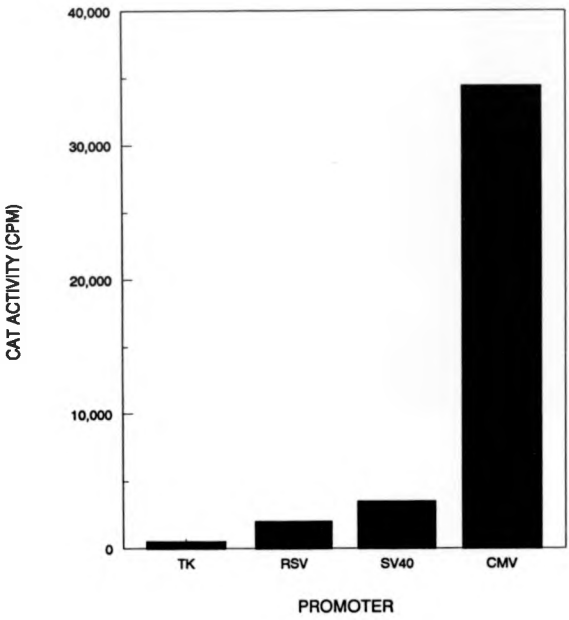
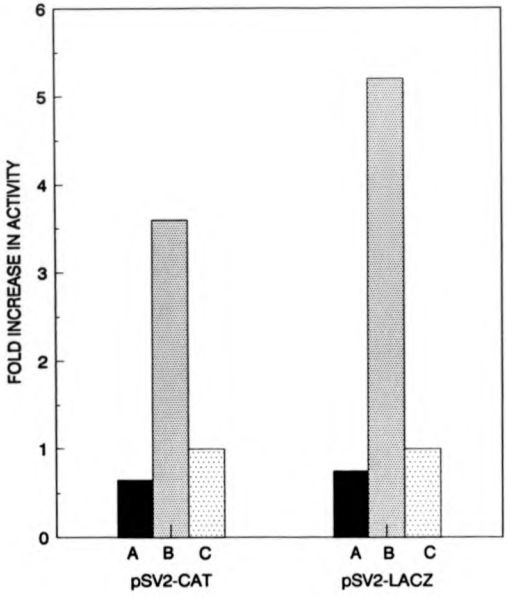

40,000
30,000
20,000
10,000
0
CAT ACTIVITY (CPM)
TK
RSV
SV40
CMV
PROMOTER

FIGURE 54: THE EFFECT OF σ3 ON *LACZ* AND CAT GENE EXPRESSION

COS-1 cells were co-transfected with 10 μg of SV40-1 (column A), SV40-3 (column B) or pEXP6 (column C) and with 1 μg of either pSV_2CAT or 1 μg pSV_2*Lac*Z. Reporter gene expression was assayed as described in Methods. The results are expressed as the stimulation of reporter gene expression over that found in cells co-transfected with pEXP6 and the relevant reporter gene as a control, which is taken as 1.


FOLD INCREASE IN ACTIVITY
6
5
4
3
2
1
0
A B C
pSV2-CAT
A B C
pSV2-LACZ

CHAPTER 9

THE CONSTRUCTION OF S4 HYBRIDS AND THEIR EFFECT ON REPORTER GENE EXPRESSION

9.1 Introduction

The work discussed in the previous chapters presented evidence to suggest that the effect of the two σ3 proteins on reporter gene expression differed. Considering the high homologies of the two proteins (Atwater *et al.*, 1986) it was also surprising to note that type 1 σ3 was not detected by immunofluorescence (figure 47, chapter 7), and in only very small amounts by immunoprecipitation (figure 43, chapter 7). Previous evidence based on *in vitro* translation assays suggested that type 1 σ3 was translated with the same efficiency as σ3 type 3 (figure 32, chapter 7). It therefore seemed reasonable to assume that type 1 σ3 was not recognised by the type 3 polyclonal serum used in the immunofluorescence and immunoprecipitation studies. In light of the findings in chapter 8, that is, the apparent stimulation of reporter gene expression by type 3 σ3 but not by that from type 1 σ3, polyclonal serum against type 1 reovirus was raised to check that our hypothesis for the failure to detect high level expression of type 1 σ3 following its transfection into cells was correct. During the time that this sera was being raised, hybrids between the two S4 genes were constructed in an attempt to localise the region of σ3 responsible for the differential effects the two proteins have on reporter gene expression.

9.2 Construction of S4 hybrids

These were constructed between the HPA-1 and HPA-3 clones (illustrated in figure 31, chapter 7). To do this simply required unique restriction enzyme sites at corresponding positions in the two genes and in the terminal additions generated during cDNA manipulation. *KpnI* and *StyI* were chosen. *KpnI* cuts both clones uniquely at the 5' end of the HIV-LTR and *StyI* cuts both S4 genes at nucleotide 824 and does not have a second site in either HPA-1 or HPA-3. The

cloning strategy used is outlined in figure 55. This strategy dictated that complete digestion of each plasmid DNA was essential, to ensure that the vector containing fragments used (B and D figure 55) did not contain any full length S4 gene sequences. Two hybrid clones were isolated: one containing the 5' portion of S4-1 (HIV-LTR -S4-1 (824 bp)) and the 3' portion of S4-3 (Rec-1), the other containing the 5' portion of S4-3 and the 3' portion of S4-1 (Rec-3). Due to the high homology of the S4 genes a range of digests were required to identify the recombinants (figure 56 and 57). These were *PstI*, *HincII*, *AwaI* and *PvuII*, each of which cut either once or twice at different positions within the two S4 genes and once or not at all within the vector sequence (figures 56 and 57). This allowed for identification of the hybrids by comparison with the parent plasmid. For example, the parent plasmid of Rec-1 is HPA-3 and the insert is from HPA-1. HPA-1 and HPA-3 can be readily identified by *PstI* digestion as S4-1 contains an internal *PstI* site at nucleotide 489 (figure 57(a)) which is absent in S4-3. Therefore Rec-1 would be expected to have the same restriction enzyme profile as wild type HPA-1 and not its parent plasmid HPA-3. The same argument applies for analysis with *HincII*. S4-3 contains an internal *HincII* site at nucleotide 438 which is absent from S4-1 (figure 56(a)). On the other hand *PvuII* digestion of Rec-1 would be expected to give the same restriction enzyme profile as the parent plasmid HPA-3, as HPA-1 contains a *PvuII* site at nucleotide 848 (i.e. at the 3' end downstream of the *StyI* junction) which is absent in HPA-3 (figure 57(a)). Finally, *AwaI* cuts at two different positions in HPA-1 and HPA-3, therefore two unique restriction enzyme profiles would be expected for the hybrids (figure 57(a)). When the restriction enzyme profiles of the hybrids were analysed on 1 % agarose gels (figure 56(b) and 57(b)) all of the above predictions were met.

These constructs were generated prior to the discovery of the requirement of the SV40 origin in the plasmid to achieve detectable levels of σ3 expression.

FIGURE 55: CONSTRUCTION OF S4 HYBRIDS: REC-1 AND REC-3

HPA-1 and HPA-3 (illustrated in figure 30, chapter 7) were digested with *KpnI/StyI* to generate two fragments: a 1.37 Kb insert, containing the HIV-LTR and relevant part of the S4 gene (bases 0-824), and a 3.28 kB fragment containing the rest of the vector:

A = HPA-1 *KpnI/StyI*

B = Remaining HPA-1 vector

C = HPA-3 *KpnI/StyI*

D = Remaining HPA-3 vector

The 4 fragments were isolated from a 1 % agarose gel and the 5' phosphate groups of B and D removed by treatment with CIP as described in Methods.

A was then ligated with D and B with C. The ligation(s) mix was transformed into MC1061. Colonies containing the hybrids were identified by mini-plasmid preparations and restriction enzyme analysis.

KEY

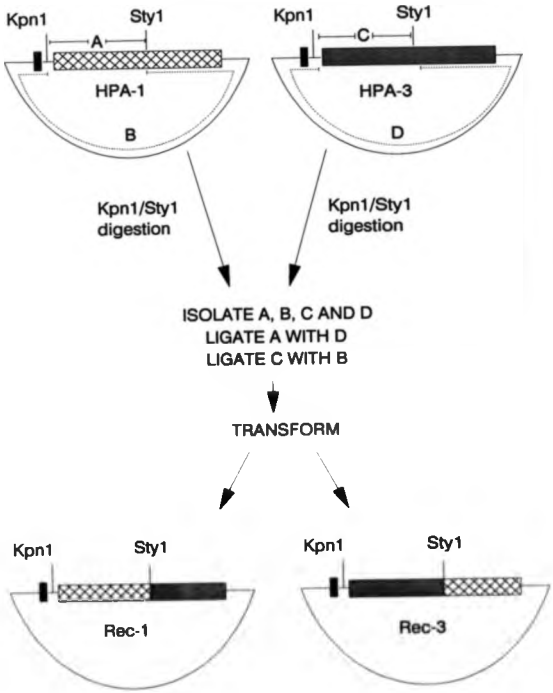

Kpn1
Sty1
A
HPA-1
B
Kpn1
Sty1
C
HPA-3
D
Kpn1/Sty1
digestion
Kpn1/Sty1
digestion
ISOLATE A, B, C AND D
LIGATE A WITH D
LIGATE C WITH B
TRANSFORM
Kpn1
Sty1
Rec-1
Kpn1
Sty1
Rec-3

FIGURE 56a: PREDICTION OF RESTRICTION ENZYME DIGESTS FOR S4 HYBRIDS I.

As discussed in the text the enzymes *AvaI*, *HincII*, *PvuII* and *PstI* were chosen to identify the hybrids. The positions where these enzymes cut in HPA-1 and HPA-3 are shown in this figure (*AvaI* and *HincII*) and in figure 57a (*PvuII* and *PstI*).

Note: HPA clones contain 5' HIV-LTR-S4-Poly A 3', and the only variables in these digests come from the S4 gene.

The predicted restriction enzyme profile of the hybrids, when compared to the wild type HPA-1 and HPA-3 are as follows:

AvaI: Digests S4-3 at nucleotide 764 and S4-1 at nucleotide 848, i.e. on either side of the *StyI* site used to generate the hybrids , therefore Rec-1 and Rec-3 would be expected to have two different restriction enzyme profiles when cut with the enzyme.

HincII: Digests S4-3 at nucleotide 438 and 857 and S4-1 at nucleotide 857. Therefore Rec-1 would be expected to have the same restriction enzyme profile as HPA-1 and Rec-3 the same profile as HPA-3.

FIGURE 56b: IDENTIFICATION OF S4 HYBRIDS (I).

Hybrids Rec-1 and Rec-3 were identified by digestion with *AvaI*, *HincII* and analysed on 1 % agarose gels. The restriction enzyme profiles agreed with those predicted in figure 56a:

AvaI: Two unique restriction enzyme profiles (track C is not fully digested).

HincII: Rec-1 has the same profile as HPA-1 and Rec-3 as HPA-3.

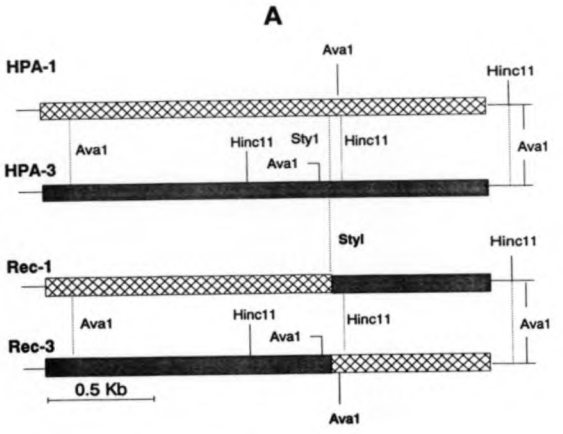
A = HPA-1

B = Rec-1

C = HPA-3

D = Rec-3

M = 1kb BRL ladder marker


A
HPA-1
Ava1
Hinc11
Ava1
Hinc11
Sty1
Hinc11
Ava1
Ava1
HPA-3
StyI
Hinc11
Rec-1
Ava1
Hinc11
Hinc11
Ava1
Ava1
Rec-3
0.5 Kb
Ava1

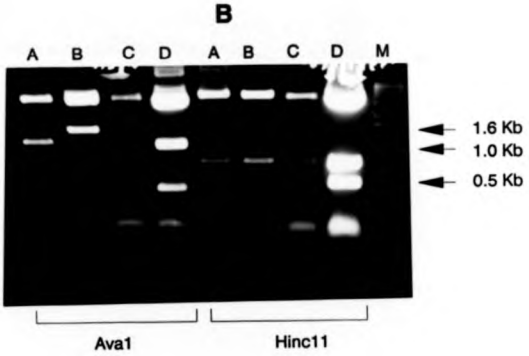

B
A
B
C
D
A
B
C
D
M
1.6 Kb
1.0 Kb
0.5 Kb
Ava1
Hinc11

FIGURE 57a: PREDICTION OF RESTRICTION ENZYME DIGESTS FOR S4 HYBRIDS (II)

The positions where *PvuII* and *PstI* cut in HPA-1 and HPA-3 shown in the figure.

PvuII: Digests S4-1 at nucleotide 838 and S4-3 does not have a *PvuII* site. Therefore Rec-1 would be expected to have the same restriction enzyme profiles as HPA-3 and Rec-3 the same profile as HPA-1.

PstI: Digests S4-1 at position 489 and S4-3 does not have a *PstI* site, therefore Rec-1 would be expected to have the same restriction enzyme profile as HPA-1 and Rec-3 the same profile as HPA-3.

FIGURE 57b: IDENTIFICATION OF S4 HYBRIDS (II)

Hybrids Rec-1 and Rec-3 were further identified by digestion with *PstI* and *PvuII* and analysed on 1 % agarose gels. The restriction enzyme profiles agreed with those predicted in figure 57a.

PvuII: Rec-1 has the same restriction enzyme profile as HPA-3 and Rec-3 as HPA-1. It should be noted that these are only partially digested by the enzyme, however the main band differentiating the serotypes (1 kb) can be clearly seen. When this was reanalysed all the correct bands were present (data no shown).

PstI: Rec-1 has the same restriction enzyme profile as HPA-1 and Rec-3 as HPA-3.

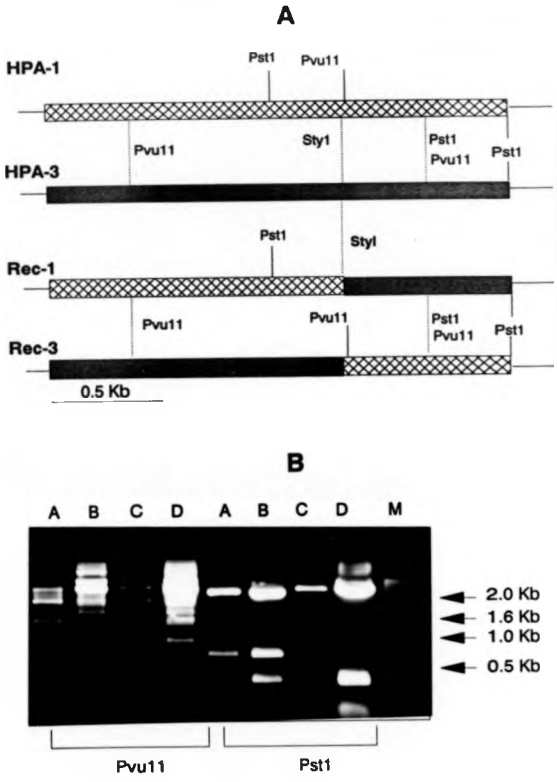
A = HPA-1

B = Rec-1

C = HPA-3

D = Rec-3

M = 1kb BRL ladder marker


A
HPA-1
Pst1
Pvu11
Pvu11
Sty1
Pst1
Pvu11
Pst1
HPA-3
Pst1
Styl
Rec-1
Pvu11
Pvu11
Pst1
Pvu11
Pst1
Rec-3
0.5 Kb
B
A B C D A B C D M
2.0 Kb
1.6 Kb
1.0 Kb
0.5 Kb
Pvu11
Pst1

The SV40 origin was therefore inserted into the *SalI* site present in the polylinker of Rec-1 and Rec-3 exactly as described for the construction of HIV-1 and HIV-3 clones from HPA-1 and HPA-3 (figure 30). The resulting clones were termed HY-1 (derived from Rec-1) and HY-3 (derived from Rec-3) and were identified by *HindIII* and *PstI* digestion as shown in figure 58.

9.3 The effect of σ3 hybrid expression on reporter gene expression

Figure 59 shows that expression of hybrid σ3 from HY-1 resulted in a 5-7 fold increase in ß-gal activity, similar to that produced by parental type 3 σ3. By contrast cells expressing the hybrid σ3 from HY-3 gave no stimulation in ß-gal activity, similar to the situation observed for parental type 1 σ3. Since HY-1 contains the 5' 800 bases of S4-1 and the 3' 400 bases of S4-3 these results suggested that the 3' end of S4-3 contains the domain responsible for stimulation of reporter gene expression.

When σ3 expression from the hybrids was analysed by immunofluorescence using type 3 polyclonal serum, σ3 expression was detected in cells transfected with HY-1 but not in those transfected with HY-3 (figure 60). The fact that expression of parental type 1 σ3 could not be detected by fluorescence but that from HY-1 (containing the 5' 800 bases of S4-1 and 3' 300 bases of S4-3) was, suggested that the 3' end of the S4-3 gene contained the region required for immunological recognition of the σ3 protein

9.4 Analysis of type 1 σ3 expression.

To ascertain if the lack of detection of type 1 σ3 by reovirus type 3 antibody was indeed due to the serum not recognising the protein, polyclonal serum was raised against reovirus type 1 as described in Methods. The serum was initially checked for activity by immunofluorescence of mouse L-cells

FIGURE 58: IDENTIFICATION OF HYBRIDS CONTAINING THE SV40 ORIGIN

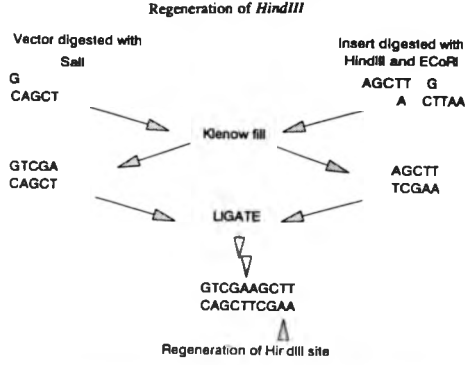
A = Rec-3

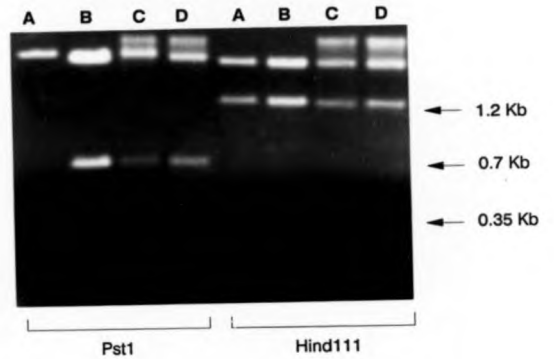
B = Rec-1

C = Hy-1

D = Hy-3

The S4 hybrid(s) was digested with *SalI* and Klenow filled. A small DNA fragment containing the SV40 origin was released from pSVE1 by *ECoRI/HindIII* digestion and the ends of the fragment Klenow filled. The fragment was ligated into the vectors containing the hybrid S4 genes and transformed into MC1061 as illustrated in figure 44. Antibiotic resistant colonies containing the SV40 origin were screened by mini-plasmid preparations and restriction analysis. *PstI* digestion released the expected 700 bp insert. *HindIII* digestion released a 350 bp insert which was due to the regeneration of the *HindIII* site by ligation of *HindIII/SalI* blunt ends. The diagram beneath the photograph represents the full length inserts and positions where the enzymes cut. The enzymes used did not digest the rest of the vector.




A
B
C
D
A
B
C
D
1.2 Kb
0.7 Kb
0.35 Kb
Pst1
Hind111

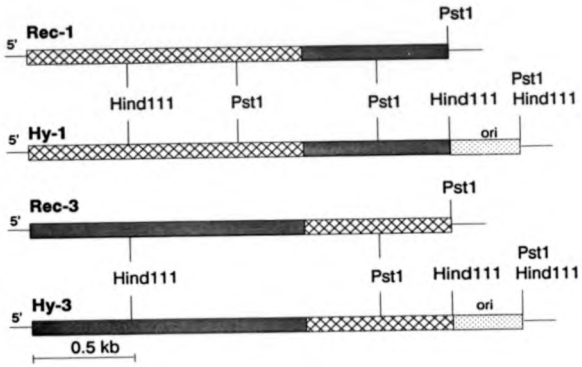
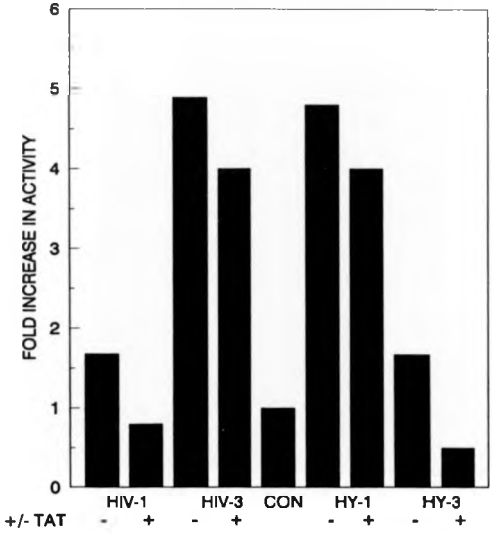

Rec-1
5'
Pst1
Hind111
Pst1
Pst1
Hind111
Pst1
Hind111
Hy-1
5'
ori
Rec-3
5'
Pst1
Hind111
Pst1
Hind111
Pst1
Hind111
Hy-3
5'
ori
0.5 kb

FIGURE 59: THE EFFECT OF σ3 ON ß-GAL GENE EXPRESSION

COS-1 cells were co-transfected with 10 μg of plasmid containing the S4 gene, 1 μg of TAT and 1 μg of pCH110. Reporter gene activity was analysed as described in Methods. The results are expressed as the fold increase in ß-gal activity compared to the control sample (HPA-3) which is taken as 1. The various S4 containing plasmids are shown below the graph.


FOLD INCREASE IN ACTIVITY
0
1
2
3
4
5
6
HIV-1
HIV-3
CON
HY-1
HY-3
+/- TAT
- + - + - + - +

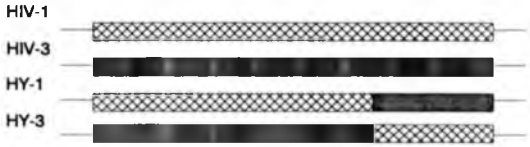

HIV-1
HIV-3
HY-1
HY-3

FIGURE 60: DETECTION OF σ3 EXPRESSION IN CELLS TRANSFECTED WITH HY-1 AND HY-3 WITH TYPE 3 ANTIBODY.

A = HY-1 + TAT (phase)

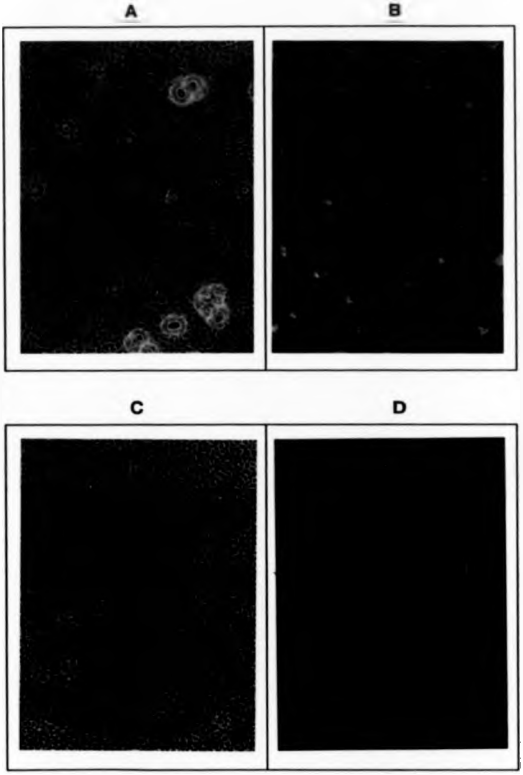
B = HY-1 + TAT (fluorescence)

C = HY-3 + TAT (phase)

D = HY-3 + TAT (fluorescence)

Subconfluent monolayers of COS-1 cells in 12 well tissue culture dishes were co-transfected with 5 μg of the relevant S4 containing DNA and 0.5 μg of TAT. 48 hours post transfection the cells were fixed and prepared for immunofluorescence as described in Methods.

magnification x100


A
B
C
D

infected at a low m.o.i with reovirus type 1 or 3 as previously described. As expected the serum was active against both type 1 and type 3 reovirus (figure 61).

In order to analyse σ3 expression using the polyclonal sera raised against type 1 virus, COS-1 cells were transfected with 5 μg of HIV-1, HIV-3, HY-1 or HY-3 in the presence of 0.5 μg of TAT and were prepared for immunofluorescence as described in Methods. Positive fluorescence was obtained from cells transfected with HIV-3. However, σ3 expression was not detected in HIV-1 transfected cells (figure 62). In view of the high homology of the two proteins it was not surprising that the type 1 serum was cross reactive with type 3 σ3 as was initially expected. However, it was surprising that no detectable type 1 σ3 expression was seen using this homologous sera. Positive fluorescence was also obtained in cells transfected with HY-1 (figure 62(c)), but no detectable σ3 expression was detected in HY-3 transfected cells as had previously been found using type 3 serum (figure 60).

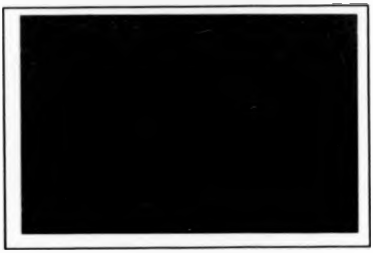
These results and those using SV40 driven σ3 expression (section 7.5.1) suggested that lack of detection of type 1 σ3 was either due to some problem in synthesis of the protein, which seemed unlikely from the *in vitro* translation data, or alternatively that the type 1 protein was unstable in comparison to that from type 3 in transfected cells.

9.5 Analysis of σ3 stability

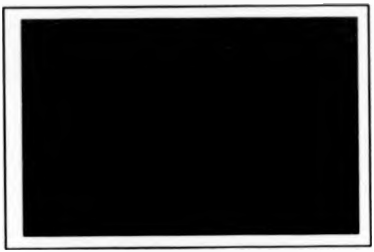
To analyse the stability of σ3 in transfected cells pulse chase experiments were carried out. The rationale was that if σ3 was stable the intensity of σ3 immunoprecipitated from pulse and chase samples would be the same. If on the other hand the protein was not stable the chased samples would be expected to give less protein than the pulsed samples. To do this experiment duplicate six well dishes of subconfluent monolayers of COS-1 cells were transfected with 10

FIGURE 61: ACTIVITY OF REOVIRUS TYPE 1 POLYCLONAL SERUM AGAINST REOVIRUS TYPE 1 AND TYPE 3

A. L-cells infected with reovirus type 1 (m.o.i 0.1)



B: L-cells infected with reovirus type 3 (m.o.i 0.1)



Subconfluent monolayers of L-cells were infected with reovirus type 1 or type 3 at an m.o.i of 0.1 and were prepared for immunofluorescence as described in methods. The cells were viewed under phase/fluorescence

Magnification x100

FIGURE 62: DETECTION OF σ3 EXPRESSION WITH REOVIRUS TYPE 1 POLYCLONAL SERUM

A = HIV-1 + TAT (phase/fluorescence)

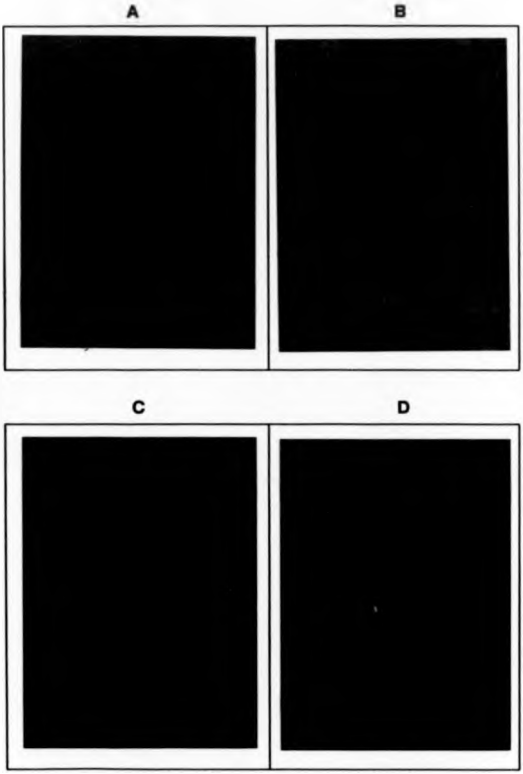
B = HIV-3 + TAT (phase/fluorescence)

C = HY-1 + TAT (phase/fluorescence)

D = HY-3 + TAT (phase/fluorescence)

Subconfluent monolayers of COS-1 cells in 12 well tissue culture dishes were co-transfected with 5 μg of the relevant S4 containing DNA and 0.5 μg of TAT. 48 hours post transfection the cells were fixed and prepared for immunofluorescence as described in Methods.

magnification x 100


A
B
C
D

μg of HIV-1, HIV-3, HY-1 or HY-3 and 1 μg of TAT or with 10 μg of SV40-1 or SV40-3. 48 hours post transfection the cells were starved of methionine for 1 hour and then pulse labelled for 6 hours as described in Methods. After this pulse labelling period, one well of each sample was harvested and the other chased by overlaying the cells with 1 ml of 100 x met-containing media for a further 6 hours. 1 μl aliquots of the cell extracts were TCA precipitated and equal amounts of labelled protein immunoprecipitated using type 1 serum as described in Methods.

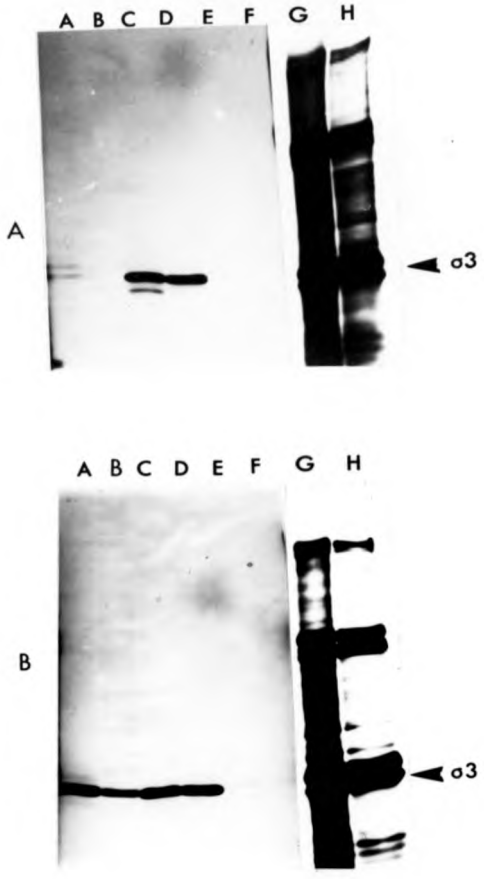
9.5.1 Results

The immunoprecipitated proteins were analysed on 10 % polyacrylamide gels as described in Methods. Figure 63(a) shows that only very small amounts of type 1 σ3 (tracks A and B) were immunoprecipitated in comparison to those from type 3 σ3 (tracks C and D). In the chase samples (track B) even less type 1 σ3 is detected than in the pulse labelled samples suggesting that type 1 σ3 is indeed unstable. It is also evident that the lower band associated with pulse labelled type 3 σ3 expression (track C) is not present in the chase track (track D). This suggests that this shorter protein product is a protein which is fully processed during the chase period. In contrast to type 3 σ3, cells transfected with the S4 gene from type 1 virus do not appear to express this smaller protein. When protein expression from the hybrids was examined (figure 63(b)) it was evident that the protein synthesised from HY-1 (containing amino acids 1-264 of σ3 type 1 and amino acids 265-365 of σ3 type 3) was as stable as that from the plasmid containing the parental type 3 S4 gene. However, the protein expressed by HY-3 (containing amino acids 1-264 of σ3 type 3 and amino acids 265-365 of σ3 type 1) had a stability comparable to that of type 1 σ3 (figure 63(b), tracks E and F). These results suggest that the 3' end of S4-3 confers stability to σ3. It is also evident that the lower band associated with pulse labelled type 3 σ3

FIGURE 63: ANALYSIS OF σ3 STABILITY

A	B
A = SV40-1 pulse	A = HIV-3 pulse
B = SV40-1 chase	B = HIV-3 chase
C = SV40-3 pulse	C = HY-1 pulse
D = SV40-3 chase	D = HY-1 chase
E = HIV-1 pulse	E = HY-3 pulse
F = HIV-1 chase	F = HY-3 chase
G = Type 3 pulse	G = Type 3 pulse
H = Type 3 chase	H = Type 3 chase

Duplicate subconfluent monolayers of COS-1 cells in six well dishes were transfected with 10 μg of the relevant DNA and 1 μg of TAT if required. 48 hours post transfection the cells were starved of methionine for 1 hour followed by a 6 hour pulse with ^{35}S-met. One set of samples was harvested and the other chased for a further 6 hours with 100x methionine as described in the text and in Methods. The cells were subjected to freeze thawing and sonication as described in Methods and the cell debris was removed by microfugation. 1 μl aliquots of the supernatants were TCA precipitated and equal amounts of labelled protein were immunoprecipitated with reovirus type 1 polyclonal serum. The proteins were analysed on 10 % polyacrylamide gels.


A B C D E F G H
A
σ3
A B C D E F G H
B
σ3

expression (track A) is not synthesised in cells transfected with HY-1 DNA but it is synthesised in cells transfected with HY-3 DNA. This suggests that the lower band present in the $\sigma 3$ tracks from the type 3 S4 gene is produced by the 5' end of the S4 gene of type 3.

9.6 Summary

Hybrids between S4-1 and S4-3 were constructed containing the 5' 800 bases of one serotype and the 3' 400 bases of the other serotype. The SV40 origin of replication was added to these plasmids to allow for replication of plasmid DNA in transfected cells. HY-1, containing the 3' 400 bases of S4-3, produced a marked stimulation of ß-gal expression in co-transfected cells, comparable to that given by wild type S4-3. These results suggested that the domain for this activity may lie in the 3' end of the S4-3 gene. Type 3 and type 1 polyclonal antibodies detected $\sigma 3$ by immunofluorescence in HY-1 but not HY-3 transfected cells.

When the stability of $\sigma 3$ was investigated it was found that type 1 $\sigma 3$ is much less stable than type 3 $\sigma 3$ and that the 3' domain of S4-3 is responsible for this stability. These results suggest that the ability of HY-1 to stimulate reporter gene expression is due to an increase in the stability of the protein. The significance of these results and a possible mechanism by which it may operate will be discussed in chapter 10.

CHAPTER 10
GENERAL DISCUSSION

10.1 The effect of σ3 on eukaryotic gene expression in *in vitro* and *in vivo* assay systems

The aim of the work presented in this thesis was to gain a greater insight into the role of reovirus in controlling host cell protein synthesis, a function of the S4 gene of the virus (Sharpe & Fields, 1982). Initial work confirmed the findings of Munemitsu and Samuel (1984), namely that type 3 reovirus does indeed inhibit host protein synthesis with greater efficiency than reovirus type 1. To further investigate this intracellular pathogenic property of the virus, *in vitro* and *in vivo* assay systems were designed in an attempt to model the effect of the S4 gene on host cell protein synthesis. The basic reagents for both these assay systems were full length cDNA clones of the S4 gene from serotypes 1 and 3 of the virus (S4-1 and S4-3) cloned into the versatile Bluescribe vector (discussed in chapter 4).

Two *in vitro* translation assay systems were developed. The first was based on the rabbit reticulocyte lysate system and the second made use of S-10 cell extracts prepared from infected and uninfected L-cells. The σ3 produced in these systems had no effect on either σ3 or ß-actin translation in the reticulocyte lysate system, or on endogenous mRNA translation in the S-10 cell extract system. One possible reason for the lack of effect of σ3 in both cases may have been that insufficient σ3 was synthesised to have any effect on translation. In the case of the reticulocyte lysate system, discussed in chapter 5, this could possibly have been overcome by adding exogenous σ3, prepared from infected cells as described by Huisman and Joklik (1976), to the system.. Another and possibly more likely explanation for the lack of effect of σ3 *in vitro* may be that it requires several other viral and/or cellular proteins to cause a full inhibitory effect. Indeed, the ability of reovirus type 3 σ1 to inhibit cellular DNA synthesis

has recently been shown to require other viral proteins (Fajorado & Shatkin, 1990).

The *in vitro* assay system was developed in an attempt to provide a quick assay in which to test hybrids between S4-1 and S4-3 and thereby domain map the region of S4-3 responsible for inhibition of host protein synthesis, prior to studying the effect of the protein *in vivo*. However the results obtained in this piece of work did not provide any evidence to suggest that σ3 has any direct effect on host cell protein synthesis in an *in vitro* situation.

To develop an *in vivo* assay system σ3 could be expressed from either a constitutive promoter or an inducible promoter, the latter being favoured to allow for the possibility that σ3 was toxic to the cells. One obvious way to study the effect of σ3 on host protein synthesis *in vivo* would have been to transfect cells with a DNA construct from which σ3 would be expressed and about 48 hours post transfection to label the cells with ^{35}S-met. Then, to determine if σ3 did indeed inhibit host protein synthesis, the extent of ^{35}S-met incorporation into protein by TCA precipitation could have been measured . However the transfection efficiency is rarely greater than 10 % and therefore it would be difficult to measure the extent of inhibition of host protein synthesis in cells expressing σ3 compared to the background of total cell protein synthesis. Indeed, Lemay and Shatkin (1986) studied the effect of σ3 in an assay system similar to this and observed that no direct effect on host protein synthesis could be related to σ3 expression. To overcome this problem we developed a system involving the use of reporter genes, as a marker for eukaryotic protein synthesis. For these assays σ3 synthesis was under the control of the tat inducible HIV-LTR to allow for analysis of reporter gene expression in the presence or absence of σ3 expression. However, as discussed in chapter 7, in order to produce a detectable level of σ3 in transfected cells the SV40 origin of replication had to be added to the plasmids and transfection carried out in COS cells which express the T-

antigen of SV40 and consequently amplify by replication any plasmid containing the SV40 origin. This change also increased the amount of HIV-LTR activity such that σ3 was detected in both the presence and absence of co-transfection with TAT. This suggested that the use of the inducible HIV-LTR/TAT system proposed by Sun and Baltimore (1989), is not as generally applicable as they initially suggested. Using polyclonal antibody against reovirus type 3 to detect σ3 expression in transfected cells, type 3 σ3 was readily detected by both immunofluorescence and immunoprecipitation. Type 1 σ3 was however not detected by immunofluorescence and only a very faint signal was detected by immunoprecipitation (figure 43 chapter 7). Restriction enzyme analysis and *in vitro* transcription-translation analysis showed that the σ3 from the type 1 virus was synthesised, and hence it was initially assumed that the lack of detection of type 1 σ3 was due to a failure of the protein to be recognised by the antibodies raised against type 3 virus.

The effect of σ3 on two different reporter genes (used as markers for host cell protein synthesis), CAT and ß-gal, was examined. σ3 expressed from Type 3 caused a 5-7 fold increase in ß-gal gene expression and in CAT gene expression when such expression was under the control of the herpes simplex TK promoter, the LTR of rous sarcoma virus or the early promoter of SV40. However, when CAT expression was under the control of the immediate early promoter of CMV then type 3 σ3 had no effect on its expression. One reason for this may be that the CMV promoter was the strongest used in these assays and consequently the levels of CAT produced may have been too high for σ3 produced from either the SV40 or HIV-LTR (in the presence of the SV40 origin of replication) promoters to have any effect on it. In contrast to these findings the σ3 of type 1 had no effect on reporter gene expression irrespective of which promoter it was being expressed from.

The results of the *in vivo* experiments therefore suggest that type 3 σ3 causes a stimulation of non-viral mRNA translation whilst that from type 1 σ3 does not. However, it must be remembered that when studied in isolation the effects produced by σ3 may not be a true reflection of what occurs in infected cells. It is possible that the S4 gene product controls the level of inhibition of protein synthesis but requires other proteins from either type 1 or type 3 to complex with it to cause a full inhibitory effect. In fact Lee *et al.* (1981) showed that σ3 is complexed to μ1c *in vivo* by using monoclonal antibodies to σ3. Conversely, the effects observed in this work may be related to the way the virus controls host protein synthesis and/or how it controls its own protein synthesis at the expense of cellular protein synthesis.

10.2. Current hypotheses for reovirus control of cellular protein synthesis.

As discussed in the Introduction there have been various proposals as to how reovirus controls host cell protein synthesis. Skup et al. (1981) initially hypothesised that control of host cell protein synthesis by reovirus occurred by a switch from cap dependent to cap independent translation at late times in viral infection. However this mechanism has been controversial for many years and recent evidence suggests that it may not be the mechanism of control. Using S-10 cell extracts prepared from uninfected and type 3 infected L-cells Lemieux *et al.* (1984) showed that capped reovirus and L-cell mRNA were both translated with greater efficiency in uninfected extracts than in infected extracts. By comparison uncapped reovirus mRNA was translated with greater efficiency in infected cell extracts. However, the theory that all uncapped mRNA would be translated with greater efficiency in the infected extracts did not hold true in the case of uncapped poliovirus mRNA, which was not translated efficiently in the virus infected extracts. As a consequence of these observations Lemieux *et al.*

(1984) proposed that a viral factor may be involved in promoting the efficient translation of viral mRNA late in infection. Indeed Lemieux *et al.* (1987) showed that σ3 purified from infected cells, according to the protocol described by Huisman and Joklik (1976), stimulated the translation of late viral mRNA. Further contradiction to this mechanism has recently been reported by Feduchi *et al.* (1988) who demonstrated that co-infection of cells with poliovirus shuts-off reovirus protein synthesis. Our results based on *in vitro* and *in vivo* assay systems did not provide any support for Millward's hypothesis.

The results presented in this work suggest that σ3 causes an increase in protein synthesis. Other workers have also found σ3 to have similar effects on both viral and non-viral mRNAs. Lemieux *et al.* (1987) showed that σ3 stimulated the translation of late viral mRNA. Work by Giantini and Shatkin (1989) also demonstrated that co-transfection of cells with a cDNA copy of the S4 gene of type 3 virus and a reporter gene resulted in a 10 fold stimulation in the reporter gene expression. In this case σ3 expression was driven by the RSV promoter and the reporter gene used was RSV-CAT, with transfections being carried out in COS-7 cells. To investigate the level at which this stimulation occurred and to see if this effect was due to intracellular competition between the co-expressing vectors, steady state CAT mRNA levels were examined by Northern blot analysis. This showed that there was only slight intracellular competition between the vectors and that stimulation of RSV-CAT by S4-3 occurred largely at the post transcriptional level.

This phenomenon of stimulation of non-viral mRNA translation has also been shown to be a function of the VAI RNA of adenovirus (previously discussed in the Introduction). Svensson & Akusjärvi (1984) demonstrated that VAI stimulates the translation of both early and late viral mRNA. To investigate the effect of VAI on non-viral mRNAs Svensson and Akusjärvi (1985) studied the levels of CAT gene expression in cells expressing VAI. They found a 5-6

fold stimulation of CAT protein synthesis, suggesting that adenovirus VAI mediates a translational stimulation which is not restricted to viral mRNAs (Svensson & Akusjärvi, 1985). The mechanism of control of translation by VAI has been discussed in detail in section 1.4. Briefly, ds RNA is produced during adenovirus replication. This activates a kinase, DAI, which in turn phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF-2α) thereby blocking the recycling and dephosphorylation of eIF-2 required for continuation of initiation of protein synthesis. VAI is produced in large amounts at late times in infection and complexes with DAI, blocking the activation of the kinase and thus allowing recycling of eIF-2 and the translation of late viral mRNAs (Katze *et al.*, 1987; Galabru *et al.*, 1989). Akusjärvi *et al.* (1987) have proposed that a similar mechanism operates in cells transfected with plasmid DNA, that is, dsRNA may be produced by base pairing of complementary plasmid transcripts which may then activate DAI and cause a decrease in protein synthesis in the transfected cells. VAI counteracts this response and restores translational activity resulting in an apparent stimulation of reporter gene expression.

10.3 A possible mechanism for σ3 action

The results obtained by Svcenson and Akusjärvi (1985) bear a close resemblance to the results obtained with σ3 both by ourselves and others. It is conceivable that σ3, in isolation from other viral factors, may control protein synthesis by a mechanism similar to adenovirus. Another important property of σ3 is that it has an affinity for dsRNA (Huisman & Joklik, 1976). In the transfected cells DAI may be activated by dsRNA or other RNA structures produced during the transcription and processing of the reporter gene mRNA which is foreign to the cell. The effect of type 3 σ3 produced following co-transfection of cells may involve association with these RNA structures thus

preventing DAI activation and allowing protein synthesis to continue. This may also be related to the events occurring in infected cells. At late times in viral infection, when inhibition of host protein synthesis is evident in type 3 infected cells, $\sigma 3$ may prevent the activation of DAI by binding to dsRNA structures produced during viral replication, thus allowing the translation of late viral mRNAs which, being the dominant mRNA in the cells, out-compete the host mRNA for the ribosomes. Therefore in the transfected cells, in the absence of other viral proteins, reporter gene expression may be stimulated by $\sigma 3$ in the same way as it stimulates the synthesis of late viral protein synthesis.

In recent years there has been other evidence to support the hypothesis that $\sigma 3$ acts as an antagonist to DAI. Bischoff and Samuel (1989) studied the activation of human Pi/eIF-2α protein kinase (DAI) by S1 and S4 reovirus mRNA to gain a further insight into the mechanism of Interferon action which is induced by viral infection (section 1.2.4). They observed that reovirus S1 mRNA was a potent activator of DAI where as reovirus S4 mRNA was a very poor activator. Kitajeski *et al.* (1986) had previously shown other RNAs such as VAI, tRNA and 5S ribosomal RNA to be poor activators .

As mentioned in the Introduction there is a translational discrepancy between the S class mRNAs. S4 mRNA is translated 10 times more efficiently than S1 mRNA. To further investigate the mechanism by which this may occur, Samuel and colleagues (1990) investigated the effect of 2 aminopurine on the translation efficiency of S1 and S4 mRNA. 2 aminopurine (2AP) is a potent inhibitor of DAI and acts in a similar way to VAI (Kaufman & Murtha, 1987). They showed that 2AP treatment of cells transfected with S1 or S4 cDNA increased the translation of S1 mRNA, possibly by inhibiting the activation of DAI, but failed to have any effect on the translation of S4 mRNA. This may have been because $\sigma 3$ had already inhibited the activation of DAI. Imani and Jacobs (1988) have also suggested that $\sigma 3$ may be an antagonist of DAI.

The results described in the previous paragraph were all obtained using the S4 gene of type 1 reovirus. Our results showed distinct differences between the ability of the σ3 proteins of the two serotypes to stimulate translation: σ3 from type 3 stimulated reporter gene expreasion whereas that from type 1 did not cause any stimulation. These findings could, in turn, be related to apparent differences the two genes exhibit on host cell protein synthesis. In an attempt to begin the localisation of the region(s) of S4 responsible for these differences, hybrids between the two S4 genes were constructed. One of the hybrids, Hy-1, contained the first 800 bp of S4-1 joined to the 3' terminal 400 bp of S4-3 whilst the other, Hy-3, was the converse i.e: the 5' end of type 3 joined to the 3' end of type 1. Co-transfection experiments showed that Hy-1 stimulated ß-gal gene expression to the same extent as wild type S4-3 and that Hy-3 had no effect on reporter gene expression, similar to that of wild type S4-1. This result initially suggested that the domain of the type 3 gene causing stimulation of reporter gene expression lay in the 3' terminal third of the S4-3 gene.

As discussed in chapter 9 type 1 σ3 was found to be much less stable in transfected cells than that from type 3. Analysis of the stability of the proteins synthesised from the hybrids indicated that the 3' end of S4-3 conferred stability on the σ3 of type 1 and also increased its ability to stimulate reporter gene expreasion, suggesting that these two properties of the protein are closely related.

In the region of S4 (824-1196 bp) where the difference in the properties of the two genes appears to lie there are 27 base changes between two viruses, 7 of which result in 6 amino acid changes. The amino acid changes are scattered throughout the domain with the exception of 2 changes at amino acids 300 and 301.

The non-homologous amino acid positions of the domain are listed below.

	Amino Acid		Bases	
Position	Ty1	Ty3	Ty1	Ty3
300	Asp	Glu	GAT	GAG
301	Ser	Ala	TCA	GTC
325	Asp	Asn	GAC	AAT
345	Ser	Pro	TCA	CCA
348	Thr	Ile	ACC	ATC
353	Asp	Asn	GAT	AAT

As mentioned earlier an important property of σ3 is that it binds to dsRNA. Schiff *et al.* (1988) investigated the structural basis of this binding activity. σ3 was functionally dissected by proteolytic cleavage with S.aureus V8 protease, which generated two major fragments of 24K and 16K. The carboxy terminal fragment was found to contain the dsRNA- binding domain. We have shown that the carboxy terminus of type 3 protein is responsible for both stability in transfected cells and its ability to stimulate reporter gene expression. One explanation for the differences in the two σ3s with respect to their ability to stimulate reporter gene expression may be that the type 3 protein is more stable, allowing it to bind to more dsRNA structures, thus causing the proposed effects discussed in section 10.3. The observations made with hybrid S4 genes i.e. the ability of HY-1 (containing the 3' end of S4-3) to stimulate reporter gene expression provides evidence to support the argument that σ3 regulates protein synthesis by its ability to bind dsRNA.

σ3 is the most abundant protein in the infected cells and it is mostly found complexed with μ1c, both constituents of the viral outer capsid shell. Huismans and Joklik (1976) showed that only free σ3 was able to bind to double stranded RNA structures. The ability of the virus to stimulate late viral mRNA protein

synthesis may be related to the amount of free σ3 present in the cell throughout the infection cycle.

10.4 Future Experiments

Further work is required to confirm the proposed mechanism of S4 control of host protein synthesis. Firstly, the effect of σ3 on reporter gene expression could be examined in the presence of other viral proteins, for example, μ1c, to see if this still causes stimulation of reporter gene expression. Secondly the levels of DAI in the transfected cell extracts could be assayed to see if they are different in cells transfected in the presence or absence of the reporter gene.

10.5 Concluding remarks

The results presented in this thesis have provided evidence to support the idea that the reovirus protein σ3 controls protein synthesis by antagonising DAI activation, thus allowing the expression of late viral proteins at the expense of cellular protein synthesis, resulting in an apparent inhibition of host cell protein synthesis. There is a difference in the ability of type 1 and type 3 reovirus to do this. These results suggest that σ3 from type 3 is stable when expressed on its own in transient assay systems, and is capable of binding to dsRNA structures, resulting in the inhibition of DAI activation which in turn stimulates reporter gene expression and late viral protein synthesis. In contrast to type 3, type 1 reovirus does not inhibit host cell protein synthesis and its σ3 protein does not stimulate reporter gene expression. We have shown that type 1 σ3 is unstable when expressed on its own in transient assays. It may be that this protein is only stable when it is complexed with μ1c on the outer capsid shell. Only free σ3 binds to dsRNA and hence σ3 type 1, being unstable, cannot bind to it and thus may not activate DAI kinase activity. Type 1 reovirus does not grow as quickly

as type 3 in tissue culture, which may in part be due to the inability of its $\sigma 3$ protein to promote late viral protein synthesis at the expense of cellular protein synthesis. Further work will hopefully clarify the mechanism by which reovirus controls protein synthesis.

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