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TITLE STUDIES ON THE CELL-BINDING PROPERTIES OF
THE REOVIRUS o1 PROTEIN

AUTHOR S.
 MASON

DEGREE Ph.D

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**STUDIES ON THE CELL-BINDING PROPERTIES OF
THE REOVIRUS σ 1 PROTEIN**

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**A thesis submitted for the degree of
Doctor of Philosophy
at the University of Warwick**

**Virus Group
Department of Biological Sciences
University of Warwick
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November 1992

To Mum, Ray and Alicia

"Though this be madness, yet there is method in't....."

Hamlet, William Shakespeare (1564-1616)



The Far Side. Gary Larson 1992

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DECLARATION

All the results presented in this thesis were obtained by the author unless specifically acknowledged and no part of this thesis has been previously presented in application for a degree.

All sources of information and materials are indicated in the text.

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ABBREVIATIONS

A_x	absorbance of a solution at x nm.
Å	Angstrom
aa	amino acid
2AP	2-aminopurine
APS	ammonium persulphate
(d)ATP	(deoxy)adenosine triphosphate
bp	base pair
BMV	brome mosaic virus
BSA	bovine serum albumin
cDNA	complementary DNA
Ci	Curie
cpm	counts per minute
(d)CTP	(deoxy)cytidine triphosphate
DIF	deionised formamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
eIF	elongation initiation factor
EMC	encephalomyocarditis virus
Et Br	ethidium bromide
FSB	formamide stop buffer
g	gram
(d)GTP	(deoxy)guanosine triphosphate
HA	haemagglutinin
HIV	human immunodeficiency virus

HRR	(major) human rhinovirus receptor
Ig	immunoglobulin
IHYP	iodohydroxybenzyl-pindolol
IPTG	isopropyl- β -thiogalactopyranoside
Kb	kilobase
kDa	kiloDalton
LD ₅₀	lethal dose 50
Mab	monoclonal antibody
MBN	mung bean nuclease
⁵ mC	5-methyldeoxycytosine
β -Me	beta-mercaptoethanol
min	minute
ml	millilitre
moi	multiplicity of infection
mRNA	messenger RNA
ng	nanogram
NP40	nonidet P40
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PCR	polymerase chain reaction
pfu	plaque forming unit
pmole	picomole
PVR	poliovirus receptor
RF	replicative form
RNA	ribonucleic acid
rpm	revolutions per minute

SDS	sodium dodecyl sulphate
ss	single-stranded
TEMED	N,N,N'N'-tetramethylethylenediamine
TMV	tobacco mosaic virus
Tris	tris(hydroxyl)aminomethane
tRNA	transfer RNA
T _s	temperature sensitive
(d)TTP	(deoxy)thymidine triphosphate
UTP	uridine triphosphate
uv	ultraviolet
VSV	vesicular stomatitis virus
v/v	volume for volume
w/v	weight for volume
μCi	microCurie
μg	microgram
μl	microlitre
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

SUMMARY

Mammalian reoviruses are ds RNA viruses that are not associated with any specific disease process in man. They can cause disease in mice where the type 1 (T1) and type 3 (T3) viruses exhibit markedly different tissue tropism and virulence properties. The different disease patterns of these serotypes are due in large part to the viral cell-attachment protein, $\sigma 1$, specifically targetting the virus to either neuronal cells (T3) or ependymal cells (T1). The main aim of this project was to locate the cell-binding domain of protein $\sigma 1$ in order to further characterise the virus-cell attachment process.

The T1 and T3 $\sigma 1$ proteins were expressed *in vitro* from cloned DNA copies of the respective S1 genes. *In vitro* translations were carried out in a rabbit reticulocyte lysate system optimised with respect to salt and s1 RNA concentration to maximise the efficiency of translation. The s1 mRNAs were poorly translated in the lysate (in agreement with *in vivo* and *in vitro* studies of other groups). Two different methods were therefore employed to effect an increase in the synthesis of the respective $\sigma 1$ proteins: (i) Oligonucleotide directed mutagenesis of the cloned S1-1 gene was utilized to simultaneously improve the weak sequence context of the $\sigma 1$ initiation codon and reduce the length of the 5' untranslated (leader) region. The resulting "mutant" transcripts possessed a leader sequence of the same length as those synthesized *in vivo*. (ii) The inclusion of 2-aminopurine in the *in vitro* translation reactions improved the efficiency with which $\sigma 1$ of both serotypes was synthesized. This was due, presumably, to an inhibition of the RNA dependent protein kinase, P1/eIF-2 α , which is involved in the control of protein synthesis.

A cell-binding assay was developed in which oligomerization of type 3 $\sigma 1$ was found to be critical for its ability to bind to monolayers of L-cells. It was found that the formation of oligomeric species required an *in vitro* translation temperature of 37°C instead of the standard 30°C. Protein $\sigma 1$ -1 was neither able to oligomerize or bind to cells. Specific, saturable binding of $\sigma 1$ -3 was demonstrated. These studies also revealed that the T1 and T3 virus probably bind to different receptors on L-cells.

Deletion mutants of $\sigma 1$ -3 were created by oligonucleotide directed mutagenesis as a means of locating the cell-binding domain of this protein. None of the mutant proteins were found to be capable of binding to L-cells. However, a conformational effect could not be discounted as the primary reason for their inability to bind, as opposed to the removal of a critical binding domain.

CHAPTER 1

Introduction

1.1 General Characteristics of Reovirus

1.1.1 Reovirus Classification

Mammalian reoviruses belong to the Orthoreovirus genus of the Reoviridae family. They were originally members of the ECHO 10 group of viruses but Sabin (1959) proposed that they be reclassified into a new group as they had several anomalous characteristics compared to other known enteroviruses: they were larger, produced cytoplasmic inclusions in monkey kidney cells, were pathogenic in newborn but not adult mice and caused haemagglutination in human type O erythrocytes. The name reovirus is an acronym for respiratory, enteric, orphan viruses as they were (and are) not associated with any known disease in humans. The prototypic type 1 reovirus is the Lang strain and was first isolated by Ramos-Alvarez and Sabin (1954) from a healthy child. Two other viral isolates from the stools of children with diarrhoea subsequently became the prototypic strains for the other serotypes of reovirus and are the type 2 Jones (or D5) and the type 3 Dearing viruses (Sabin, 1959). Another viral isolate, Abney, also became a prototype for reovirus type 3 (Rosen, 1960 and Rosen *et al.*, 1960).

The three serotypes can be distinguished on the basis of haemagglutination-inhibition and neutralization assays (Rosen, 1960; Rosen, 1962 and Sabin, 1959).

1.1.2 The Structure and Morphology of Reovirus

Reovirions are non-enveloped particles, 75-80nm in diameter and consist of two concentric layers, the inner and outer capsids, both of which are icosahedral in shape (Jordan and Mayor, 1962; Luftig *et al.*, 1972 and

Harvey *et al.*, 1981). An electron micrograph of a reovirus type 3 particle is shown in Figure 1.1.1 (reproduced from Schiff and Fields, 1990). The inner core is 50-60nm in diameter and encloses the double-stranded (ds) RNA genome (Luftig *et al.*, 1972 and Harvey *et al.*, 1981) which is tightly packed into a spherical structure organized such that the adjacent stretches of helix are parallel to each other (Harvey *et al.*, 1981). This was the first natural example of ds RNA and was identified when mammalian cells infected with reovirus stained orthochromatically to give a pale green colour when treated with acridine orange, instead of the intense red colour associated with single-stranded (ss) nucleic acids (Gomatos *et al.*, 1962). The genome consists of ten discrete segments of ds RNA (Shatkin *et al.*, 1968) which can be divided into three size classes, L (large), M (medium) and S (small) (Bellamy *et al.*, 1967 and Watanabe and Graham, 1967).

Reoviral proteins can also be divided into three size classes - λ , μ and σ - and comprise both structural and non-structural species (Smith *et al.*, 1969; Zweerink *et al.*, 1971; Both *et al.*, 1975 and McCrae and Joklik, 1978; Table 1). The outer capsid consists of the major proteins σ_3 and μ_{1c} and the minor protein σ_1 (Smith *et al.*, 1969) whilst the core is composed of the major polypeptide species λ_1 , λ_2 and σ_2 and the minor protein λ_3 and possibly μ_2 (Smith *et al.*, 1969). Polypeptide λ_2 forms a pentameric spike structure (Ralph *et al.*, 1980) which is located at the vertices of the core icosahedron. Antibodies directed against λ_2 were found to react with intact virions, suggesting that λ_2 is exposed on the surface of the virus particle (Hayes *et al.*, 1981). Protein σ_1 is thought to be located close to λ_2 since λ_2 specific antibodies prevent anti- σ_1 antibodies from binding virions, an effect not observed with antibodies directed against the other outer capsid proteins (Lee *et al.*, 1981b).

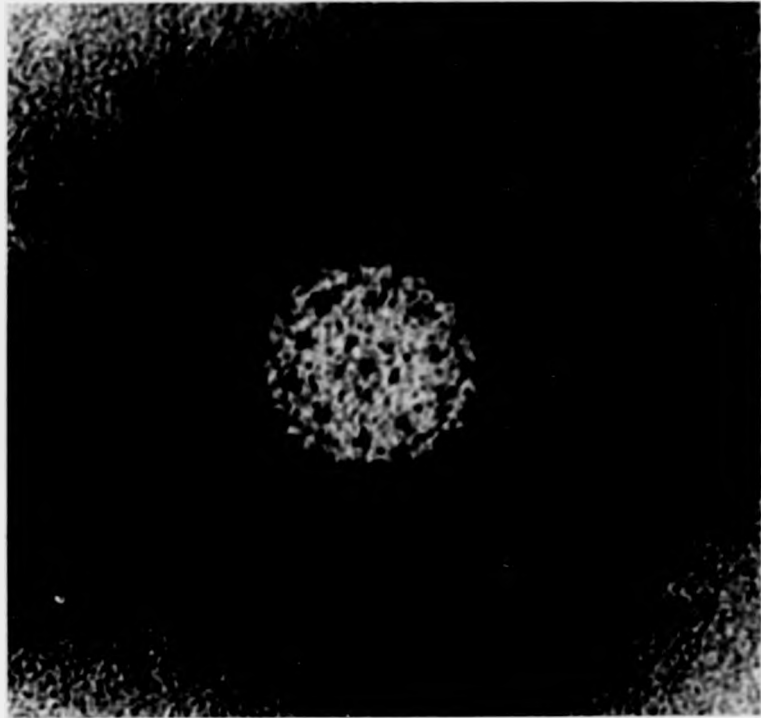


Figure 1.1.1 Electron Micrograph of a Reovirus Type 3 Particle
Reproduced from Schiff and Fields (1990).

dsRNA	Gene Size (Kb)	Protein	Protein RMM (kDa)	Location of Protein
L1	3.85	λ 3	135	Core
L2	3.91	λ 2	140	Core Spike
L3	3.89	λ 1	155	Core
M1	2.3	μ 2	70	Core
M2	2.2	μ 1/ μ 1c	80/72	Outer Capsid
M3	2.24	μ NS	75	Non-structural
S1	1.42	σ 1	49	Outer Capsid
	0.36	σ 1s	14	Non-structural
S2	1.33	σ 2	38	Core
S3	1.2	σ NS	36	Non-structural
				structural
S4	1.2	σ 3	34	Outer Capsid

Table 1 Properties of the Genes and Proteins of Reovirus

Adapted from Schiff and Fields (1990).

1.1.3 Coding Assignments of the Genome RNAs

Two different approaches to the problem of assigning the protein coding potential of each gene were pursued in independent laboratories and both gave similar answers. A direct methodology, where the individual ds RNA segments were isolated, denatured and then translated *in vitro* resulted in the identification of the polypeptide encoded by each gene (McCrae and Joklik, 1978; Figure 1.1.2); it also formally demonstrated that the plus strand of the genome RNA could act as mRNA. At the same time, Mustoe *et al.* (1978) used a genetic approach. It was known that the ds RNA and protein profiles of each serotype were different when examined by polyacrylamide gel electrophoresis (PAGE). Recombinant viruses were derived from pairwise crosses of different serotypes and their genome and protein profiles examined; comparisons between the recombinants and parental viruses enabled unambiguous coding assignments for the M and S sized genes. (This could not be achieved for the L genes as the λ protein profiles of the three serotypes could not be sufficiently resolved).

Cleavage of $\mu 1$ to $\mu 1c$ (Smith *et al.*, 1969 and Zweerink and Joklik, 1970) by the removal of a small amino-terminal fragment (Jayasuriya *et al.*, 1988) accounted for the initial identification of eleven protein products from ten genes. However, a series of investigations led to the discovery of a third non-structural protein, $\sigma 1s$, found to be encoded by the S1 gene. Two unique 80S ribosome protected fragments were isolated from s1 mRNA (Kozak, 1982a). The s1 message was also shown to direct the synthesis of two formylmethionine dipeptides *in vitro* (Cenatiempo *et al.*, 1984). Sequencing of S1 cDNA clones from all three serotypes revealed the existence of a second open reading frame (ORF) initiating about 70 nucleotides downstream and out of frame with

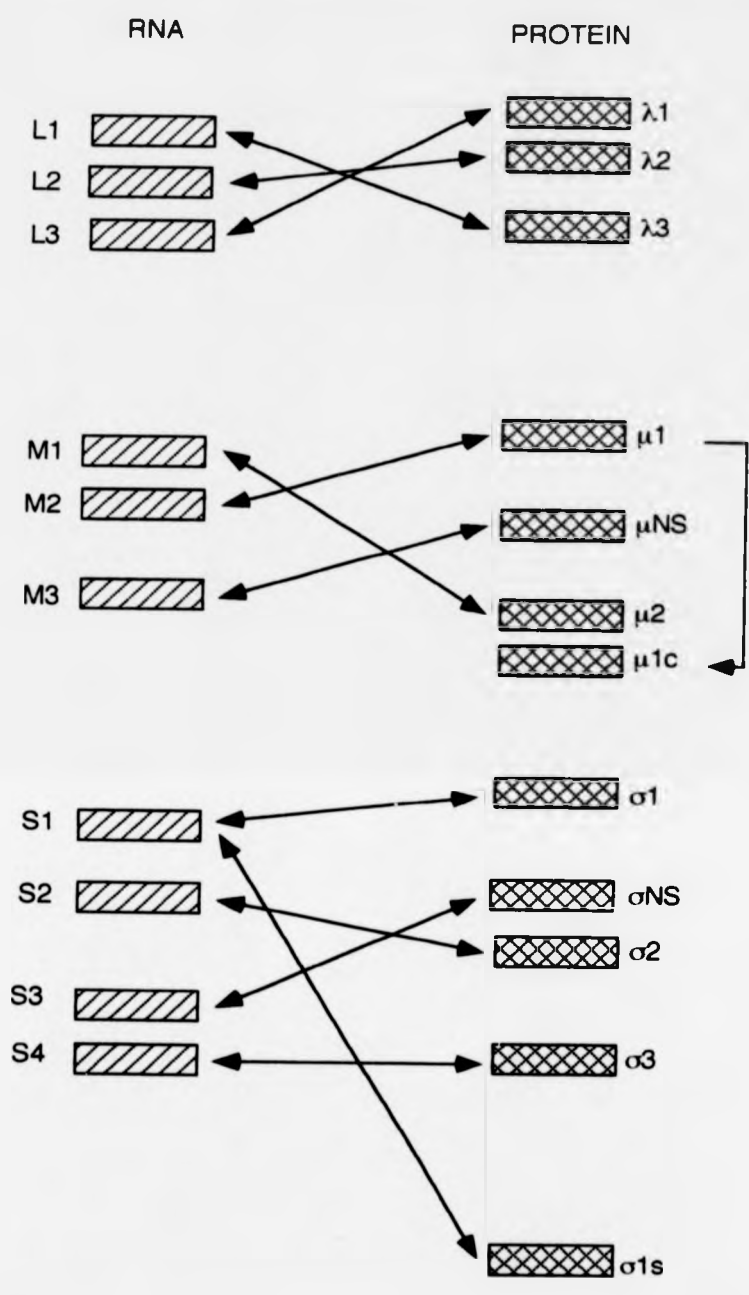


Figure 1.1.2 Gene - Protein Coding Assignments for Reovirus
 Adapted from McCrae and Joklik (1978).

the start codon for $\sigma 1$ (Nagata *et al.*, 1984; Bassel-Duby *et al.*, 1985; Cashdollar *et al.*, 1985 and Munemitsu *et al.*, 1986). The second ORF was predicted to form a small highly basic protein of approximately 14 kilodaltons (kDa). This has been expressed from s1 mRNA *in vitro* (Ernst and Shatkin, 1985; Jacobs *et al.*, 1985 and Jacobs and Samuel, 1985) and has been identified in the cytoplasm of reovirus infected cells but is not associated with virus particles (Ernst and Shatkin, 1985; Jacobs and Samuel, 1985 and Ceruzzi *et al.*, 1986). Expression in *Escherichia coli* (*E. coli*) has also been achieved (Ceruzzi *et al.*, 1986 and Pelletier *et al.*, 1987). The S1 gene, unlike the others, is therefore functionally dicistronic, but the functional significance of $\sigma 1$ s protein is unknown.

1.2 An Overview of the Reovirus Replicative Cycle

1.2.1 Adsorption/Penetration

Reoviruses attach to specific receptors on the cell surface by means of the outer capsid protein $\sigma 1$ (Lee *et al.*, 1981b). Once adsorbed, the virus particles are taken up into phagocytic vesicles which then fuse with lysosomes. This appears to be a fairly rapid process as virus particles have been observed in these endocytic vesicles as early as 15 minutes post-adsorption (Silverstein and Dales 1968; Sturzenbecker *et al.*, 1987 and Georgi *et al.*, 1990).

Evidence that viral entry is a receptor mediated endocytotic event, was obtained recently by Georgi *et al.* (1990) who followed the initial events in reovirus infection by means of individually labelled fluorescent virions. Virus particles initially attached over the entire surface of the cell, were internalized as small clusters of virions which then migrated to a perinuclear localization. At this point the label became dispersed, probably owing to fusion of the vesicles containing the internalized clusters of virions with lysosomes. This strongly resembles the characteristic sequence of events involved in the receptor mediated endocytosis of various cellular ligands (Goldstein *et al.*, 1985).

1.2.2 Uncoating

Chymotrypsin digestion of reovirus particles *in vitro* produces viral cores that are biochemically and morphologically similar to those produced *in vivo*. Digestion *in vitro* in low salt buffer yields viral cores lacking all outer shell proteins (Smith *et al.*, 1969 and Joklik, 1972) whilst digestion in high salt conditions gives a structure that more closely resembles the

subviral particle (SVP) formed *in vivo* as it contains polypeptide δ , one of the digestion products of polypeptide $\mu 1c$ (Joklik, 1972; Shatkin and LaFiandra, 1972; Silverstein *et al.*, 1972 and Borsa *et al.*, 1973).

Uncoating appears to be an essential step in the reovirus life cycle since lysosomotropic agents, which raise the pH of acidic vesicles, prevent degradation of the outer coat and inhibit the replicative cycle (Canning *et al.*, 1983).

1.2.3 Transcription

Digestion of the virus outer capsid proteins with chymotrypsin was shown to activate the transcriptase of reovirus (Shatkin and Sipe, 1968; Skehel and Joklik, 1969 and Banerjee and Shatkin, 1970). Specifically, cleavage of $\mu 1c$ appears to be the step responsible for activation of the viral transcriptase since removal of $\sigma 3$ (the other major outer capsid polypeptide) is not concomitant with the onset of transcription (Joklik, 1972 and Drayna and Fields, 1982). However, Borsa *et al.* (1973 and 1974) reported that transcriptional activity was not manifest unless the digested particles were in the presence of monovalent cations. (These cations were not removed in the viral purification procedure of the other authors mentioned above).

Virus specific mRNA can be isolated from cytoplasmic extracts of infected cells (Bellamy and Joklik, 1967 and Shatkin and Rada, 1967) and separated into three size classes (l, m and s) corresponding to the genome segments from which they are derived (Bellamy and Joklik, 1967; Watanabe and Graham, 1967 and Watanabe *et al.*, 1967). All ten segments of ds RNA are transcribed and only one strand of the ds RNA template is copied (Skehel and Joklik, 1969; Levin *et al.*, 1970 and Banerjee and Shatkin, 1970).

Transcripts produced *in vitro* were shown to be identical in nature to their *in vivo* counterparts (Hay and Joklik, 1971). Both *in vivo* and *in vitro* messages were described as full length copies of their respective genomic segments. This resulted from hybridization experiments in which they had appeared to form perfect hybrids with denatured ds RNA (Skehel and Joklik 1969; Levin *et al.*, 1970 and Hay and Joklik 1971). This postulation was confirmed later from sequencing data, in which the 3' termini of all ten species of mRNAs and plus strand RNAs were shown to possess a short consensus sequence (McCrae, 1981 and Antczak *et al.*, 1982). Translation *in vitro* confirmed that these molecules were messenger RNAs (McDowell *et al.*, 1972 and Graziadei *et al.*, 1973). Sequencing experiments of McCrae (1981) and Antczak *et al.* (1982) revealed that the minus strands also shared a common 3' terminal sequence, which was different to that of the plus strands.

The transcriptase reaction is fully non-conservative in that the genomic ds RNA remains within the viral core and is never detected amongst the transcription products (Skehel and Joklik, 1969; Banerjee and Shatkin, 1970; Silverstein *et al.*, 1970 and Schonberg *et al.*, 1971).

Early transcription occurs in parental SVPs (uncoated, infectious virions) and yields capped mRNA (Desrosiers *et al.*, 1976; Furuichi *et al.*, 1975; Galster and Lengyel, 1976 and Skup *et al.*, 1981). The 5' terminal cap has the structure: $^7\text{mG}(5')\text{ppp}(5')\text{G}^{(m)}\text{pCp}$ (Furuichi *et al.*, 1975; Faust *et al.*, 1975 and Skup *et al.*, 1981). The plus strand of the genome RNA also has the same cap structure as early mRNA and mRNA produced from viral cores via *in vitro* reactions (Furuichi *et al.*, 1975)

Late transcripts, however, are produced from progeny virion particles and have been reported by one group to have uncapped 5' ends - pGpC....- owing to latent activity of the capping enzymes in these particles (Skup and Millward, 1980b; Zarbl *et al.*, 1980 and Skup *et al.*, 1981).

The early transcripts are thought to act as templates for minus strand synthesis, but it has been suggested that some late transcripts may also perform a similar role (Morgan and Zweerink, 1977).

The transcriptional activity of the progeny virions is abrogated by assembly of the the outer shell to form whole virions (Astell *et al.*, 1972) with $\sigma 3$ implicated as the protein responsible for the inactivation. This was taken to mean that the transcriptase and capping enzymes were latent in whole virions. However, Stolfus *et al.* (1974) demonstrated an oligoadenylate activity in whole virions. Yamakawa *et al.* (1982) also showed that intact virions do have transcriptase and capping activities but that only short oligonucleotides are produced, probably owing to abortive initiation. This correlates well with earlier observations that reovirions contain single stranded (ss) as well as ds RNA. The ss RNA is present as short oligoadenylate molecules and truncated 5'-G- terminated molecules (Bellamy and Joklik, 1967; Bellamy *et al.*, 1972; Nichols *et al.*, 1972 and Stolfus and Banerjee, 1972).

Physical constraints imposed by the outer capsid proteins of the maturing virion have been proposed to convert the transcriptase to an oligoadenylate synthetase (Silverstein *et al.*, 1976). The G- terminated molecules appear to be products of abortive initiation (Bellamy *et al.*, 1972 and Yamakawa *et al.*, 1982) since they contain the consensus sequence -GCUA- found at the 5' end of reovirus mRNAs (Nichols *et al.*, 1972) and genes (McCrae, 1981 and Antczak *et al.*, 1982).

Recently, biochemical evidence has been obtained to suggest that $\lambda 2$ is the reovirus guanylyltransferase as it is the only protein able to bind radiolabelled GTP (forming a $\lambda 2$ -GMP complex) and catalyse the transfer of the guanylyl group to short reovirus oligonucleotides (Cleveland *et al.*, 1986). Sequencing of the L2 gene (which encodes $\lambda 2$) and subsequent

analysis of the data has also revealed some homology to the consensus sequence of GTP binding proteins (Seliger *et al.*, 1987).

1.2.4 Replication to Double-Stranded RNA

The capped message sense transcripts produced early in infection serve as templates for minus strand synthesis in nascent, progeny SVPs (Acs *et al.*, 1971; Schonberg *et al.*, 1971 and Sakuma and Watanabe, 1971). Replication is fully conservative as the parental ds RNA remains within the SVP throughout the infectious cycle (Silverstein *et al.*, 1970; Acs *et al.*, 1971 and Schonberg *et al.*, 1971). The replication step is also thought to proceed asynchronously in that plus sense RNA is synthesized before the minus strand (Acs *et al.*, 1971 and Schonberg *et al.*, 1971), but some evidence exists to suggest that late mRNA (i.e. that synthesized late in the infectious cycle) may also act as minus strand template (Morgan and Zweerink, 1977). Whichever is correct, only a single round of replicase activity apparently occurs (Zweerink, 1974).

1.2.5 Translation

Newly synthesized viral proteins can be detected two hours post-infection and by 10 hours most of the proteins synthesized in infected cells are viral (Zweerink and Joklik, 1970). This is in accordance with earlier observations that following reovirus infection, a gradual decrease in host cell protein synthesis is observed with a concomitant increase in viral polypeptide synthesis (Gomatos and Tamm, 1963 and Ensminger and Tamm, 1969).

The underlying mechanism causing the switch to viral protein synthesis and hence a preference for the translation of uncapped RNA is

as yet unknown. Several theories have been proposed to account for this phenomenon and only a brief description of these will be given.

ai) Modification of the cap dependent host cell translation system.

Skup and Millward (1980a) and Skup *et al.* (1981) have proposed that the cap dependence of the host cell translation apparatus undergoes a modification to allow the translation of capped mRNA early in infection and uncapped message at later stages. They demonstrated that early mRNA was capped and could be translated efficiently only in extracts from uninfected cells, whilst late mRNA was uncapped and could only be translated in extracts prepared from infected cells.

aii) A variation on the above theme has been forwarded by Lemieux *et al.* (1984) in which the host translation machinery is not modified *per se* but instead a factor present in infected cells aids the translation of uncapped mRNA. Two other studies have even proposed that $\sigma 3$ may function in just this manner, Lemay and Millward (1986) and Lemieux *et al.* (1987).

b) Inactivation of a host cell cap binding protein.

Zarbl and Millward (1983) suggest that a host cell cap binding protein necessary for the translation of capped RNA is inactivated and thus allows translation of uncapped mRNAs.

c) Competition of viral mRNA for a message discriminating factor.

A completely different mechanism to those suggested by the authors in (a) and (b) has been proposed in which the ability of viral and cellular mRNAs to compete for a message discriminatory factor determines the efficiency with which a message is translated (Walden *et al.*, 1981). In this model the discriminatory factor must bind to the message before complexing with 40S ribosomal subunits. *In vitro* studies by Brendler *et al.* (1981a;b) determined that under a particular set of magnesium and potassium ion concentrations, the hierarchy of translation efficiencies

owing to competition faithfully reproduced that of the *in vivo* experiments of Walden *et al.* (1981). Also, no difference in this order was noted for uncapped compared to capped reovirus messages. Further work by Detjen *et al.* (1982) disputed the takeover of the host translation system by uncapped viral message as it was seen that: infected and uninfected cell extracts translated capped globin mRNA equally well, there was no visible increase in decapped globin mRNA translation in reovirus infected cells and translation of cellular and viral messages were sensitive to inhibition by the cap analogue 7-methyl guanosine triphosphate. Also, eIF 4A and the cap binding protein complex have been proposed by Ray *et al.* (1983) to be candidates for these message discriminatory factors .

1.2.6 Regulation of Translation

Viral protein synthesis is a highly regulated process in reovirus with control exerted at both transcriptional and translational levels.

1.2.6.1 Transcriptional Control

Viral mRNA synthesis is regulated not only with respect to the frequency of transcription but also in a qualitative manner with a set of so-called "early mRNAs" preferentially transcribed at early times in infection. Between 0-2 hours post-infection, only 4 mRNAs are produced, namely, 11, m3, s3 and s4 (Watanabe *et al.*, 1968; Nonoyama *et al.*, 1974 and Lau *et al.*, 1975).

Nonoyama *et al.* (1974) showed that if protein synthesis is inhibited at the time of infection by the addition of cycloheximide, then only the 4 early viral mRNAs are produced. By performing the block at various times post-infection a gradual increase in the number of gene segments

transcribed was observed with all 10 ds RNAs being transcribed by 4-6 hours post-infection. Similarly, Shatkin and LaFiandra (1972) showed that viral cores produced by chymotrypsin digestion can synthesize all ten species of mRNA *in vitro* but are restricted to the early pattern when used to infect cycloheximide treated cells.

Skehel and Joklik (1969) and Banerjee and Shatkin (1970) demonstrated that the rate of transcription *in vitro* was inversely proportional to the size of the ds RNA segment being transcribed, i.e. 2x and 4x as many s sized transcripts are formed as m and l respectively in accordance with their gene sizes. This situation differs *in vivo*. Zweerink and Joklik (1970) found that although the relative rates of transcription were broadly similar between size classes there were differences among some of the individual class members. However, it was reported in a later study by Nonoyama *et al.* (1974) that early in the infectious cycle there were some differences in the transcription rates of individual messages within a size class but this changed late in infection to each message having a similar transcription frequency. It is possible that the differences in the methods of analysis contribute to some of the discrepancies between the latter two studies. A list of transcription frequencies is given in Table 1.2.1.

Gene	Transcription Frequency	Translation Frequency	<u>Translation</u> <u>Transcription</u> Frequency
L1	0.05	0.03	0.6
L2	0.05	0.15	3
L3	0.05	0.1	2
M1	0.15	0.03	0.2
M2	0.3	1.0	3.3
M3	0.5	0.5	1
S1	0.5	0.05	0.1
S2	0.5	0.2	0.4
S3	1.0	0.3	0.3
S4	1.0	0.7	0.7

Table 1.2.1 Approximate Relative Transcription and Translation Frequencies of the Reovirus Genes

Taken from Joklik (1981).

1.2.6.2 Control of Translation Frequencies

The ten species of reovirus mRNA are translated with widely varying efficiencies (Table 1.2.1), a phenomenon first reported by Zweerink and Joklik (1970). These differences remain constant throughout the infectious cycle (Zweerink and Joklik, 1970; Joklik, 1981 and Gaillard and Joklik, 1985) despite capping of the early messages and non-capping of the late transcripts. The relative translation efficiencies are approximately equivalent for each serotype (Gaillard and Joklik, 1985). Different turnover rates of the various mRNAs do not account for the varying translation efficiencies as each message appears to be equally stable (Skehel and Joklik, 1969 and Zweerink and Joklik, 1970) and the different abundancies of individual viral transcripts only partially accounts for this effect. The S4 gene is transcribed twice as frequently as the S1 gene but translated fourteen times as frequently. The s4 message is therefore translated seven times more efficiently.

Remarkably similar results to the *in vivo* studies have been produced *in vitro* (Levin and Samuel, 1980). For example, Levin and Samuel (1980) found that the s4 mRNA was translated about 8x as efficiently as the s1 mRNA *in vitro* in a wheat germ system correlating with a sevenfold difference between them *in vivo* (Gaillard and Joklik, 1985 and Atwater *et al.*, 1987). The polypeptides synthesized *in vitro* migrate to the same relative positions as native reovirus proteins on sodium dodecyl sulphate (SDS) polyacrylamide gels (Graziadei and Lengyel, 1972; McDowell *et al.*, 1972; Levin and Samuel, 1977 and Skup and Millward, 1977) and would therefore appear to be authentic products.

Altogether, this data suggested that control of translation frequency is probably directed by an inherent property of each message; i.e. resides within sequence information. To this end, the 5' terminal sequences of the

reovirus mRNAs and genes were determined (Kozak, 1977; Kozak and Shatkin, 1977a,b; Kozak, 1982b; McCrae, 1981 and Antczak *et al.*, 1982) and analysed for features that may be important in regulating the translational efficiencies of the reovirus messages. No obvious feature, such as leader length or sequence symmetry was found that correlated with the ability of a message to be well or poorly translated. The only feature apparently associated with efficiently translated messages is an AG rich area upstream of and surrounding the initiation codon, the significance of which is unknown (Antczak *et al.*, 1982).

Kozak (1981; 1983 and 1984) proposed the "-3 +4" rule for translation, based on comparisons between numerous eukaryotic mRNA sequences in which initiation of translation is efficient if the -3 nucleotide (with respect to the initiating AUG codon) is a purine - usually an "A"- and the +4 a "G". This is generally obeyed in reovirus with the exception of the m1 message; here the start codon is in a favourable context GUCAUGG, but the message is translated poorly (Antczak *et al.*, 1982).

1.2.7 Morphogenesis and Release

Free ds RNA has never been found in the cytoplasm of reovirus infected cells. Minus sense RNA is formed from a plus sense template with which it remains associated in a progeny particle and so is not found free in the cytoplasm either (Acs *et al.*, 1971). Assembly of reovirus RNA is therefore purported to be at the level of single stranded, plus sense RNA and the earliest particles detected contain ss RNA associated into a protein complex that is RNase sensitive (Acs *et al.*, 1971; Morgan and Zweerink, 1974; 1975 and Zweerink *et al.*, 1976). Replication of the ss RNA then occurs to give particles containing ds RNA, that are RNase resistant and manifest transcriptional activity (Acs *et al.*, 1971; Sakuma and Watanabe,

1971 and Morgan and Zweerink, 1975); these are the progeny transcriptase particles. It was suggested by Acs *et al.* (1971) and Sakuma and Watanabe (1971) that the RNase resistance of these particles was the result of a conformational change (in the replicase particles) that accompanied the replication step. They further suggested that the transcriptase may be an alternative form of the replicase enzyme but no direct evidence was reported.

Although replicase and progeny transcriptase particles could be isolated from infected cells, pure populations of these two species were never obtained (Zweerink *et al.*, 1972; Zweerink, 1974; Morgan and Zweerink, 1975 and Zweerink *et al.*, 1976). However, the progeny transcriptase particles were shown to be morphologically different from the SVPs formed when infectious virions are uncoated (Morgan and Zweerink, 1975 and Zweerink *et al.*, 1976). In the literature to date, there appears to have been little further elaboration on either the nature of the replicase and transcriptase particles or their respective enzyme activities. Hence, it is still unknown if the viral replicase and transcriptase are two different enzymes or alternative activities of the same protein (or protein complex).

One of the last steps in reovirus morphogenesis appears to be the formation of the short oligonucleotides present in mature virus particles (Zweerink *et al.*, 1976), previously described in section 1.2.3.

A characteristic of reovirus infected cells is that they develop dense granular particles in the cytoplasm known as inclusion bodies or factories (Gomatos *et al.*, 1962). During the infection, they coalesce and form a collar around the nucleus. Viral factories have been shown to contain ds RNA (Gomatos *et al.*, 1962 and Silverstein and Schur, 1970) and mature and incomplete virions (Gomatos *et al.*, 1962 and Dales and Gomatos, 1965). Isolation of the cytoplasmic fraction containing viral factories demonstrated that it contained ss RNA and ds RNA synthesis activity *in*

vitro (Acs *et al.*, 1971) and that all ten ds RNA segments can be synthesized from it (Watanabe *et al.*, 1968). No ribosomes are found within the factories; this necessitates the export of RNA to the cytoplasm for translation and import of proteins for virion assembly. Taken together, the above studies suggest that the viral factories are the sites of virus replication and assembly.

1.3 Reovirus as a Pathogen

1.3.1 The Mouse Model of Reovirus Pathogenesis

Reoviruses are widely distributed in nature, being found in both vertebrates and invertebrates. Approximately 80% of the human population is estimated to be seropositive for reovirus but there is little evidence of association with disease or illness (Sharpe and Fields, 1985). The same is true for most animals with the important exception of mice. Reovirus T3 causes a spontaneous infection in the mouse, characterized by jaundice, oily hair, diarrhoea and growth retardation, symptoms also seen in systemically infected newborn mice (Walters *et al.*, 1963 and Stanley *et al.*, 1964). Infection of the central nervous system (CNS) is also possible with reovirus serotypes 1 and 3, when they are inoculated intracerebrally; this mode of infection also reveals highly specific differences in the pattern of disease and neurotropism exhibited by these two serotypes. Type 1 infects ependymal cells (which line the ventricles of the brain) causing a non-fatal hydrocephalus from which the mice recover (Kilham and Margolis, 1969; Margolis and Kilham, 1969 and Weiner *et al.*, 1977); type 3 however, attacks the neurones and invariably leads to a fatal encephalitis (Walters *et al.*, 1963; Margolis *et al.*, 1971; Gonatas *et al.*, 1971; Raine and Fields, 1973 and Weiner *et al.*, 1977). In each case the brain cell/tissue type attacked is specific to the respective serotype; type 1 does not infect neurones and type 3 does not invade the ependymal cells.

There are also striking differences in the virulence of these two serotypes when inoculated in this manner. The type 3 virus can have a lethal dose 50 (LD₅₀) of only 10 pfu whereas doses of up to 10⁶ pfu of type 1 are non-lethal; correspondingly, the yield of virus from the brains of such mice is 10²-10³ -fold greater for type 3 than for type 1 (Raine and

Fields, 1973; Weiner *et al.*, 1977; Weiner *et al.*, 1980b and Hrdy *et al.*, 1982).

Inoculation of newborn mice by the oral route does not lead to clinical symptoms of these characteristic disease patterns with either T1 (Walters *et al.*, 1965) or T3 virus (Rubin and Fields, 1980) although the former is more virulent than the latter in this case, as shown by an increase in viral titre in the stomach for type 1 and a decrease for type 3 (Rubin and Fields, 1980). This is discussed more fully in section ii.

After oral inoculation, reovirus initially concentrates in intestinal M (microfold) cells, specialized cells that overlie Peyer's patches and then the Peyer's patches themselves (Wolf *et al.*, 1981; Wolf *et al.*, 1983 and Kauffman *et al.*, 1983b). Transport through the cells of Peyer's patches allows access to the underlying tissue, from which the virus can spread to more peripheral organs. Reovirus type 3 has a limited capacity to spread, reaching only the Peyer's patches in adult mice and the mesenteric lymph nodes in suckling mice. Type 1 can spread to the mesenteric lymph nodes and spleen in both adult and suckling mice (Kauffman *et al.*, 1983b).

1.3.2 The Use of Reassortants in the Genetic Analysis of Reovirus Pathogenesis

The nature of the genetic material of reovirus, i.e. discrete segments of ds RNA, has greatly aided the study of many aspects of reovirus biology as it confers upon the virus the ability to form reassortants. During replication each gene segment segregates independently of the others. Therefore, mixed infections, that is infection of the same cell with two different serotypes, results in a high proportion of the progeny virions having some genome segments derived from each parental serotype; these are the reassortant viruses (Fields and Joklik, 1969; Fields, 1971; Sharpe *et*

al., 1978 and Ramig *et al.*, 1978). Some of these reassortants contain a genome where only one gene segment has been replaced and are called monoreassortants, (Figure 1.3.1). Investigations could therefore be carried out to ascertain the biologic effects of individual genes.

1.3.2.1 The Role of the S1 Gene

As reovirus types 1 and 3 exhibit such distinct patterns of tissue tropism and neurovirulence, reassortants were made between these two serotypes and their virulence properties examined by intracranial inoculation of suckling mice. The results suggested that the specific virulence properties correlated with the presence of the appropriate type S1 gene (Weiner *et al.*, 1977). In a further study, a recombinant clone containing the S1 gene of reovirus type 3 and nine genes from type 1 - clone 1.HA3 - was shown to cause a necrotizing encephalitis and killed the mice (as did the type 3 virus). Infection with the reciprocal clone - 3.HA1 - (containing 9 genes from type 3 and the S1 of type 1) was not fatal to any of the mice and showed the same pattern of tropism as type 1 virus, i.e. damage to ependymal cells, not the neurones (Weiner *et al.*, 1980b). Thus both tissue tropism and virulence are determined by the S1 gene (Weiner *et al.*, 1977 and Weiner *et al.*, 1980b). It was thought that the $\sigma 1$ protein (encoded by the S1 gene) was probably responsible for targeting the virus to the different cell types and hence producing the separate patterns of tropism and virulence. This was shown to be the case in two separate studies, one by Tardieu and Weiner (1982) and the other by Dichter and Weiner (1984). Respective binding studies *in vitro* demonstrated the absolute specificity of type 1 and clone 3.HA1 attachment to ependymal cells and of type 3 and clone 1.HA3 to neural cells. Similar studies *in vivo*,

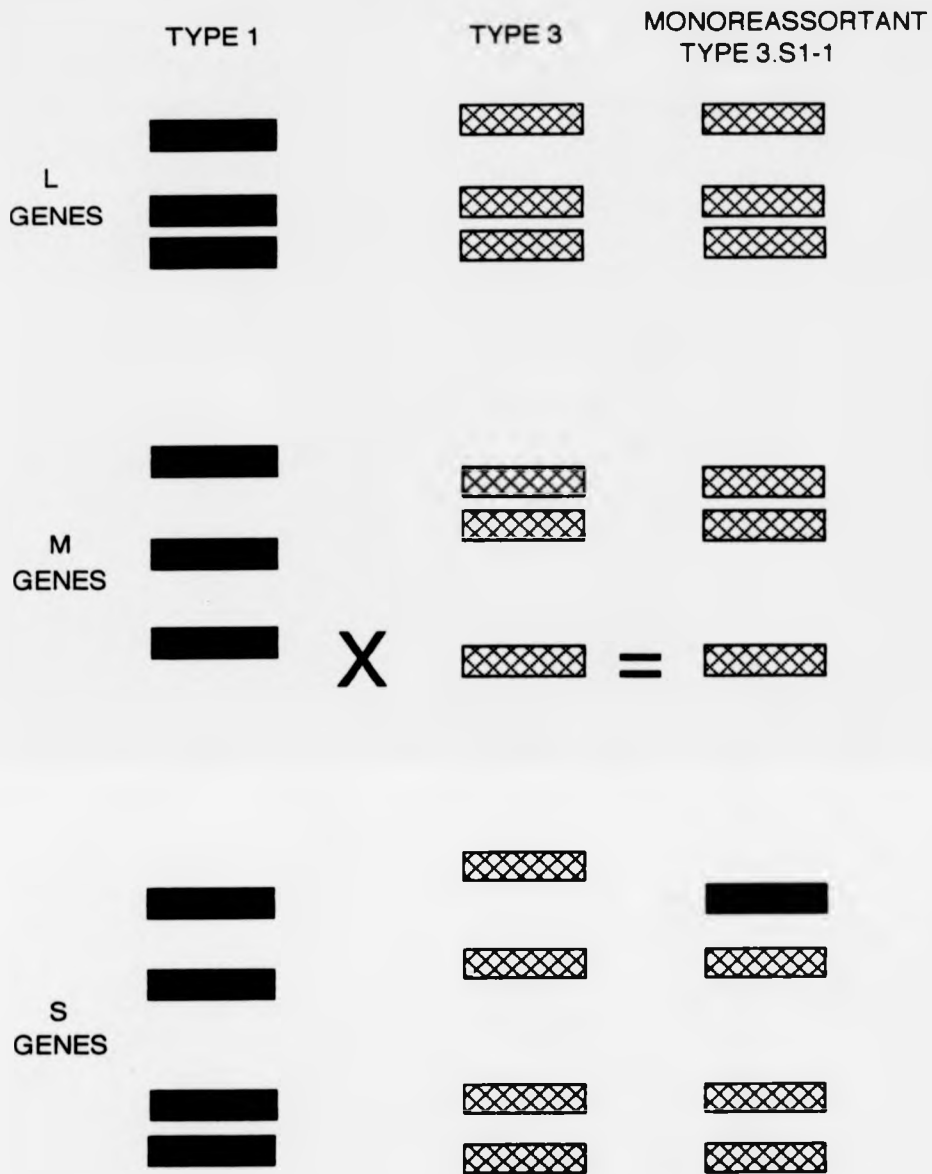


Figure 1.3.1 Schematic Representation of Reassortant Formation

Here, a genetic cross between type 1 and type 3 reovirus is shown, resulting in a monoreassortant where the S1 gene of type 1 has replaced that of type 3.

on sections of infected mouse brains showed type 1 viral antigen on ependymal cells and type 3 on neuronal cells (Weiner *et al.*, 1980b).

i) S1 and Attenuation

Studies on antigenic variants of reovirus further revealed the involvement of the S1 gene in the virulence process. Spriggs and Fields (1982) generated reovirus type 3 antigenic variants by growing stock type 3 virus in the presence of a neutralizing, anti-haemagglutinin (anti-HA) monoclonal antibody (Mab). The virulence properties of these variants were subsequently investigated. In each case the variants were found to be far less virulent than the parental type 3 Dearing strain (requiring an LD₅₀ dose at least 10,000 times higher) and had a greatly reduced ability to replicate in the brains of the mice into which they were injected (Spriggs and Fields, 1982). Subsequent investigations (Spriggs *et al.*, 1983a; Kaye *et al.*, 1986 and Bassel-Duby *et al.*, 1986) revealed that the variants could only infect a subset of neurones and were completely impaired in their ability to spread to the brain after peripheral inoculation.

Genes from a selection of these and other similarly generated variants were sequenced and found to contain only a single amino acid substitution in the carboxy-terminal half of the $\sigma 1$ protein, where the cell-binding domain has been postulated as being located (Bassel-Duby *et al.*, 1986). A reassortant virus containing the S1 gene from one of the variants clearly showed the single amino acid substitution in $\sigma 1$ to be responsible for the attenuated virulence and growth characteristics of these variants (Kaye *et al.*, 1986).

ii) S1 and Viral Spread in the Host

Reovirus T1 and T3 also use distinct pathways of spread once they have invaded the host, a property again related to the origin of the S1 gene. Type 1 virus spreads to the CNS via lymphatic and haematogenous routes, whereas type 3 disseminates via neural pathways and in particular appears to utilize the system of fast axonal transport (Tyler *et al.*, 1986). Studies on reassortant viruses demonstrated that this was specifically owing to the parental origin of the S1 gene. Similarly, the degree to which reovirus will spread to other organs after intragastric inoculation is also influenced by the nature of the S1 segment (Kauffman *et al.*, 1983b).

1.3.2.2 The Role of the M2 Gene

The M2 gene encodes the viral protein $\mu 1$ whose cleavage product $\mu 1c$ is a major component of the viral outer capsid. Studies involving various type 3 field isolates and reassortant clones have illustrated the marked effect on virulence that the M2 gene can have. Reovirus type 1 and 3 differ greatly in their sensitivity to chymotrypsin (and other proteases including those found in gastric fluids) and in the subsequent effect it has on their ability to replicate. Type 1 is relatively insensitive to protease action and can replicate as ably as intact virions but type 3 is extremely sensitive and loses this ability (Rubin and Fields, 1980). This characteristic was found to map to the M2 RNA segment and resulted in T1 virus being able to replicate in the intestine of perorally infected mice whilst T3 was rapidly cleared from this site. However, recombinant viruses containing a type 1 M2 gene and a type 3 S1 gene were lethal when inoculated by the same route (Rubin and Fields, 1980).

Recently, evidence has been obtained that questions this particular function of the M2 gene. Bodkin and Fields (1989) noted that in the study

of Rubin and Fields (1980), the reassortant viruses utilized to assign the protease sensitivity of the virus to the M2 gene were generated by crossing mutagenized, temperature sensitive type 3 virus with wild type stocks of type 1 virus. It was also noted that the amount of virus excreted from the intestines of infected mice and the ability to be transmitted amongst littermates of newborn mice mapped to the L2 gene (Keroack and Fields, 1986); furthermore, the reassortant viruses utilized in this study were derived from non-mutagenized parental stocks. Bodkin and Fields (1989) therefore employed similar reassortant viruses created from crossing wild type stocks of T1 and T3 and found that the growth and survival properties of reovirus in the intestinal environment of mice were determined by the L2 and S1 genes. Thus, reassortants containing the T1 L2 and S1 genes behaved as type 1 reovirus and grew well in the intestinal environment whereas the converse was true for type 3 virus and reassortants with a type 3 L2/S1 pairing. Furthermore, there was no correlation of these growth and survival properties with the presence of the M2 RNA segment.

Apart from its possible role in conferring protease sensitivity, the M2 gene affects reovirus virulence in a less well understood manner. Hrdy *et al.* (1982) examined various field isolates of reovirus type 3 and found a highly attenuated clone - T3(H/Ta). The greatly reduced virulence of this isolate mapped to the M2 gene but it retained a chymotrypsin sensitivity comparable to the T3 Dearing laboratory strain and displayed a pattern of tissue tropism that was similar to all of the other T3 isolates. Thus, it was suggested that the M2 segment may affect the virus' growth properties (Hrdy *et al.*, 1982).

1.4 The $\sigma 1$ Protein of Reovirus

The previous sections have shown that $\sigma 1$ plays a crucial role in the replication of reovirus and in determining the tropism and virulence properties of the virus. The following sections describe the structure and other functions of this protein.

1.4.1 The Physical and Morphological Properties of $\sigma 1$

The S1 gene of reovirus, which encodes the $\sigma 1$ protein (Weiner *et al.*, 1978) has been cloned and sequenced by several groups (Nagata *et al.*, 1984; Bassel-Duby *et al.*, 1985; Cashdollar *et al.*, 1985 and Munemitsu *et al.*, 1986). The gene is approximately 1.4 Kb long and encodes a $\sigma 1$ protein of approximately 450 amino acids, 49 kDa in molecular mass.

The predicted T3 $\sigma 1$ protein sequence was analysed and predicted to form two structurally distinct regions (Bassel-Duby *et al.*, 1985). The amino-terminal third of the protein (aa 28-158) was seen to consist of a heptapeptide repeat pattern (*a-b-c-d-e-f-g*) in which the amino acids occupying positions *a* and *d* are primarily hydrophobic. Proline and aromatic residues are absent in the repeat which led to the suggestion that this region would form an α -helical coiled-coil. By analogy with other coiled-coil protein structures the hydrophobic residues at positions *a* and *d* would form the interface between adjacent helices and provide the major stabilising force for this region of $\sigma 1$ through hydrogen bonding (Bassel-Duby *et al.*, 1985).

The carboxy-terminal half of the protein was predicted to have no regular structure. It was therefore proposed to have a globular configuration composed of short α -helices and β -sheets with many turns.

Sequence determination of all three S1 genes and computer analysis of the predicted amino acid residues coupled with computer processed electron micrographs of virion released type 2 σ 1 proteins have refined this model, resulting in a predicted structure that can be divided into several morphological domains [Ti, Tii, Tiii, Tiv and H] (Nibert *et al.*, 1990 and Fraser *et al.*, 1990), which are shown schematically in Figure 1.4.1. The amino-terminal twenty or so aa (Ti) are proposed to form a short hydrophobic tail and a hinge that joins this region to the α -helical coiled-coil (Tii). Immediately carboxy-terminal to the coiled-coil, an area of cross- β sheet is proposed (Tiii) and this is joined to the C-terminal globular head (H) by means of a flexible neck (Tiv). The latter is thought to consist of an area of β -sheet separating two short coiled-coils. Fraser *et al.* (1990) and Nibert *et al.* (1990) have suggested that the neck provides flexibility, which may be important in the attachment process and could also contribute to the formation of the extended and retracted states of σ 1 seen in the electron micrographs of Furlong *et al.* (1988), (described below). A slight anomaly was also cleared by the complementary studies of Fraser *et al.* (1990) and Nibert *et al.* (1990) in that the region of coiled-coil identified by Bassel-Duby *et al.* (1985) did not fully account for the length of the fibrous portion of σ 1 particles seen in electron micrographs (Furlong *et al.*, 1988). However, the region of heptad repeats was extended and the cross- β sheet of domain Tiii was proposed to form a rod-like structure (Nibert *et al.*, 1990) thus reconciling the predicted length of the fibrous section to that observed.

Experiments using Mabs directed against various reovirus proteins (Lee *et al.*, 1981b) and a separate study with a particular reovirus temperature sensitive (Ts) mutant (McPhillips and Ramig, 1984) have suggested that σ 1 is closely associated with the λ 2 spike. Bassel-Duby



1-27	28-187	188-255	256-333	334-478
Ti	Tii	Tiii	Tiv	H
NH ₂				COOH
Tail + Hinge	Coiled-coil	Cross-β sheet	Neck	Globular head

Figure 1.4.1 The Proposed Morphological Domains of the $\sigma 1$ Protein of Reovirus

Adapted from Nibert *et al.* (1990). Protein $\sigma 1$ can be divided into several discrete structural regions, Ti -Tiv + H, the approximate amino acid alignment positions of which (for all three serotypes) are given above the boxed areas.

et al. (1985) have further proposed that the coiled-coil of $\sigma 1$ may extend into the channel of the spike formed by $\lambda 2$.

Smith *et al.* (1969) originally proposed that $\sigma 1$ existed in virions as a dimer. Evidence has recently been presented that suggests it may exist as a tetrameric structure (Bassel-Duby *et al.*, 1987 and Banerjea *et al.*, 1988) and can exist in an extended or retracted state (Furlong *et al.*, 1988). Polypeptide $\sigma 1$ expressed in *E.coli*, isolated from reovirus infected cells or when released from intact virions formed an approximately 200,000 kDa band when subjected to SDS-PAGE under non-reducing conditions (Bassel-Duby *et al.*, 1987). Similarly, SDS-PAGE analysis of $\sigma 1$ expressed from a vaccinia virus recombinant gave a band of the same size (Banerjea *et al.*, 1988).

A dimeric or tetrameric structure has also been proposed by Yeung *et al.* (1987) on the basis of chemical crosslinking experiments of $\sigma 1$ purified from virions, whilst Fraser *et al.* (1990) have suggested a tetramer on the basis of their computer enhanced electron micrographs. Electron microscopy studies have also visualized $\sigma 1$ both attached to and in isolation from virus particles. They have confirmed the lollipop nature predicted for this protein in that it can be seen as a fibre or rod topped with a globular head (Banerjea *et al.*, 1988; Furlong *et al.*, 1988 and Fraser *et al.*, 1990). However, the proposed extended/retracted forms of $\sigma 1$ (Furlong *et al.*, 1988) were only noted for virion associated protein; free $\sigma 1$ was only seen in an extended state. The physiological importance of these two states (if any) has yet to be determined although Furlong *et al.* (1988) have suggested that it may be the extended form that is involved in the attachment to host cells. In this state, the likelihood of $\sigma 1$ binding to a receptor molecule(s) is probably increased.

1.4.2 The Functions of $\sigma 1$

Although $\sigma 1$ constitutes only 1-2% of the total viral protein (Smith *et al.*, 1969; Lee *et al.*, 1981b and Bassel-Duby *et al.*, 1987) it performs a wide variety of functions (Table 1.4.1), many of which can be related to the fact that it is the host cell attachment protein (Lee *et al.*, 1981b). It can also interact with the host cell in a less direct manner, an example of which is to alter certain aspects of host macromolecular synthesis (see below). A further property of $\sigma 1$ is that it is the viral haemagglutinin (Weiner *et al.*, 1978) where glycoprotein has been shown to be the moiety to which it binds (Paul and Lee, 1987).

Weiner and Fields (1977) demonstrated that $\sigma 1$ is responsible for eliciting virus serotype specific neutralizing antibodies and must therefore contain type-specific antigenic determinants. Further evidence for the type-specificity of this protein is that antibodies directed against $\sigma 1$ generally: precipitate only homologous type $\sigma 1$ molecules (Gaillard and Joklik, 1980 and Lee *et al.*, 1981a), neutralize only homologous type virus (Hayes *et al.*, 1981) and will only inhibit the haemagglutination properties of the corresponding virus serotype (Hayes *et al.*, 1981). The type-specificity is not absolute however, as of four Mabs generated against $\sigma 1-3$, three neutralized the homologous type virus, but the fourth demonstrated high activity against type 3 and type 1 virus (Hayes *et al.*, 1981). Also, whereas antiserum against type 3 virus was able to precipitate only homologous type $\sigma 1$, anti-type 2 virus antiserum precipitated type 1 as well as type 2 $\sigma 1$, albeit less efficiently (Gaillard and Joklik, 1980).

These observations are consistent both with ds RNA hybridisation studies (Gaillard and Joklik, 1982) which demonstrated that S1 has diverged the most out of the ten gene segments and with sequence comparison data (Cashdollar *et al.*, 1985) which show there to be very

Table 1.4.1 Functions of the $\sigma 1$ Protein of Reovirus

FUNCTION	REFERENCES
Cell attachment	Lee <i>et al.</i> 1981b
Haemagglutinin	Weiner <i>et al.</i> (1978)
Tissue tropism and neurovirulence	Weiner <i>et al.</i> (1977) and Weiner <i>et al.</i> (1980b)
Pathways of spread in the host	Tyler <i>et al.</i> (1986)
Role in viral growth in the intestine	Bodkin and Fields (1989)
Antigen responsible for type-specific neutralizing antibody production	Weiner and Fields (1977)
Cytotoxic T-cell generation	Finberg <i>et al.</i> (1979; 1982)
Suppressor T-cell generation	Fontana and Weiner (1980)
T-cell dependent delayed-type hypersensitivity	Weiner <i>et al.</i> (1980a)
Interaction with host cell microtubules	Babiss <i>et al.</i> (1979) and Sharpe <i>et al.</i> (1982)
Inhibition of host cell DNA replication	Sharpe and Fields 1981

little homology between the S1 genes of the three virus serotypes. A curious point emerging from these studies is that although T1 and T3 reoviruses exhibit the greatest overall genome homology, in the case of S1 and hence $\sigma 1$, the highest homology is between T1 and T2.

Cell-mediated immune responses are also induced by $\sigma 1$, with cytotoxic T-cell responses (Finberg *et al.*, 1979;1982), suppressor T-cell generation (Fontana and Weiner, 1980) and delayed hypersensitivity reactions (Weiner *et al.*, 1980a) all elicited against this protein. A further important feature of this protein is that it determines tissue tropism and virulence patterns (as previously described in section 1.3.2.1) (Weiner *et al.*, 1977 and Weiner *et al.*, 1980b).

Two other properties of $\sigma 1$ are (a) that it is proposed to be responsible for the inhibition of host cell DNA synthesis (Sharpe and Fields, 1981) with T3 $\sigma 1$ being a more potent inhibitor than that of T1 and (b) it is also thought to control the association of virus particles with microtubules (Babiss *et al.*, 1979 and Sharpe *et al.*, 1982). A recent study has elaborated on the inhibition of cellular DNA synthesis associated with the S1 gene segment (Fajardo and Shatkin, 1990). It was found that the inhibition exhibited by T1 reovirus was accelerated when it was used to infect cells co-expressing the T3 $\sigma 1$ and $\sigma 1s$ proteins but not $\sigma 1$ by itself. Inhibition was not effected merely by the expression of $\sigma 1$ or $\sigma 1$ and $\sigma 1s$ together. The initial conclusion drawn by Sharpe and Fields (1981) was made before the discovery of the second ORF in the S1 gene encoding the $\sigma 1s$ product.

1.4.3 Localisation of the Various Functions of $\sigma 1$ to Distinct Domains on the Protein

Reassortant viruses (see section 1.3.2) have proved to be an extremely useful tool in the study of reoviruses and in the assignment of many functions to protein $\sigma 1$. Antibody mapping has demonstrated that at least some of these functions exist as distinct domains on the $\sigma 1$ protein. Monoclonal antibodies were raised against the T3 $\sigma 1$ protein and found to have either: neutralizing activity, haemagglutination inhibition (HI) activity, weak neutralizing and HI activity or neither of these properties (Burstin *et al.*, 1982). From this work, it was deduced that there were at least three antigenically distinct sites on $\sigma 1$. Further evidence for distinct domains on $\sigma 1$ was provided by Spriggs *et al.* (1983b) in radioimmunoassay competition experiments where those Mabs showing strong neutralizing activity did not compete with those exhibiting HI activity in binding assays with $\sigma 1$.

1.5 Examples of Virus - Receptor Systems

It is possible that viruses bind to host cells either specifically or in a non-specific manner. The former case would imply that the initial interaction is through a specific set of receptor molecules, whose cell-surface expression would be a major factor in determining the cell types permissive for productive infection. Non-specific binding would intimate that permissiveness is determined at the level of virus entry or at some stage in replication. (These factors also apply when viruses interact with a specific receptor moiety). It has become clear that specific binding does occur in a number of virus systems. In the following sections some virus-receptor interactions will be described, from which it can be seen that a broad spectrum of approaches have been employed to identify specific viral receptors. This has shed further light on the various ways in which tissue tropism and viral virulence is governed in the host.

1.5.1 Non-Enveloped RNA Viruses

Two viruses from the Picornaviridae family - poliovirus and rhinovirus - will be used as examples of the interaction of non-enveloped viruses with host cells.

1.5.1.1 Poliovirus

Poliovirus, the causative agent of poliomyelitis, belongs to the enterovirus genus of the family Picornaviridae. It is 30 nm in diameter, icosahedrally shaped and possesses a single stranded, positive sense RNA genome of approximately 7.4 Kb. The virion capsid is composed of 60

copies of proteins VP1, VP2, VP3 and VP4 which are derived by proteolytic cleavage of a large (~ 220 kDa) polyprotein (reviewed by Rueckert, 1985).

Cell surface expression of the poliovirus receptor (PVR) was thought to be a major determinant in confining poliovirus replication to a limited number of sites in its human or primate hosts. This view arose from the observation that transfection of the viral RNA into non-susceptible cells relieved the inhibition of viral replication, suggesting that the block to infection was at the binding, entry or uncoating stage (Holland *et al.*, 1959). More recently, further supporting evidence was obtained by Mendelsohn *et al.* (1986) who rendered non-permissive L-cells susceptible to poliovirus infection by transforming them with human DNA leading to the expression of the PVR at the cell surface.

A 100 kDa protein was identified in HeLa cells that was proposed to be involved in poliovirus attachment (Shepley *et al.*, 1988). Mendelsohn *et al.* (1989) used a molecular cloning and expression approach to identify and isolate the PVR gene. Sequencing of the cDNA lead to a predicted size for the PVR of 45 kDa. Comparative analysis of the predicted amino acid sequence showed the PVR to be a new member of the immunoglobulin superfamily, having a three domain Ig-like extracellular structure, a transmembrane region and a short cytoplasmic tail. In fact, two cDNA clones were obtained which diverged at their COOH termini; one possessed a longer tail than the other leading to predicted 45 kDa and 43 kDa forms that could arise from alternative splicing or polyadenylation events (Mendelsohn *et al.*, 1989).

Koike *et al.* (1990) have also cloned the PVR and found that it exists as four different mRNA isoforms generated by alternative splicing pathways giving rise to four different PVR molecules. Two of the four are membrane bound and two are soluble as they lack the transmembrane domain. The two membrane bound forms were identical to those cloned by

Mendelsohn *et al.* (1989). Transformants expressing the soluble forms of the PVR were hardly permissive for poliovirus infection whereas those with the membrane bound form were fully permissive.

The relevance of these different forms *in vivo* is unknown as three of the mRNA isoforms can be detected in many human tissues including those that do not normally support poliovirus infection. All four isoforms were not detected as the means of assay was by the polymerase chain reaction and the primers utilised in the reactions did not enable selection of the fourth isoform (Koike *et al.*, 1990). Mendelsohn *et al.* (1989) also detected PVR mRNA in many tissues not all of which sustain virus replication *in vivo*. It has therefore been suggested that certain cell types may still specifically regulate the production of one particular mRNA (by splicing for example) or that some form of translational regulation may limit PVR expression at the cell surface. Alternatively, post-translational modification could produce tissue specific expression (Mendelsohn *et al.*, 1989). A monoclonal antibody against the PVR only stained a subset of cells when used to histochemically stain human brainstem (Shepley, 1988), implying that only a limited number of cells express the PVR protein.

Recently, the PVR gene was used to create a mouse transgenic for PVR (Ren *et al.*, 1990). Inoculation of these mice with poliovirus type 1 Mahoney (P1/Mahoney) by the intracerebral, intravenous or intraperitoneal route leads to the expected paralysis in nearly all of the cases. Inoculation with the live attenuated Sabin type 1 vaccine strain did not result in clinical disease. P1/Mahoney was not pathogenic when inoculated orally. The cloning of the PVR has therefore led to the development of a potential mouse model of a clinically important human disease (Ren *et al.*, 1990).

Once bound, poliovirus is thought to gain entry to the cell by the process of receptor mediated endocytosis whereby its genome is liberated

to the genome from acidic vesicles (Madshus *et al.*, 1984 and Zeichhardt *et al.*, 1985). Increasing the pH of the intracellular acidic vesicles prior to or at the time of infection prevented viral replication by inhibiting the virus uncoating step as neither attachment, penetration or the infectivity of the virus was shown to be affected by the weak bases used to raise the pH.

1.5.1.2 Rhinovirus

Rhinovirus also belongs to the Picornaviridae family but is a member of the rhinovirus genus. It therefore possesses the same general physical and chemical characteristics as poliovirus, i.e. icosahedral shape, single strand of positive sense RNA, etc.

There are however, over 100 different serotypes of human rhinoviruses which can be split into two main groups according to the cellular receptor they bind. These groups were assigned according to cross competition assays between the viral serotypes and cell protection assays with a monoclonal anti-receptor antibody. Approximately 80 of the 100 serotypes have been classified into a "major rhinovirus receptor group" and bind to a single receptor entity. The remaining serotypes belong to the "minor rhinovirus receptor group" and bind to one or more receptors (Abraham and Colonno, 1984 and Colonno *et al.*, 1986).

The major human rhinovirus receptor (HRR) was initially identified as a 90 kDa glycoprotein with a broad tissue distribution (Tomassini and Colonno, 1986 and Colonno *et al.*, 1986). Two distinct approaches were used in the identification of intercellular adhesion molecule-1 (ICAM-1) as the major HRR. One group followed the same strategy to that employed in the cloning of the PVR. HeLa cell genomic DNA was used to transform non-permissive cells rendering them susceptible to rhinovirus binding after transformation. The HRR protein was purified from these transformed cells

and partially sequenced enabling it to be identified as ICAM-1 by sequence comparison. This identification was confirmed by cDNA cloning and sequencing (Greve *et al.*, 1989). The second group noticed that ICAM-1 and the proposed major HRR had a similar tissue distribution. Binding assays to purified ICAM-1 and ICAM-1 transfected non-permissive cells with two major rhinovirus serotypes, HRV 14 and HRV 3, confirmed ICAM-1 as the HRR (Staunton *et al.*, 1989).

ICAM-1 is a 90 kDa glycoprotein with an amino-terminal structure composed of five Ig-like domains, a single transmembrane segment and a short cytoplasmic tail (Simmons *et al.*, 1988 and Staunton *et al.*, 1988). It is therefore a member of the Ig superfamily. Lymphocytes bind to a variety of cell types, including epithelial cells, via the interaction of LFA-1 (lymphocyte function associated molecule-1) with ICAM-1 (reviewed by Dustin *et al.*, 1988). ICAM-1 is strongly induced by lymphokines and cytokines in inflammatory responses (Dustin *et al.*, 1988). Rhinoviruses infect the nasal epithelium *in vivo*; rhinovirus infection may therefore lead to the increased expression of its receptor (Staunton *et al.*, 1989).

The three dimensional structures of HRV14, P1/Mahoney and the attenuated P3/Sabin strain have all been determined (Rossmann *et al.*, 1985; Hogle *et al.*, 1985 and Filman *et al.*, 1989). A cleft or canyon was noticed to encircle each of the five fold axes of the viral icosahedral structure which separates a peak from a plateau structure (Figure 1.5.1); various prominent loops were found to lie in close proximity to the canyons. Rossmann *et al.* (1985) proposed the canyon to be the site of attachment for the host cell receptor. Its deepest residues would be inaccessible to an antibody whose Fab fragment ($\sim 35\text{\AA}$) would be too large to penetrate into the canyon (25\AA). Consequently, they would be free from immune pressure and enable the virus to maintain an unchanging receptor binding site.

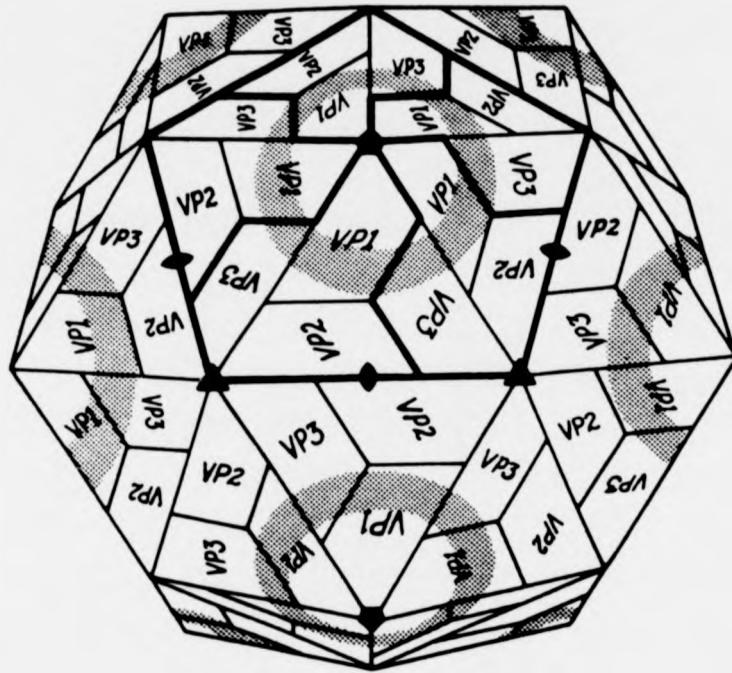


Figure 1.5.1 The Rhinovirus Canyon

Shown above is a schematic diagram of the position of the canyon structure in a picornavirion, reproduced from Rossmann (1989). The canyon is formed roughly at the junction of the clustered VP1 subunits and the surrounding VP3 proteins. Polypeptide VP4 is not evident as it is buried beneath VP1 and VP2. VP1 contributes the majority of the residues lining the canyon and VP3 most of the remainder; VP2 is barely associated with this structure (Rossmann *et al.*, 1985).

Neutralizing Mabs have been generated against both poliovirus and rhinovirus (Sherry and Rueckert, 1985; Sherry *et al.*, 1986; Minor *et al.*, 1983;1985 and Diamond *et al.*, 1985) and used to generate neutralizing escape mutants. When analyzed, the mutations could be grouped geographically into four distinct clusters which mapped to the most exposed sites surrounding the canyon (Hogle *et al.*, 1985 and Rossmann *et al.*, 1985) but not in the canyon itself.

A comparison of picornavirus sequences revealed that the canyon contains highly conserved amino acid residues (Rossmann and Palmenberg, 1988) and mutation of residues in the canyon floor abrogated efficient binding of HRV 14 to its host cell (Colonno *et al.*, 1988). Taken together, these observations provide strong evidence for the canyon being the receptor site.

1.5.2 Enveloped RNA Viruses

Influenza virus and human immunodeficiency virus-1 will be the main examples discussed in the following sections as the means of attachment and entry to the host cell are very different to the aforementioned viruses.

1.5.2.1 Influenza Virus

Influenza viruses belong to the Orthomyxoviridae family and are characterized by a lipid envelope which surrounds a helical nucleocapsid. The genome is comprised of 8 (influenza A and B) or 7 (influenza C) segments of negative sense, single-stranded RNA. Two types of spike glycoprotein are found in the the lipid envelope, the haemagglutinin (HA)

and neuraminidase (NA) (Kilbourne, 1987). These viruses are a major cause of upper respiratory tract infections in man and some animals.

i) **Structure of the HA**

The HA is formed by proteolytic cleavage of a precursor molecule called HA₀ (Lazarowitz and Choppin, 1975 and Klenk *et al.*, 1975) which divides it into two disulphide linked chains, HA₁ and HA₂ by the removal of one (or more) residues (Verhoeven *et al.*, 1980 and Wilson *et al.*, 1981; Figure 1.5.2). This proteolytic cleavage was also found to be a prerequisite for viral infectivity. Virions carrying HA₀ could attach to, but not efficiently infect cells without prior protease digestion, the effect of which was to cleave the precursor (Lazarowitz and Choppin, 1975 and Klenk *et al.*, 1975).

The three dimensional structure of bromelain released HA has been determined (Wilson *et al.*, 1981) to 3Å resolution and the HA ascertained to be composed of a long α -helical stem on top of which sits a globular head. Bromelain cleaves the HA close to the viral membrane leaving only the small COOH terminal fragment of HA₂ associated with the membrane. Three HA molecules associate to form a trimeric structure with the HA₂ chains coming together as an α -helical coiled-coil and the HA₁ chains forming the globular heads, but with far fewer inter-subunit interactions (Wilson *et al.*, 1981). A diagram of the trimeric HA structure is shown in Figure 1.5.2.

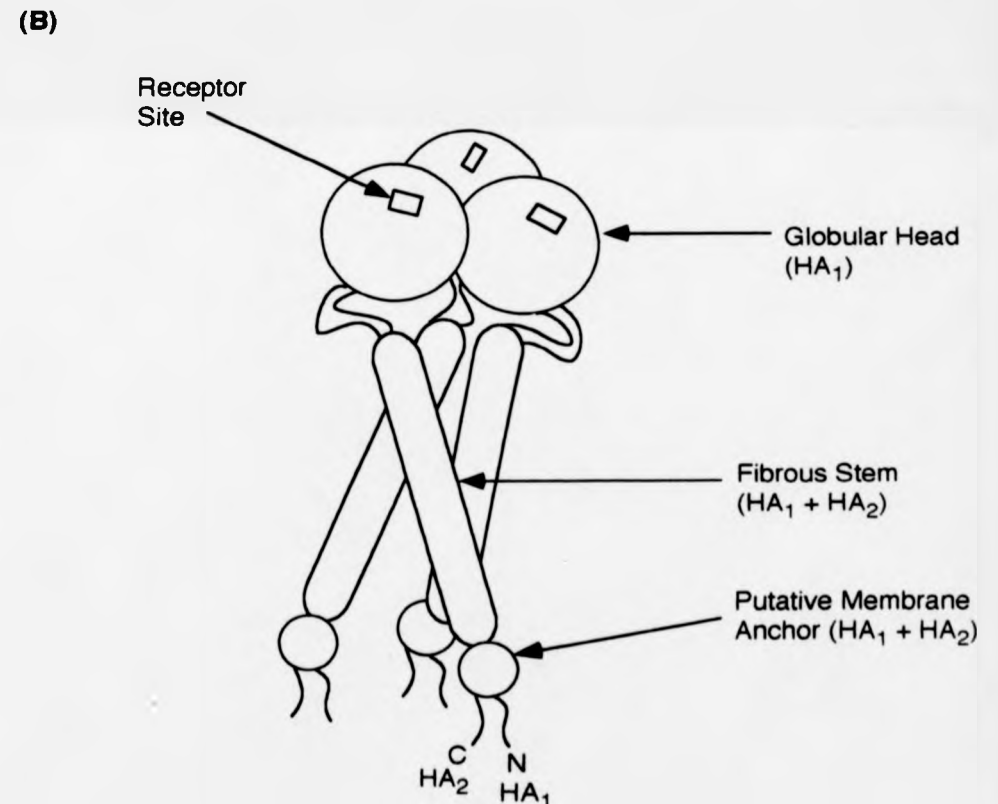
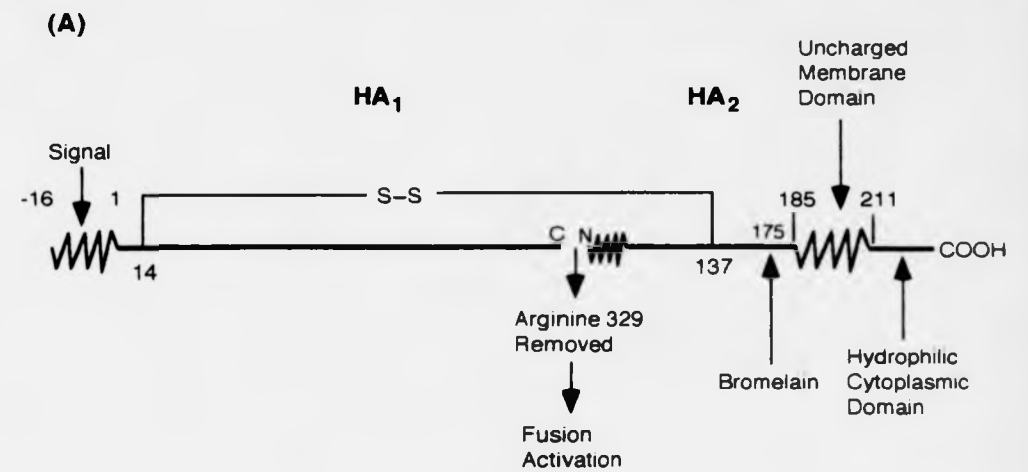
A cellular protein, BiP (Binding Protein) appears to interact with the influenza HA during the subunit folding process and/or during assembly of the subunits to form the trimeric structure. Gething *et al.* (1986) found that two different, incorrectly folded, mutant forms of the HA were retained in

Figure 1.5.2 Schematic Diagram of the 1968 Hong Kong Influenza Virus Haemagglutinin

(A) Linear map of the haemagglutinin. Shown here is the external domain (which extends to aa 184 of HA2), the membrane anchor (aa 185-211) and the cytoplasmic domain (aa 212-221). Also shown are the various processing sites and location of the fusion peptide; the only disulphide bond shown is that linking HA1 and HA2; those within HA1 and HA2 are not marked.

(B) Trimeric structure of the haemagglutinin showing the receptor binding sites.

The diagrams opposite are adapted from Wilson *et al.* (1981)



the endoplasmic reticulum (ER) whilst wild-type (correctly folded) HA forms were transported from the ER to the Golgi and cell-surface as normal. In the ER, the mutant forms were associated with BiP. It was thus suggested that BiP bound to misfolded molecules to prevent their intracellular transport and were possibly transiently associated with wild-type chains to ensure correct folding (Gething *et al.*, 1986). These functions of BiP (and BiP related) proteins were also suggested by Munro and Pelham (1986) as a general concept in the intracellular folding and association of many cellular proteins.

ii) **Influenza Virus Entry and Membrane Fusion**

The HA functions as both the cell attachment (Rogers, *et al.*, 1983 and Wiley and Skehel, 1987) and membrane fusion protein (White *et al.*, 1982a) of influenza virus. Sialic acid is the receptor moiety to which influenza binds (Paulson *et al.*, 1979) and different strains of influenza virus exhibit preferences for specific sialic acid configurations. Binding is mediated by a surface pocket on the globular head of the HA molecule into which the sialic acid residue fits exactly (Weis *et al.*, 1988). Single amino acid substitutions in the binding pocket alter the specificity for different sialyloligosaccharide structures (Rogers *et al.*, 1983) and later studies by Weis *et al.* (1988) have shown that this is likely to be a conformational effect, rather than via a direct interaction with the altered residue.

Influenza viruses enter the host cell by the process of receptor mediated endocytosis and so are delivered to acidic vesicles (Matlin *et al.*, 1981) where the viral membrane fuses with that of the vesicle, thus delivering the nucleocapsid to the cytosol. Membrane fusion is strictly dependent upon an acidic pH (White *et al.*, 1981; Huang *et al.*, 1981 and

Matlin *et al.*, 1981). The acidic environment is thought to induce a conformational change in the viral HA, causing the N-terminal hydrophobic portion of HA₂ to become exposed and mediate the fusion event (Skehel *et al.*, 1982; White and Wilson, 1987 and Weis *et al.*, 1988). Experiments involving expression of a cloned cDNA copy of the HA and the use of reassortant viruses have demonstrated that the HA is the only viral gene product necessary for fusion and that it confers the pH dependence property of the fusion event (White *et al.*, 1982b and Lenard *et al.*, 1982).

1.5.2.2 Comparison of Influenza with the Paramyxoviruses

Paramyxoviruses are similar in structure to the influenza viruses in that they consist of a lipid envelope which encloses a helical nucleocapsid whose genetic material is negative sense, single stranded RNA (reviewed by Galinski and Wechsler, 1991). However, the genome comprises a single molecule of RNA and the cell attachment and fusion functions are associated with different spike glycoproteins. The HN protein is the viral haemagglutinin and neuraminidase and the F-protein mediates membrane fusion (Galinski and Wechsler, 1991). These viruses also probably use sialic acid bearing structures as receptors (Landsberger *et al.*, 1981 and Oku *et al.*, 1982).

Homma and Ohuchi (1973) and Scheid and Choppin (1974) found that virus grown in certain cell lines was non-fusogenic and non-infectious unless treated with trypsin, a property that mapped to the F-protein. Trypsin cleaved the protein into its two subunits, F₁ and F₂, which are linked by a disulphide bond, and restored the fusogenic property and infectivity of the virus.

The F₂ fragment corresponds to the N-terminal region and F₁ to the C-terminus of F₀ (the uncleaved precursor), with the F₁ domain serving to anchor the spike to the viral membrane (Scheid and Choppin, 1977; Figure 1.5.3). Sequencing of the N-terminus of F₁ has revealed that it is highly hydrophobic (Gething *et al.*, 1978). This terminus is created upon cleavage of F₀ and is homologous to other paramyxovirus F₁ proteins and to the HA₂ of influenza virus (White *et al.*, 1983). It was suggested that this region could play a role in membrane fusion (Gething *et al.*, 1978), probably via a conformational effect exposing this hydrophobic area after cleavage of F₀. That the N-terminus of F₁ is involved in membrane fusion is supported by the experiments of Richardson *et al.* (1980) and Richardson and Choppin (1983). Various synthetic peptides that were similar in sequence to this region of F₁, but which contained a carbobenzoxy-group at their N-termini, were found to inhibit virus infectivity at the level of penetration and virus-cell membrane fusion. (The exact effect of the carbobenzoxy group was not known but its presence greatly increased the inhibitory activity of the peptides). The site of peptide action was determined to be on the cell membrane not that of the virus, leading to the suggestion that the peptides competed with the F₁ protein for receptor sites on the cell membrane, thus preventing fusion of the membranes (Richardson and Choppin, 1983).

Evidence for a conformational change in the F protein upon cleavage to the F₁.F₂ complex came principally from circular dichroism studies of the uncleaved and cleaved forms of the protein (Hsu *et al.*, 1981 and Kohama *et al.*, 1981). The two forms gave different circular dichroism spectra indicating that each had a distinct conformation. Further evidence came from the observation that the cleaved form bound more anionic detergent than the uncleaved, probably owing to the exposure of previously hidden hydrophobic residues (Hsu *et al.*, 1981). Also, cleaved

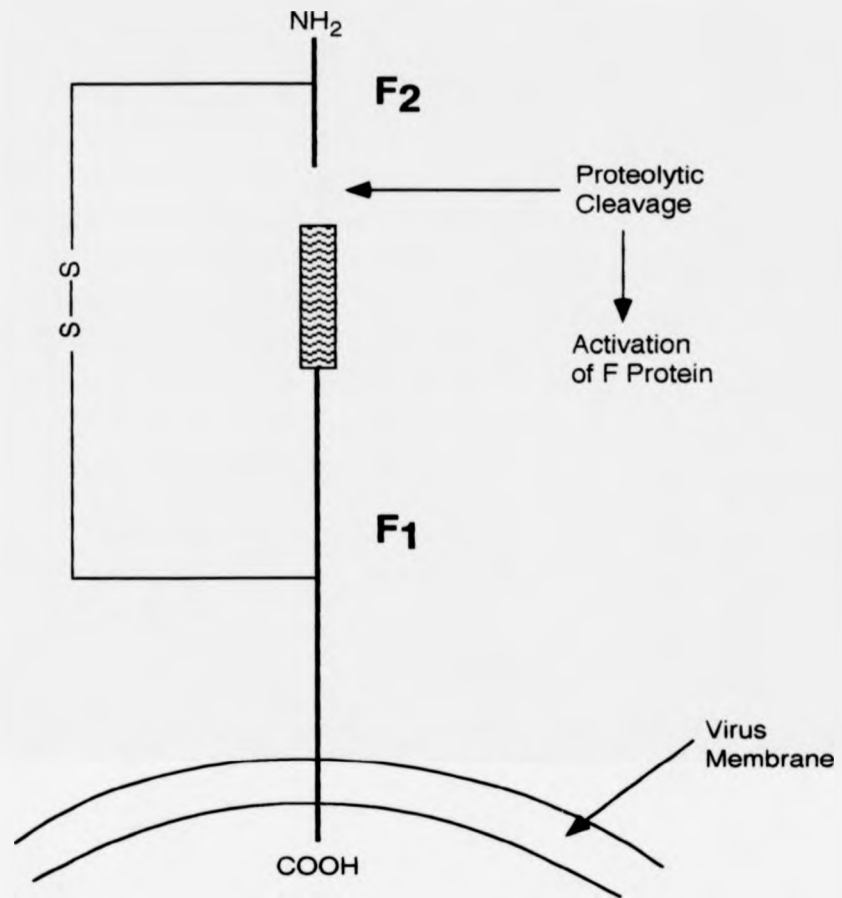


Figure 1.5.3 Schematic Representation of the F Protein of the Paramyxovirus - Sendai Virus (Adapted from White *et al.*, 1983).

The disulphide linkage shown between F_1 and F_2 is not an exact representation and only indicates that these two chains are linked in such a manner. The hydrophobic amino-terminus of F_1 exposed upon proteolytic cleavage of the F_0 protein is shown as a box.

and uncleaved F protein exhibited different isoelectric points (pIs). As the amino acid composition of each form is similar, a conformational change resulting in a rearrangement of the charged residues was suggested to account for the more acidic pI of the cleaved form (Kohama *et al.*, 1981).

A major difference in the fusion event between paramyxoviruses and influenza is that for the former it is largely a pH independent process (Clavell and Bratt, 1972 and White *et al.*, 1983). This means that fusion can occur at the cell surface and not in an acidic vesicle, leading to the direct release of the nucleocapsid into the cytosol after fusion (Fan and Sefton, 1978). It is still not clear however, if this type of fusion leads to a productive infection.

1.5.3 Human Immunodeficiency Virus and CD4

Human immunodeficiency virus type 1 (HIV-1), belongs to the lentivirus genus of the Retroviridae family. It is generally accepted as the aetiologic agent of acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984 and Popovic *et al.*, 1984) which is characterised by a depletion of T cells. It had been noticed that the virus exhibits a selective tropism for T lymphocytes bearing the surface marker CD4 (Klatzmann *et al.*, 1984a) suggesting that this glycoprotein may play a role in the entry of HIV-1 into the host cell. Dalglish *et al.* (1984) and Klatzmann *et al.* (1984b) subsequently demonstrated that only CD4 bearing T cells were susceptible to infection and that antibodies to CD4 specifically inhibited this process. Confirmation that CD4 was in fact the receptor for HIV-1, came from genetic studies in which the CD4 gene was transfected into various CD4⁻ cell lines, including epithelial and B-cells as well as T-cell lines. Expression of the CD4 protein rendered these transfected cells permissive for infection (Maddon *et al.*, 1986). The

block to productive viral infection appears to be at the step of virus binding/entry as transfection of HIV DNA clones into non-permissive cells overcomes this block (Levy *et al.*, 1986 and Adachi *et al.*, 1986).

HIV can infect other cell types including monocytes and macrophages (Gartner *et al.*, 1986; Clapham *et al.*, 1987 and Gendelmann *et al.*, 1988) and brain tissue (Koenig *et al.*, 1986; Wiley *et al.*, 1986; Cheng-Mayer *et al.*, 1987 and Chiodi *et al.*, 1987).

The viral outer envelope glycoprotein gp120 mediates binding to the host cell (McDougal *et al.*, 1986) and is produced, together with gp41, by proteolytic cleavage of the precursor molecule gp160 (Robey *et al.*, 1985 and McCune *et al.*, 1988; Figure 1.5.4). The cleavage event is necessary for viral infectivity (McCune *et al.*, 1988). The glycoproteins gp120 and gp41 are associated non-covalently and gp41 serves as the membrane anchor for the complex whilst gp120 is exposed on the membrane surface (Veronese *et al.*, 1985 and Ozel *et al.*, 1988).

Entry to the host cell is thought to occur by direct fusion of the viral and cellular membranes at the cell surface in a pH independent manner, analogous to that discussed above for paramyxoviruses (Stein *et al.*, 1987; McClure *et al.*, 1988 and Grewe *et al.*, 1990). The fusion process and uncoating of the internalised virus is not dependent upon the cytoplasmic tail of CD4, thought to be required for CD4 internalization (Bedinger *et al.*, 1988). Viral entry would not appear to be via receptor mediated endocytosis but Pauza and Price (1988) and Grewe *et al.* (1990) have provided some evidence it can occur.

Glycoprotein gp120 can be divided into several conserved (C) and variable (V) regions (Willey *et al.*, 1986), some of which have been assigned cell-binding or fusion functions. The carboxy-terminal half of gp120 harbours the CD4 binding site; mutations in the second, third and fourth conserved regions affect CD4 binding without significantly altering

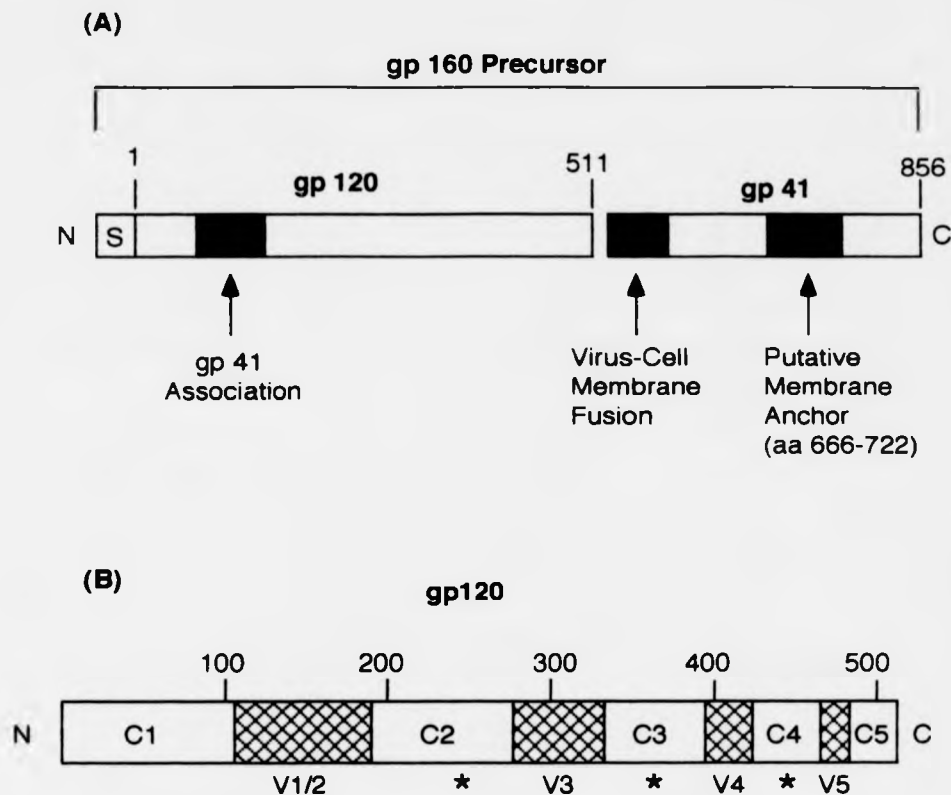


Figure 1.5.4 Schematic Representation of the HIV *env* Glycoprotein

(A) Diagram of the gp160 precursor molecule and its cleavage products gp120 and gp41. Some of the functional regions of these proteins are marked by black boxes; S = signal sequence. Diagram adapted from Burny *et al.* (1988).

(B) Representation of the conserved and variable regions of gp120. The conserved regions are denoted by unshaded boxes and the variable regions by hatched boxes; asterisks denote domains in which residues have been reported to be important in CD4 binding. These residues were assigned in mutational analyses where the mutations did not appear to affect the conformation of gp120; see text for details. The location of the conserved domains is based on gp120 maps in Lasky *et al.* (1987) and Olshevsky *et al.* (1990).

the conformation of the viral protein (Kowalski *et al.*, 1987; Cordonnier *et al.*, 1989a;b and Olshevsky *et al.*, 1990; Figure 1.5.4). Similar studies delineated conserved regions C1, C3, C4 and C5 as important for CD4 interaction but the interpretation of these results is less clear as the mutational analysis was by the introduction of insertions or deletions within these areas and the overall effect on conformation was not determined (Kowalski *et al.*, 1987; Lasky *et al.*, 1987; Linsley *et al.*, 1988 and Cordonnier *et al.*, 1989a;b). The gp120 sequences important in CD4 binding would therefore appear to be discontinuous.

The non-covalent association between gp120 and gp41 is mediated by an amino-terminal domain of gp120 whilst a hydrophobic region in the carboxy-terminus of gp41 acts to anchor the complex to the surface of the infected cell and virion (Kowalski *et al.*, 1987). Another hydrophobic domain has been located in the amino terminal half of gp41 and proposed to be important in the fusion process owing to its homology with the fusion peptides of paramyxoviruses (Gallaher, 1987). Mutations introduced into this domain were found to inhibit fusion, demonstrating its importance (Kowalski *et al.*, 1987). It has been proposed that a conformational change in the gp120/gp41 complex on binding to the host cell could lead to exposure of the fusogenic peptide of gp41 and its insertion in the cell membrane, leading to virus-cell fusion (Burny *et al.*, 1988). This is analogous to the fusion event observed during influenza infection. However, with influenza, the conformational change required for exposure of the fusion peptide is driven by the acid environment of the endosome (Skehel *et al.*, 1982 and White and Wilson 1987). In contrast, virus-cell fusion occurs at the cell surface for HIV (Stein *et al.*, 1987) and there is no direct evidence of a conformational change in the gp120/41 complex taking place upon binding to the host cell membrane.

Regions within gp120 may be important in the fusion event as shown by studies using antibodies raised against peptides derived from the V3 and C2 areas. Antibodies against the V3 region (which contains the V3 loop, a major neutralizing antibody epitope) inhibit fusion induced by HIV-1 (Palker *et al.*, 1988 and Skinner *et al.*, 1988) but not binding to CD4. The polyclonal sera directed against the C2 peptide was able to neutralize several HIV isolates but also did not affect binding, implying that C2 may be involved in fusion or another postbinding event (Ho *et al.*, 1988).

A characteristic of cells infected *in vitro* with HIV-1 is the formation of multinucleate giant cells, or syncytia (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Levy *et al.*, 1984 and Popovic *et al.*, 1984). Syncytia formation is also observed when infected, HIV-1 producing cells are mixed with uninfected CD4 bearing cells. This phenomenon is the result of the interaction of gp120 exposed on the surface of infected cells with cell surface CD4 on uninfected cells; CD4⁻ cells do not undergo fusion when similarly analysed (Lifson *et al.*, 1986a;b; Sodroski *et al.*, 1986 and Kowalski *et al.*, 1987). Syncytia die soon after formation and so the involvement of uninfected CD4 bearing cells in syncytia formation may contribute to the characteristic depletion of T cells expressing this marker protein seen in AIDS patients (Lifson *et al.*, 1986a;b).

1.6 The Reovirus Receptor

1.6.1 The Nature of the Reovirus Receptor

Much effort has been expended in attempts to characterize and identify the reovirus receptor and although progress has been made, the precise receptor molecule has not been unequivocally defined.

Reovirus T1 and T3 bind to, and infect different cells *in vivo*; the former binds to ependymal cells (Kilham and Margolis, 1969 and Margolis and Kilham, 1969) and the latter to neuronal cells (Margolis *et al.*, 1971; Gonatas *et al.*, 1971 and Raine and Fields, 1973). Their ability to specifically bind to these respective cell types has also been demonstrated *in vitro* (Tardieu and Weiner, 1982 and Dichter and Weiner, 1984). However, *in vitro*, all three serotypes can bind to the same cell type -mouse L-cells - which are routinely used for the purification of reovirus.

Radiolabelled reovirus has been utilized in many cell-binding assays to ascertain the presence of saturable, high affinity receptors on the host cell. In many cases L-cells were used (Armstrong *et al.*, 1984; Epstein *et al.*, 1984; Gentsch and Hatfield, 1984 and Gentsch and Pacitti, 1985), although other cell types have been investigated, including rat pituitary cells (Maratos-Flier *et al.*, 1983), murine splenic lymphocytes (Epstein *et al.*, 1984), HeLa cells (Gentsch and Hatfield, 1984) and rat endothelial cells (Verdin *et al.*, 1989). Roughly similar values have been obtained for the number of receptor sites/cell and the affinity of binding and these are approximately 80,000-100,000 binding sites and a dissociation constant (K_d) of approximately 0.8-3.0nM (Table 1.6.1).

It is not known if T1 and T3 reovirus utilize the same receptor on L-cells. Epstein *et al.* (1984) found that only homologous serotype virus could prevent binding in competition assays performed with whole virus.

Table 1.6.1 Reovirus Cell Binding Characteristics

Cell Type	No. Binding Sites/Cell	K _d Value (nM)	Binding Ligand	Reference
L-Fibroblast	3-5 x 10 ⁵	3	Reovirus T3	Armstrong <i>et al.</i> 1984
L-Fibroblast	2.5 x 10 ⁴	0.45	Reovirus T1	Epstein <i>et al.</i> 1984
L-Fibroblast	8.5 x 10 ⁴	0.8	Reovirus T3	Epstein <i>et al.</i> (1984)
L-Fibroblast	3.0 x 10 ⁴	0.5	Clone 3.HA1	Epstein <i>et al.</i> (1984)
L-Fibroblast	0.86-1.1 x 10 ⁵	N.D.	Reovirus T3	Gentsch and Hatfield, (1984)
L-Fibroblast	7.8 x 10 ⁴	N.D.	Reovirus T3	Gentsch and Pacitti, (1985)
L-Fibroblast	1.6 x 10 ⁴	0.25	Reovirus T3	Sawutz <i>et al.</i> (1987)
Rat Pituitary	4.2 x 10 ³	*	Reovirus T3	Maratos-Flier <i>et al.</i> (1983)
HeLa	1.26 x 10 ⁵	N.D.	Reovirus T3	Gentsch and (Pacitti) 1984
Murine Splenic Lymphocytes	6 x 10 ⁴	0.6	Reovirus T3	Epstein <i>et al.</i> (1984)
Smooth Muscle DDT1	1.45 x 10 ⁴	0.03	Reovirus T3	Sawutz <i>et al.</i> (1987)
Rat Endothelial	6.8 x 10 ⁴	0.63	Reovirus T1	Verdin <i>et al.</i> (1989)
Rat Endothelial	3.44 x 10 ⁴	0.43	Reovirus T3	Verdin, (1989)

Lee *et al.* (1981b), however, were able to inhibit the attachment of $\sigma 1$ protein using reovirus of homologous and heterologous serotypes; thus, T1, T2 and T3 virus were all effective in competing the binding of $\sigma 1$ derived from any of the three virus serotypes.

The precise role played by sugar residues in the virus-cell attachment process is also unclear. In two separate studies neuraminidase treatment of the target cells had no effect on the cellular binding capacity of T1 or T3 reovirus; neither did the presence of various sugars (Armstrong *et al.*, 1984 and Epstein *et al.*, 1984). However, in experiments by Gentsch and Hatfield (1984) and Gentsch and Pacitti (1985), neuraminidase digestion decreased reovirus binding by 50-70%; the residual binding was still saturable and specific. Whether neuraminidase affects the specificity of reovirus attachment is therefore unclear.

Sialic acid containing glycoproteins were found to inhibit reovirus attachment as were several sialyloligosaccharides (Gentsch and Pacitti, 1985) indicating that it is the sialic acid component that causes the inhibitory effect. Cell surface, sialic acid components were thus suggested to play a role (as yet undefined) in the interaction of reovirus with the host cell.

1.6.2 The Use of Anti-Idiotypic Antibodies as Probes for the Reovirus Type 3 Receptor

An alternative approach to characterizing and identifying the reovirus receptor was to generate anti-reovirus T3 (anti-R3) antibodies which could subsequently be used as probes for the reovirus receptor.

Nepom *et al.* (1982b) used antibodies directed against the HA ($\sigma 1$) of reovirus T3 to generate a rabbit, xenogeneic, anti-idiotypic antiserum. A neutralizing, anti- $\sigma 1$, monoclonal antibody, 9BG5, (Burstin *et al.*, 1982)

was shown to specifically inhibit the binding of the xenogeneic antisera with the anti-HA antibodies, suggesting that an idiotypic, HA binding determinant had been identified. The generation of such anti-idiotypic antibodies is shown schematically in Figure 1.6.1. Other $\sigma 1$ Mabs did not have this effect and inhibition was shown to be type specific. The idiotypic HA T3 binding determinant was thus designated Id3 to signify its association with the binding of the type 3 HA (Nepom *et al.*, 1982a;b).

The xenogeneic antisera was then used to probe for the presence of the Id3 determinant on a number of cell types and the resultant pattern of attachment corresponded with that shown by reovirus (Nepom *et al.*, 1982a;b). R1.1 cells (a T cell thymoma cell line) and mouse neural cells (from explanted nervous tissue) specifically bound the rabbit antisera and reovirus T3 whereas ependymal (T1 virus specific cells) and YAC (lymphoma) cells did not.

Following this, an anti-idiotypic monoclonal antibody - 87.92.6 - was prepared against Id3 using the Id3 bearing 9BG5 hybridoma (Noseworthy *et al.*, 1983). This antibody also mimicked the characteristic patterns of reovirus T3 binding but there were some exceptions; two cell lines P815 (mastocytoma) and EL-4 (lymphoma) can bind and are permissive for reovirus T3 yet do not bind and are not protected by 87.92.6 (Noseworthy *et al.*, 1983 and Kauffman *et al.*, 1983a) suggesting that there are two structurally distinct receptors, only one of which is recognized by the Id3 antigenic site on the viral HA.

Several other pieces of evidence indicated that the anti-idiotypic antibodies would be useful anti-R3 receptor antibodies. Monoclonal 87.92.6 has comparable binding parameters to T3 virus in that there are 79,000 binding sites for this antibody on R1.1 cells with a K_d of approximately 0.96 nM (Gaulton *et al.*, 1985) which agrees very well with that determined by Epstein *et al.* (1984). Reovirus T3 blocks the

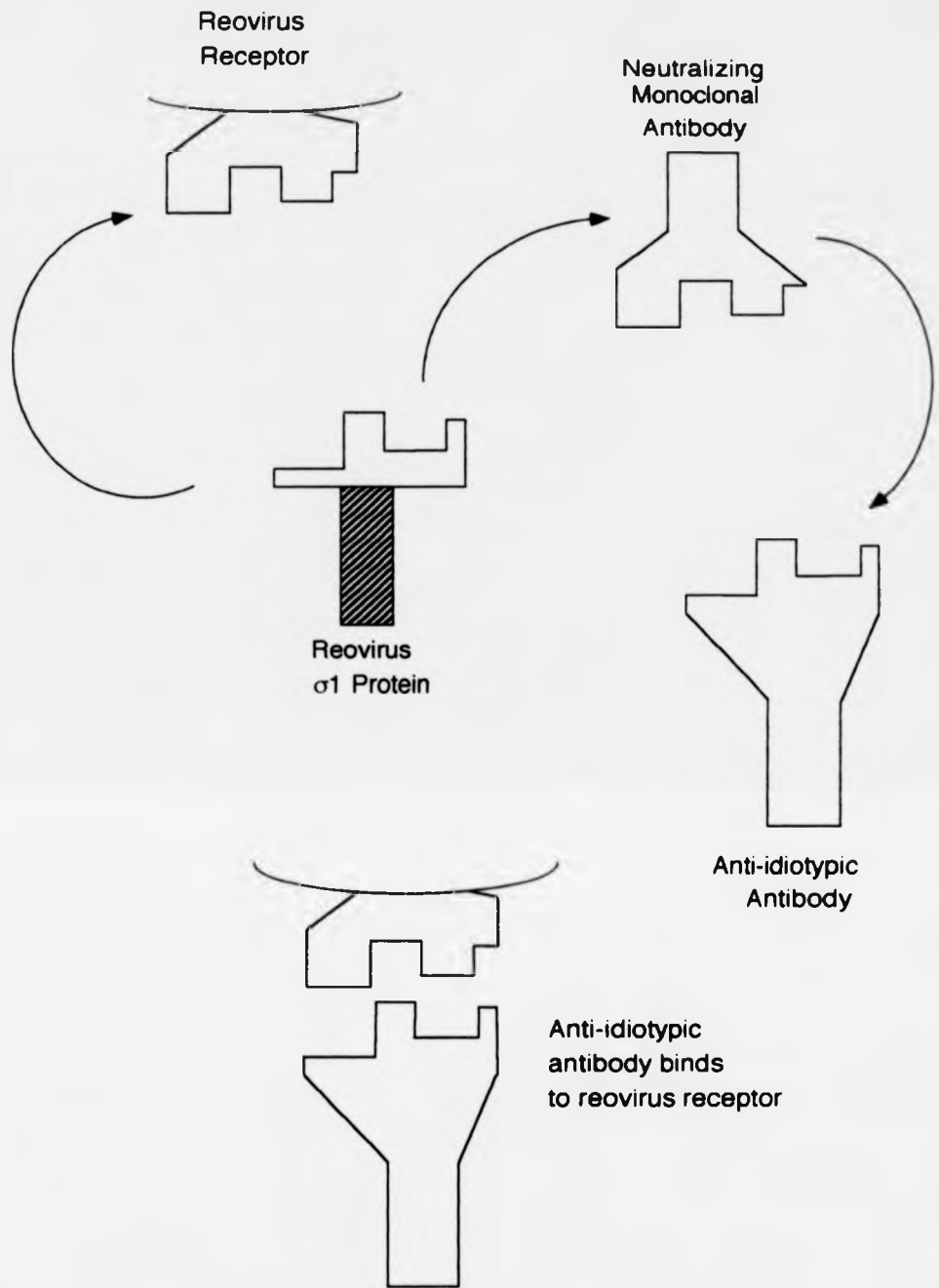


Figure 1.6.1 Schematic Representation of the Anti-idiotypic Approach to Identifying the Reovirus Receptor

attachment of 87.92.6 to R1.1 and rat B104 neuroblastoma cells (Gaulton *et al.*, 1985 and Kauffman *et al.*, 1983a) and conversely, 87.92.6 blocks the serotype specific binding of T3 reovirus to a thymoma line BW5147 (Kauffman *et al.*, 1983a). This anti-idiotypic antibody also prevents viral attachment and infection of neurones grown in cell culture (Dichter *et al.*, 1986).

1.6.3 Putative Identification of the Reovirus Receptor

Analysis of R1.1 thymoma and B104 neuroblastoma membrane extracts by immunoprecipitation and Western blotting techniques revealed that the xenogenic rabbit antisera and reovirus T3 bound to a single glycoprotein of 67 kDa with a heterogeneous isoelectric point of 5.8-6.0. Various other murine B and T lymphoid cell lines as well as L 929 fibroblasts also expressed this protein. This 67 kDa polypeptide was therefore proposed to be the reovirus T3 receptor (Co *et al.*, 1985a).

The tissue distribution of β -adrenergic receptors was observed to be similar to that for reovirus binding and infection (Co *et al.*, 1985b and Cohen *et al.*, 1989 unpublished results). Purified β 2-adrenergic receptor (β 2AR) was specifically precipitated by the rabbit anti-idiotypic antibodies and had a similar M_r and pI as the reovirus receptor (67 kDa and 5.8-6.0 respectively). Tryptic digests of the two receptors revealed an identical pattern and the reovirus T3 receptor was shown to specifically bind the β -antagonist IHYP (iodohydroxybenzyl-pindolol). Hence, the two receptors were deemed to be structurally and antigenically similar (Co *et al.*, 1985b).

However, although similar, the two receptors are not identical as shown by the fact that the β -adrenergic ligand and reovirus binding sites are distinct entities. Anti-R3 antibodies and β -adrenergic ligands do not compete for the same binding sites on either purified reovirus T3 receptor

or β 2AR (Liu *et al.*, 1988 and Sawutz *et al.*, 1987. Further evidence came from Choi and Lee (1988) who used the human epidermoid carcinoma A431 cell line to investigate the relatedness of the two receptors. This cell line possesses a large number of functional β -adrenergic receptors and was susceptible to reovirus T3 infection. Four pieces of evidence pointed to the receptors being distinct moieties:-

a) reovirus did not stimulate adenylate cyclase activity whereas a β -agonist did, b) reovirus T3 did not inhibit the dose dependent stimulation of adenylate cyclase even at saturating concentrations and so was not acting as an antagonist, c) the binding of a β -adrenergic antagonist could not be competed by R3 and d) internalization of the β -adrenergic receptors did not affect Reovirus T3 binding and *vice versa*. The same results were seen for mouse L 929 fibroblasts which were also shown to possess few β -adrenergic receptors (Choi and Lee, 1988).

1.6.4 Structural and Functional Determinants Shared by σ 1 and the Anti-idiotypic Antibody 87.92.6

Bruck *et al.* (1986) determined the nucleic acid and predicted protein sequence of the the 87.92.6 antibody and then compared it to the already available σ 1 T3 predicted protein sequence. A significant area of homology was detected between aa 317-332 of σ 1 and the combined determinant of the complementarity determining region II (CDRII) of Mab 87.92.6. Amino acids 45-51 of the antibody V_H (variable region of the heavy chain) exhibited high homology with 317-323 of σ 1, as did a second slightly larger area, comprising amino acids 47-54 of the antibody V_L (variable region of the light chain) and 324-331 of σ 1.

A series of synthetic peptides were built corresponding to these regions of homology in order to test their functional relevance. Peptide V_H

corresponded to aa 43-56 of the V_H CDRII of Mab 87.92.6; peptide V_L corresponded to aa 39-55 of the V_L CDRII of Mab 87.92.6 and the reo peptide to aa 317-332 of σ 1 (Williams *et al.*, 1988). The latter peptide was extremely hydrophobic and so was of limited use in most of the assays in which it was to be tested. A list of the properties of these peptides is given in Table 1.6.2 and are described below.

The neutralizing anti-HA3 Mab 9BG5 could bind V_L peptide, reo peptide and V_H+V_L peptide (i.e. peptides V_H and V_L linked by an amino terminal cysteine residue) but not to V_H. This same peptide reactivity pattern was evident when they were used in competition assays to inhibit the binding of reovirus T3 with 9BG5, suggesting that the area of sequence similarity between the V_L of Mab 87.92.6 and reo peptide (aa 323 -332 of σ 1) may be involved in the neutralizing epitope.

The effects of the peptides in binding assays between reovirus T3 and L-cells and between 9BG5 and the anti-idiotypic antibody 87.92.6 were also investigated. It was thus determined that V_L peptide and hence aa 323-332 of the HA were involved specifically in reovirus T3 receptor recognition but that V_H and V_L of Mab 87.92.6 were important in the interaction of the idiotypic and anti-idiotypic Mabs.

A reovirus neutralizing escape mutant, K, created by the growth of reovirus T3 in the presence of Mab 9BG5 was investigated to establish how it escapes neutralization. Peptide V_L prevented variant K (and reovirus T3) from binding to L-cells; K also had a greatly diminished capacity to bind to 9BG5. From this, it was concluded that variant K attaches to L-cells via the same residues as the parental virus but escapes neutralization by 9BG5 by diminishing the Mab's ability to interact with this site on the haemagglutinin. The only alteration in K was an amino acid change at position 419 of σ 1 (Bassel-Duby *et al.*, 1986). From the above work it is possible that amino acid 419 is involved in 9BG5 recognition.

Table 1.6.2 Properties of the Synthetic Peptides Derived from the Anti-idiotypic Antibody 87.92.6

Peptide	Property						
	Homology to $\sigma 1-3$ residues	Inhibition of 87.92.6/R1.1 cell binding	Inhibition of reo 3/L-cell binding	Interaction with 9BG5	Inhibition of 87.92.6/9BG5 binding	Inhibition of reo 3/9BG5 binding	Inhibition of reo1/L-cell binding
V _H	aa 317-324	No	No	No	Yes	No	Not tested
V _L	aa 323-332	Yes*	Yes	Yes	Yes	Yes	No
V _{L+H}		Yes*	Yes	Yes	Yes	Yes	Not tested

* = When peptide is coupled to bovine serum albumin

reo. 3 = reovirus type 3

9BG5 = the neutralizing monoclonal antibody raised against $\sigma 1-3$

87.92.6 = the monoclonal antiidiotypic antibody raised against 9BG5

The above table lists the main properties of the synthetic peptides derived from homologous areas of the reovirus type 3 $\sigma 1$ protein and the antiidiotypic antibody 87.92.6. Data taken from Williams *et al.* (1988). See text for details.

1.6.5 Summary

- 1) Serotype-specific tissue tropism of reovirus T1 and T3 appears to be largely due to the cell attachment protein $\sigma 1$, targetting each virus serotype to a different cell-type.
- 2) The type 1 and type 3 virus both bind to L-cells, but it is unclear if they attach to the same receptor. Sialic acid may be involved in the receptor recognition process for the T3 virus.
- 3) An antiidiotypic monoclonal antibody (87.92.6) was generated in an attempt to identify the reovirus receptor. The β -2 adrenergic receptor was subsequently proposed to be the receptor on L-cells but a later study proved this to be unlikely.
- 4) A combined determinant of Mab 87.92.6 shows sequence homology to aa 317-332 of protein $\sigma 1$ -3. (a) Amino acids 317-332 of $\sigma 1$ have been proposed to constitute a receptor binding site. (b) Amino acids 323-332 of $\sigma 1$ have been identified as a putative neutralization epitope. Amino acid 419 was defined as a site of interaction with the neutralizing Mab, 9BG5.

1.7 AIMS

- 1) Express the $\sigma 1$ protein of reovirus *in vitro* from cloned copies of the S1 type 1 and type 3 genes.
- 2) Develop a cell-binding assay utilizing the *in vitro* expressed $\sigma 1$ proteins.
- 3) Map the cell-binding domain(s) of the $\sigma 1$ polypeptides by effecting a mutational analysis of the respective cloned genes.

The *in vitro* approach taken in this project was designed to further characterize the reovirus-receptor binding process by utilizing the virus cell-attachment protein $\sigma 1$. It was hoped that this would clarify the controversial subject of the type 1 and type 3 viruses binding to the same or distinct receptors on mouse L-cells. Also, by attempting to map the binding domain of $\sigma 1$, some molecular detail of the binding process would be gained, which could have implications with regard to the serotypic differences in tissue tropism seen in the reovirus-mouse model.

CHAPTER 2

Materials and Methods

2.1 MATERIALS

2.1.1 A list of the biochemicals, chemicals and radiochemicals utilized in this study is given below:

Amersham International plc., Amersham, Buckinghamshire:

a ^{35}S dATP (44TBq/mmol), Amplify, 5, 6 ^3H -UTP (1.92 TBq/mmol), L- ^{35}S methionine (30TBq/mmol).

BDH Chemicals Ltd., Poole, Dorset:

Ammonium acetate, β -mercaptoethanol, Bromophenol blue, Caesium chloride, Calcium chloride, Crystal violet, EDTA, Glucose, Glycerol, Lithium chloride, Nonidet P40 (NP40), Phenol, PEG 6000, Potassium chloride, Sodium acetate, Sodium chloride, Sodium dihydrogen phosphate, Sodium dodecyl sulphate, Sodium hydroxide, Sucrose, Tris-base.

Bethesda Research Laboratories UK Ltd., P.O. Box 145, Cambridge:

Restriction enzymes, T4 DNA Ligase, Urea.

Bio-Rad, Richmond, California:

Ammonium persulphate, N, N'-methylene-bis-acrylamide, TEMED.

Boehringer Mannheim UK Ltd., Lewes, East, Sussex:

Calf intestinal phosphatase, Mung bean nuclease.

Difco Laboratories, Basingstoke, Hampshire:

Bacto-agar, Bactotryptone, Yeast extract.

Eastman Kodak Co., Rochester, New York:

LX 24 X-ray developer, FX 40 X-ray fixer.

FSA, Loughborough, Leicestershire:

Acids, Acrylamide, Formaldehyde, Formamide, Glycine, Magnesium chloride, Xylene cyanol.

Fuji Photo Film Co. Ltd., Japan:

RX X-ray film.

May and Baker Ltd., Dagenham:

Diethyl ether, Trichloroacetic acid.

Northumbria Biologicals Ltd., Cramlington, Northumberland:

Foetal calf serum, Tissue culture dishes.

Pharmacia Biotechnology, Uppsala, Sweden:

DNA polymerase I (Klenow fragment), 5' methyl cap ($m^7G5'ppp5'G^m$), 5-methyl dCTP.

Polaroid UK Ltd., St Albans, Hertfordshire:

Type 665 (positive/negative) and 667 (positive) film.

Promega, Madison, Wisconsin, USA:

1mM amino acid mixture (minus methionine), BMV RNA, Rabbit reticulocyte lysate (nuclease treated), RNAsin, T3 RNA polymerase, T7 RNA polymerase.

Sigma Chemical Co. Ltd., Poole, Dorset:

Agarose medium EEO (type II), 2-aminopurine, Ampicillin, BSA, Chloramphenicol, Deoxynucleoside triphosphates, Dideoxynucleoside triphosphates, DTT, EtBr, IPTG, Lysozyme, Manganese chloride, MOPS, Ribonucleotide triphosphates, Rubidium chloride, Spermidine.

United States Biochemicals, Cleveland, Ohio, USA:

Sequenase version 2.0, Sequencing handbook.

Whatman International Ltd., Maidstone, Kent:

3MM Filter paper (No.1).

2.1.2 Media and Solutions

The following media and solutions were prepared by the media preparation staff in the Department of Biological Sciences, University of Warwick :

2x Agar, 2x GMEMS, 200mM Glutamine, 200mM Leucine, MEMS, NaHCO₃ (5% w/v), Phosphate buffered saline (PBS; 8g NaCl, 0.2g KCl, 1.15g Na₂HPO₄, 0.2g KH₂PO₄ per litre of sterile, distilled water), Penicillin/Streptomycin stock solution (100g and 100,000,000 units per 2l sterile, distilled water respectively), Sterile distilled water, Trypsin solution (0.25% in versene), 200mM Valine, Versene.

2.2 STORAGE OF BACTERIAL STOCKS

The bacterial strains utilized in this study were maintained in the short term on minimal agar plates (see below for recipe) or in the long term by mixing an overnight culture of bacteria (grown as in section 2.7.1) with sterile glycerol in an 85:15 ratio and then freezing and storing in liquid nitrogen.

Minimal agar plates consisted of : 1x "A" salts (5x = 5.25g K₂HPO₄, 2.25g KH₂PO₄, 0.5g (NH₄)₂ SO₄, 0.25g sodium citrate per 100ml of sterile, distilled water), 0.5ml of 1M MgSO₄, 0.25ml of 1% thiamine HCl, 10ml of 20% glucose per 500ml sterile, distilled water, solidified with noble agar to a final concentration of 1.5%.

2.3 TISSUE CULTURE

2.3.1 Recovery of Cells from Liquid Nitrogen

An ampoule of L-cells was removed from liquid nitrogen and immediately thawed at 37°C. The cells were then diluted into 20ml. of MEMS1 media (MEMS + 5% FCS, 0.001% streptomycin, 100,000 units penicillin and 2mM glutamine), centrifuged at 1.5K for 5 minutes (mins) and resuspended in 10ml media before being transferred to a 75 cm² Flow bottle. Further media was added and the cells allowed to grow to confluency by incubation in a 37°C, 5% CO₂ incubator.

2.3.2 Growth and Maintenance of L-cells

2.3.2.1 In Monolayer Culture

L-cells were grown in 75cm² Flow bottles or 1.5 litre roller bottles using MEMS1 medium. When confluent, the cells were passaged at a ratio of 1:4. Monolayers were rinsed twice with versene and detached with a small volume (3-5ml) of a trypsin/versene mixture. The trypsin was inactivated by the addition of approximately 10mls of media, the cells pipetted vigorously and reseeded. (Roller bottles were initially seeded with the contents of two Flow bottles).

2.3.2.2 In Suspension Culture

Cells from 6 confluent roller bottles were harvested, pooled and counted using a haemocytometer before being diluted into the appropriate volume of media to give a cell density of 8×10^5 - 1×10^6 cells/ml. The culture was incubated in a flat bottomed, round flask, with stirring, at 37°C. Incubation was continued for 6-8 hours before the cells were split 1:2; thereafter the cells were split 1:2 every 24 hrs to maintain the cell density between 5×10^5 - 1×10^6 cells/ml.

2.4 VIRUS GROWTH AND PREPARATION

2.4.1 Preparation of Virus Inocula

Confluent Flow bottles of L-cells were inoculated with virus at a multiplicity of infection (moi) of 0.1 pfu/cell in 10ml. of MEMS1. One bottle was mock inoculated with MEMS1 as a control. Cells were incubated at

37°C for 3-4 days until the cytopathic effect (cpe) was clearly evident as compared to the control. The contents of each Flow were harvested, pooled and stored at -70°C. The stock was freeze-thawed 3x and sonicated before being titrated as described in 2.4.4.

2.4.2 Large Scale Growth of Virus

This was essentially performed as described by McCrae (1985). Four or eight litre spinner cultures of L-cells (cell density 1×10^6 /ml) were incubated at 34°C for 1-2 hrs before reovirus inoculum was added at a moi of 10 pfu/cell. (The culture temperature must be lowered as reovirus is cytotoxic to L-cells at 37°C). Incubation was continued at 34°C for 36-42 hrs, after which time the culture was transferred to 4°C and the cells allowed to settle for 4 -8 days.

2.4.3 Virus Purification

This procedure was carried out as described by McCrae (1985). Briefly, most of the culture medium was removed by suction and the cellular material remaining concentrated by centrifugation at 2K, 4°C for 20mins. Cells were resuspended in reovirus resuspension buffer, homogenized with Arcton (trifluoro-trichloroethane) and the phases separated by low speed centrifugation. The aqueous phase was decanted and the process repeated 3-4 times. The aqueous phases were pooled and back extracted with Arcton. Virus was concentrated from the aqueous phase by centrifugation at 20K, 4°C for 2hrs in a Beckman SW 28 rotor, the resulting pellet overlaid with 50mM Tris pH 8.0 and stored at 4°C overnight.

After resuspension by sonication, virus was layered on a pre-formed, 1.2-1.4 g/ml density, caesium chloride gradient and centrifuged at 20K, 4°C for 2hrs in a Beckman SW 28 rotor. The virus band (grey/blue colour) was harvested, diluted in 50mM Tris pH 8.0 and re-centrifuged as described above to concentrate the virus. The viral pellet was finally resuspended in a small volume of 50mM Tris pH 8.0 and stored at -70°C.

2.4.4 Plaque Assay of Virus

Monolayers of L-cells were plated out in 24-well tissue culture dishes (2×10^5 cells/well). Serial 10 fold dilutions of virus were made in PBS; monolayers were then washed once with PBS, 100µl of the appropriate virus dilution added and the dishes incubated at 37°C for 1 hour.

After incubation, the monolayers were gently overlaid with a 1x GMEM/agar mix which was allowed to harden before the dishes were incubated in a 37°C/5% CO₂ incubator in a humidified atmosphere. Reovirus type 1 plaque assays were incubated for 4-5 days and type 3 for 3-4 days.

Monolayers were fixed with formal saline (30% formaldehyde in PBS [v/v]), the agar overlay removed and monolayers stained with crystal violet (0.5% [w/v] crystal violet in 20% [v/v] ethanol) to visualize plaques.

2.4.5 Labelling of Infected Cell Polypeptides

Confluent L- cell monolayers in 12-well tissue culture dishes (5×10^5 cells/well) were washed 1 x with PBS and inoculated with 100µl virus at a moi of 10 pfu/cell. Virus adsorption was allowed to proceed at 37°C for 1 hr, after which the monolayers were overlaid with MEMS + 2%

FCS and incubated at 37°C for 12-15 hrs. The media was then removed, the cells washed in methionine-free (met-free) GMEMS and starved of methionine by incubation in met-free GMEMS + 2% FCS for 1-2hrs. (Met-free GMEMS = 500ml 2x GMEMS minus leucine, valine, methionine and NaHCO₃, supplemented with 10ml 200mM leucine, 10ml 200mM valine, 10ml 200mM glutamine, 20ml NaHCO₃ [5% w/v], 1ml penicilin/streptomycin stock solution, and made up to a volume of 1 litre with sterile distilled water). 100µl of ³⁵S-methionine (500µCi/ml in PBS) was used to replace the media and the cells returned to 37°C for 20mins. Monolayers were scraped into 250µl 50mM Tris-HCl pH 8.5, divided into 50µl aliquots and stored at -70C.

The trichloroacetic acid (TCA) precipitable radioactivity of the samples was measured (essentially as described in 2.11.2) before analysis on SDS-polyacrylamide gels.

2.5 ROUTINE PURIFICATION OF NUCLEIC ACIDS

2.5.1 Phenol/Chloroform Extraction

An equal volume of phenol/chloroform was added to the DNA sample and inverted several times to ensure thorough mixing. The sample was then briefly spun in an Anderman microfuge to separate the phases and the upper, aqueous layer removed without disturbing the interface, to a fresh tube. If necessary, successive extractions were performed until the interface was clear. The sample was then extracted once with chloroform and the DNA precipitated as described below.

2.5.2 Precipitation of DNA

DNA was precipitated from aqueous solutions in the following manner. The sample was made 0.15M with respect to lithium chloride and 2-2.5 volumes ethanol (-20°C) added followed by incubation on ice or at -20°C for 15 minutes. DNA was pelleted by centrifugation, washed with 80% ethanol and dried under vacuum. Finally, the nucleic acid pellet was resuspended in sterile, distilled water or TE (10mM Tris-HCl pH 8.0, 1mM EDTA).

2.6 DNA RESTRICTION AND MODIFICATION REACTIONS

2.6.1 Restriction Enzyme Digests

Restriction digests were carried out according to the manufacturers instructions. Buffers used were those supplied with the enzymes (as a 10x concentrate) or were made as 10x stocks as detailed in the suppliers instructions.

Typically, DNA was digested in a 20µl reaction volume containing buffer at 1x concentration. Enzyme was added to no more than 10% of the final volume and the sample incubated at the appropriate temperature, usually 37°C.

Following digestion, the DNA was analysed by agarose gel electrophoresis or phenol/chloroform extracted and ethanol precipitated before use in further enzyme modification reactions.

2.6.1.1 Partial Digests

These were performed during subcloning procedures when it was necessary to use a restriction enzyme whose recognition sequence was present more than once in the DNA being manipulated.

A preliminary reaction was set up to determine the conditions necessary to create the correct extent of digestion. The DNA to be digested was diluted to 1mg/ml (in TE) and the enzyme concerned diluted to 1 unit/ μ l in the appropriate 1x reaction buffer and stored on ice. A reaction mix was set up where 1 μ g DNA was pre-warmed in 9 μ l of 1x reaction buffer at 37°C; 1 unit of enzyme was added to the pre-warmed mix and 1 μ l aliquots taken at various time points, typically 0', 3', 5', 7', 10', and 15' minutes. The aliquots were added to 1 μ l of 0.5M EDTA pH 8.0 and analysed on a mini-gel, as described below. The optimum time point was taken to be the one that gave the greatest amount of linearised product.

A larger reaction was then carried out by increasing the scale of the digestion and incubating for the previously determined length of time. For example, instead of 1 μ g DNA with 1 unit of enzyme for 5 minutes, 5 μ g would be digested with 5 units of enzyme for 5 minutes. The linearised fragment would then be isolated from a mini-agarose gel (section 2.9.1).

2.6.2 End-Filling Reactions

The Klenow fragment of *E. coli* DNA polymerase I was used to fill 5' overhanging ends of DNA. The reaction mixture shown overleaf was incubated at 37°C for 15 minutes then at 65°C for 5 minutes to inactivate the enzyme before precipitation of the DNA as described in section 2.5.2.

End-filling reaction mix

DNA: up to 5µg

3µl 10x Pol. 1 buffer

(10x Pol.1 buffer = 0.5M Tris-HCl pH 8.0,

0.1mM MgCl₂)

3µl 10mM DTT

1µl 20mM dNTPs

2 units Klenow polymerase 1

H₂O to 30µl

2.6.3 Removal of Overhanging Ends

3' overhanging ends were removed by digestion with mung bean nuclease (MBN). The reaction mix used was as follows :

DNA up to 5µg

5µl 10x MBN buffer

(300mM NaAc pH 5.0, 500mM NaCl,

10mM ZnCl₂, 50% glycerol)

10 units mung bean nuclease

H₂O to 50µl

The mix was incubated at 37°C for 30 minutes and then at 70°C for 10 minutes. This was followed by phenol/chloroform extraction and ethanol precipitation.

2.6.4 Phosphatase Treatment

The 5' phosphates of restriction endonuclease digested vector DNA were removed by treatment with calf intestinal phosphatase (CIP) to prevent re-ligation. CIP buffer (10 x buffer = 0.5M Tris-HCl pH 9.0, 10mM MgCl₂, 10mM ZnCl₂, 10mM spermidine) was added to a final concentration of 1x and 1unit of CIP added. Incubation followed at 37°C for 30 minutes for 5' overhanging ends or for 15 minutes at 37°C, followed by the addition of a further unit of CIP then 55°C for 45 minutes for blunt-ended or recessed 5' ends.

The CIP was inactivated by heating at 70°C for 10 minutes followed by phenol/chloroform extraction and the DNA ethanol precipitated and resuspended in H₂O.

2.6.5 Ligations

Ligation reactions were performed in a volume of 15µl containing 100-200ng of vector DNA and sufficient insert DNA such that there was a 5-10:1 ratio of molecules of the latter to the former. 1.5µl of 10x ligation buffer (660mM Tris-HCl pH 7.5, 50mM MgCl₂, 50mM DTT and 10mM ATP*) and 3 units of T4 DNA ligase were added to the DNA and the remaining volume made up with sterile, distilled water. The ligation mix was incubated at 15°C for 16 hours or room temperature for 4 hours. Half of the mix was then transformed into *E. coli*.

* For blunt ended ligations, the ligation buffer contained 5mM ATP (BRL Focus newsletter, 1988).

2.7 TRANSFORMATION AND PREPARATION OF PLASMID DNA

Two methods were used for bacterial transformation:

2.7.1 Transformation with calcium chloride prepared cells

1ml of an overnight culture of *E. coli* JM103 (grown from a single colony in minimal media, stock solutions of which comprised: 20ml 5x A salts, 0.1ml of 1M MgSO₄, 0.05ml of 1% thiamine HCl and 2ml of 20% sterile glucose solution, made up to a 100ml final volume with sterile, distilled H₂O and filter sterilised before use) was diluted 1:100 in L-broth (10g bactotryptone, 5g yeast extract, 5g sodium chloride per litre of sterile, distilled H₂O) and incubated at 37°C, 200 rpm, until the A₅₉₀ reached 0.4-0.6. The cells were then pelleted, resuspended in 0.5 volumes of ice cold 0.1M MgCl₂ and immediately re-centrifuged. The pellet of cells was then gently resuspended in 1ml of 0.1M CaCl₂ and incubated on ice for 30 minutes.

The DNA sample to be transformed was made 0.1M with respect to CaCl₂ in a volume of 100µl, added to a 200µl aliquot of the competent cells and incubated on ice for 20-30 minutes. The mix was then heat shocked at 42°C for 2 minutes and returned to ice for 30 minutes. Samples of 10, 100 and ~200µl were spread on dried Luria-Bertaini (LB) agar plates (10g bactotryptone, 5g yeast extract, 5g NaCl, 15g bacto-agar per litre of sterile, distilled H₂O). When antibiotic selection was necessary, ampicillin at a final concentration of 100µg/ml was included in the agar plates. Blue/white colour selection was made possible by including 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside (X-Gal) at 0.002% and

isopropyl- β -thiogalactopyranoside (IPTG) at 4 μ M in the plating medium. Plates were incubated overnight at 37°C.

2.7.2 The Hanahan Method (Hanahan, 1983)

E. coli JM103 cells were grown as described above (2.7.1), centrifuged, resuspended in 12.5ml of solution RF1 (Table 2.1) and incubated on ice for 15-30 minutes. Following incubation, the cells were re-centrifuged and resuspended in 2.4ml of solution RF2 (Table 2.1). They were then incubated for a further 15 minutes on ice before being aliquoted into 200 μ l amounts. Aliquots not used immediately were stored at -70°C. 10 μ l samples of DNA were added directly to the competent cells, gently mixed and returned to ice for 30 minutes. Aliquots were plated out as described above (section 2.7.1).

Table 2.1 Composition of Transformation Buffers RF1 and RF2

RF1	RF2
1.2g RbCl	0.5M MOPS (3-[N-Morpholino]propanesulphonic acid)
0.99g MnCl ₂	0.06g RbCl
3.0ml KOAc	0.55g CaCl ₂
0.15g CaCl ₂	NaOH to pH 6.8
12ml glycerol	H ₂ O to 50ml
Acetic acid to pH 5.8	
H ₂ O to 100ml	

RF1 and RF2 were filter sterilized before use.

2.7.3 Small Scale Isolation of Plasmid DNA (Mini-plasmid preparations)

This was based on the method of alkaline lysis as described in Sambrook *et al.* (1989)

Individual bacterial colonies were picked into 5ml of L-broth (containing ampicillin at a final concentration of 100µg/ml) and grown for 5-6 hours or overnight at 37°C, 200rpm. 1.5ml of culture were spun in an eppendorf centrifuge to pellet the cells. The pellets were each resuspended in 100µl of GET solution (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8.0), to which 200µl of 0.2M NaOH, 1% SDS was added. After gentle mixing, 150µl of 3M sodium acetate pH 4.8 was added, the solutions mixed and then incubated on ice for 5 minutes. Samples were then centrifuged for 10 minutes and the pellets removed with a toothpick. 2 volumes of 100% ethanol (~ 950 µl) added and samples left for 5 minutes at room temperature. DNA was pelleted by centrifugation for 10 minutes, washed in 80% ethanol and vacuum dried before being resuspended in 50µl sterile distilled water or TE.

2.7.4 Large Scale Isolation of Plasmid DNA

An overnight culture (10ml) of the desired clone (grown in L-broth containing ampicillin at 100µg/ml) was inoculated into 1 litre of L-broth / ampicillin (100µg/ml) and incubated at 37°C with shaking for 6 hours. Chloramphenicol was then added to a final concentration of 150µg/ml and incubation continued overnight.

Bacterial cells were then pelleted by centrifugation at 2K, 4°C, for 15 minutes, resuspended in 8.4ml of 25% sucrose, 50mM Tris-HCl pH 8.0 and placed in a pre-cooled 500ml conical flask. 1.4ml of lysozyme

(10mg/ml in 50mM Tris-HCl pH 8.0) was added to the cells and the mix swirled on ice for 5 minutes. A volume of 4.6ml of 0.25M EDTA was then added, the mix swirled for a further 10 minutes on ice before 9.6ml of lysis solution (2% Triton, 62.5mM EDTA, 50mM Tris-HCl pH 8.0) was slowly added.

Lysed cells were centrifuged at 16K, 4°C for 1 hour in a Beckman JA 20 rotor to pellet the chromosomal DNA. The resulting supernatant was removed, adjusted to 25 ml with sterile water and 25g of CsCl plus 1.25ml of EtBr (10mg/ml) added. This was then centrifuged at 25K, 23°C for 1 hour in a Beckman SW 28 rotor and the floating debris removed. The samples were then transferred to Beckman 38ml quick seal tubes and centrifuged in a VTi 50 rotor at 45K, 23°C for 16-18 hours.

Plasmid DNA bands were recovered by side puncture of the tubes and either dialysed against three changes of 5l of TE or diluted into 5 volumes of TE. The DNA was precipitated by making the sample 0.15M with respect to LiCl followed by the addition of 2 .5 volumes of ethanol (section 2.5.2).

After resuspending the pellet of DNA in 1ml of TE, a phenol/chloroform extraction was performed, the nucleic acid precipitated and resuspended in 0.5 ml of TE. To check the purity of the sample, optical density (O.D) measurements were made at 260 and 280 nm and the 260/280 ratio calculated; a ratio of 1.8 or greater signified that the sample contained a negligible amount of contaminating protein. The DNA concentration was then estimated from the O.D reading obtained at 260nm (1 O.D unit was taken to be equivalent to 50µg/ml DNA).

2.8 SITE DIRECTED MUTAGENESIS

2.8.1 Subcloning into M13

Type 1 and 3 reovirus S1 genes (S1 T1 and S1 T3) were subcloned from their respective transcription clones into M13mp19 in order to produce single-stranded (ss) DNA as template for oligonucleotide directed mutagenesis. Restriction enzyme digests, DNA modification reactions and ligations were performed as described in section 2.6.

2.8.2 Transformation into *E. coli* JM103

Competent cells to be used in the transformation of M13mp19 based ligation reactions were prepared as detailed in section 2.7.1. The plating procedure differed from that used for plasmid transformations in the following manner. After heat-shock treatment and incubation on ice, the ligation/competent cell mix was added to 3ml of molten (45°C) H-Top media (5g bactotryptone, 4g NaCl per 500ml distilled H₂O, solidified by the addition of 4g noble agar) and 200µl of exponentially growing JM103 cells. This mixture was then poured onto dried LB agar plates, allowed to set and incubated overnight at 37°C. When blue/white colour selection was required, 30µl of 10mM IPTG and 15µl of 2% X-Gal were added to the plating mix.

Plaques were picked and grown as described below (section 2.8.3) and the replicative form (RF) of the DNA from the mp19 clones prepared by the method of alkaline lysis as for mini- plasmid DNA preparations. Restriction digests were then carried out on 10µl of the RF DNA to screen for the desired clones.

2.8.3 Preparation of Single-Stranded Template

Individual plaques (that were well separated) were picked into 3ml of L-broth using a sterile toothpick and the cultures incubated in an orbital shaker at 37°C, 250-300 rpm for 6 hours. 1.5ml aliquots of each culture were centrifuged at 10K for 5 minutes in a Heraeus microfuge and 1ml of the supernatant removed to a fresh microfuge tube; (the remaining supernatant was stored at 4°C as it served as stock inoculum for any future preparations of ss or RF DNA). 2.5ml of 20% PEG (polyethylene glycol) 6000/2.5M NaCl was added to the supernatant and the mixture incubated at room temperature for 15 mins. The samples were spun at 10K rpm for 5 mins in a Heraeus microfuge to pellet the phage particles, the liquid removed and the last traces of supernatant wiped from the tube. After resuspending the pellets in 100µl of TNE (20mM Tris-HCl pH 7.6, 10mM NaCl, 1mM EDTA), 50µl of phenol was added, the tubes vortexed and left to stand at room temperature for 5 mins. before being centrifuged at 10K rpm for 5 mins. in an Anderman microfuge to separate the two phases. 80µl of the upper aqueous layer was removed and extracted 3x with diethylether. DNA was precipitated as described in section 2.5.2, dried and resuspended in 30µl of sterile water or TNE and stored at -20°C.

2.8.4 Kinasing the Mutagenic Oligonucleotide

Freeze-dried oligonucleotides, synthesized on a Applied Biosystems 380B DNA Synthesizer, were resuspended in 300µl of sterile water and their concentrations determined by A_{260} measurements; here an optical density value of 1 unit was taken to be equivalent to 20µg/ml single-stranded DNA.

100pmols. of oligonucleotide was kinased in a single reaction, carried out as follows : 10µl oligonucleotide (10 pmol/µl) was mixed with 2µl of 10x kinase buffer (10x buffer = 500mM Tris-HCl pH 8.0, 100mM MgCl₂), 2µl 10mM ATP, 10 units of T4 polynucleotide kinase and water to a final volume of 20µl. The reaction mix was incubated at 37°C for 30 mins and the enzyme inactivated by heat treatment at 70°C for 5 minutes. Kinased oligonucleotide was stored at -20°C.

2.8.5 Oligonucleotide Directed Mutagenesis

Mutagenesis was performed using the method of Vandeyar *et al.* (1988), in which high efficiency mutagenesis is made possible by the incorporation of 5 methyl dCTP in the mutant strand which then allows the parental strand to be digested away by restriction enzymes and exonuclease III (Chapter 10, Figure 10.2.2).

10µl of ss DNA (~1.5µg) were mixed with 3µl of kinased oligonucleotide (10-30pmol) and 2µl of 10x annealing buffer (200mM NaCl, 20mM Tris-HCl pH8.0) in a total volume of 20µl, adjusted with sterile water. The annealing mix was incubated at 65°C for 5 mins and then room temperature for 10 mins to allow the oligonucleotide to anneal to the template.

An extension/ligation reaction was then set up by addition of the reagents shown in Table 2.2 to the annealing mix.

Table 2.2 Composition of the Oligonucleotide Directed Mutagenesis Extension/Ligation Reaction Mixes

Reagent	Volume	Final Concentration
10x Annealing Buffer	3 μ l	
5mM dNTP's *	5 μ l	0.5 mM
10mM ATP	5 μ l	1mM
100mM DTT	2.5 μ l	5mM
T4 DNA Polymerase	5 units	
T4 DNA Ligase	3 units	
H ₂ O	to 50 μ l	

* = 5mM dATP, 5mM dGTP, 5mM dTTP and 5mM 5-methyl dCTP.

This mix was incubated at 37°C for 90 mins followed by 70°C for 10 mins. to destroy the polymerase and ligase activities.

Following inactivation, 10 units (1 μ l) each of *Hha*1 and *Msp*II were added to the extension/ligation mixture and returned to 37°C for 45 minutes. 100 units of exonuclease111 (1.5 μ l) were finally added to the reaction mix and digestion allowed to proceed for a further 45 mins at 37°C before being stopped by heat inactivation at 70°C for 10 minutes. Reaction volume changes were negligible upon addition of the above mentioned enzymes.

2.8.6 Transformation of the Mutagenesis Reaction

Half of the mutagenesis reaction mix (25 μ l) was transformed into competent *E. coli* RD24 cells [(Mcr A⁻B⁻) New England Biolabs] essentially as described in section 2.8.2 with *E. coli* JM103 used as the lawn bacteria.

2.8.7 Screening the Plaques

This method of mutagenesis yields a high number of mutants, enabling screening to be carried out by the direct sequencing of a small number of plaques. Typically, single-stranded DNA from 10 plaques was prepared as in section 2.8.3 and analysed by sequencing as described in section 2.8.8 below.

2.8.8 Sequencing Reactions

Sequencing reactions were performed according to the United States Biochemicals (USB) handbook (1989), based upon the dideoxy method of sequencing described by Sanger *et al.* (1977).

2.8.8.1 Single Stranded DNA

An annealing reaction was set up that contained 7 μ l (~1 μ g) ss M13 DNA template, 1 μ l (1pmol) of -20 M13 universal primer and 2 μ l of 5x reaction buffer (200mM Tris-HCl pH 7.5, 100mM MgCl₂, 250mM NaCl). The sample was heated at 65°C for 2 mins and allowed to cool slowly to room temperature over a period of 30 minutes. The following were added to the annealed primer/template :

2 μ l of 5-15x diluted labelling mix (7.5 μ M dGTP, 7.5 μ M dTTP, 7.5 μ M dCTP), 1 μ l 100mM DTT, 0.5 μ l ³⁵S dATP and 2 μ l Sequenase version 2.0 enzyme, diluted 1:8 in enzyme dilution buffer (10mM Tris-HCl pH 7.5, 5mM DTT, 0.5mg/ml BSA). After thorough mixing, the sample was incubated on ice (for short extensions of the primer) or at room temperature (for longer extensions) for 2-5 mins. Aliquots of 3.5 μ l were then added to the four termination mix tubes, each of which contained one of the relevant ddNTP solutions (i.e. ddGTP, ddATP, ddTTP or ddCTP), pre-warmed to 37°C. The tubes were centrifuged to mix the solutions and incubated at 37°C for 5 mins. Reactions were stopped by the addition of 4 μ l of formamide stop buffer (95% de-ionised formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol).

4 μ l aliquots were analysed immediately on denaturing 8% polyacrylamide gels as described in section 2.8.9. The remainder of the reaction mix could be stored at -20°C for up to 7 days for further analysis with no adverse effect on sequence integrity (USB handbook, 1989).

2.8.8.2 Double Stranded Plasmid DNA

Plasmid DNA purified by caesium chloride gradient centrifugation was used in plasmid sequencing reactions. 4 μ g of DNA was used in each sequencing reaction. The DNA template was made up to a volume of 18 μ l with TE and denatured by the addition of 2 μ l of freshly prepared 2M NaOH followed by incubation at room temperature for 10 minutes. 4 μ l of 5M ammonium acetate pH 5.4 was added, the sample vortexed and 100 μ l of 100% ethanol (-20°C) immediately added and vortexed . The sample was placed in a dry ice/industrial methylated spirits (IMS) bath for 10 mins. before being centrifuged for 10 mins at 10K in an Anderman microfuge to precipitate the DNA, which was subsequently

washed in 70% and then 100% ethanol before being air dried at room temperature.

The dried pellet of DNA was resuspended in 7 μ l of sterile water and sequenced in the same manner as for ss DNA (section 2.8.8a). Denatured DNA was not stored, but used immediately in sequencing reactions.

2.8.9 Sequencing Gels

Sequencing reactions were analysed on denaturing polyacrylamide gels. 40cm gels were cast and run using commercial (Biorad) apparatus. A 50ml gel solution consisting of 8% acrylamide (from a 38% acrylamide : 2% bisacrylamide stock) and 7M urea in 1X TBE buffer (10 x = 108g Tris base, 55g boric acid, 40ml 0.5M EDTA pH 8.0 per litre of distilled water) was made and polymerised by the addition of 0.6% (v/v) ammonium persulphate and N,N,N',N'-tetramethylethylenediamine (TEMED), each to a final concentration of 0.06%.

Gels were pre-electrophoresed in 1x TBE for 1 hour at 1700 volts. Immediately prior to loading, 4 μ l of sample was denatured by heating to 80°C for 3 mins and then electrophoresed for 1.5-6 hours depending upon the amount of sequence to be determined.

After electrophoresis, gels were fixed in 10% acetic acid/10% methanol for at least 15 minutes before being dried on Whatman 3MM filter paper under vacuum at 80°C. The dried gel was exposed to Fuji RX X-Ray film at room temperature for at least 12 hours.

2.9

AGAROSE GEL ELECTROPHORESIS

Large and small (mini) horizontal agarose gels were used for analysis of DNA after restriction digests and during subcloning procedures. Typically, the concentration of agarose used was 1% (w/v) but concentrations between 0.8 - 1.5% were also employed depending upon the size of the fragments to be resolved.

The following method describes the preparation of a mini-gel; however all gels were prepared in the same manner, taking into account any necessary differences in volumes and weights of the appropriate substances. 50 ml. gels were used for rapid analysis of DNA fragments. The appropriate amount of agarose was dissolved in 50ml. 1x TBE buffer to which 2 μ l of a 10mg/ml stock of ethidium bromide (EtBr) was added. The gel was poured in a commercial gel former containing a well-forming comb and allowed to set. Once set, the gel was submerged in 1x TBE buffer in which it was electrophoresed. Commercial apparatus used was Biorad and Uniscience for mini-gels and Scie-Plas or Koch-Light Ltd (New Brunswick Scientific Co.) for large gels.

DNA samples were prepared for electrophoresis by adding 1/5 the volume of 5x TBE gel loading buffer (50% glycerol, 5x TBE, 0.02% bromophenol blue and 0.02% xylene cyanol) before being loaded into the wells of the gel. Electrophoresis was carried out for 1 hour at 80 mA. DNA bands were then visualized on an ultraviolet (uv) illuminator and the gel photographed using a Polaroid camera and type 665 or 667 Polaroid film.

2.9.1 Isolation of DNA Fragments

Fragments of DNA to be isolated were first electrophoresed as described above and subsequently isolated from the agarose gel in the

following manner. After electrophoresis was complete, the gel was inspected on a long wavelength uv illuminator (to minimise damage to the DNA) and a slit cut in the gel in front of the desired fragment(s). A piece of dialysis tubing was then backed with Whatman 3MM paper and inserted into the slit with the paper facing the DNA fragment. The gel was replaced in the gel tank from which the buffer had been removed and the buffer replaced until a meniscus was formed with the end of the gel.

Electrophoresis then continued at 150 mA for 1 - 2 minutes and the gel and paper inspected on the illuminator to ensure that the DNA had run into the paper. (This was repeated if necessary until all of the desired band had been eluted from the gel).

The DNA was recovered from the paper by elution with TNE (200mM NaCl, 20mM Tris-HCl pH 7.5, 1mM EDTA); the Whatman paper was placed in a 0.5ml microfuge tube which had previously had a hole punctured in the bottom. This was then placed in a 1.5ml microfuge tube, centrifuged at 10K for 2 minutes and the eluant removed to a fresh tube. 150µl of TNE was added to the paper and re-centrifuged. One or two further elutions were carried out and the washes pooled before being phenol/chloroform extracted and the DNA ethanol precipitated. The dried pellet was typically resuspended in 10µl of sterile water and a 1-2 µl aliquot analysed on an agarose gel.

2.9.2 Agarose Gel Electrophoresis of RNA

RNA produced from *in vitro* transcriptions was size fractionated on 1.2% agarose gels containing 50% formamide. Gels were prepared as described in section 2.7 with the exceptions that the gel mix included 50% de-ionised formamide (DIF) and 0.1x TPE buffer (1x = 36mM Tris, 30mM NaH₂PO₄[2H₂O]), 2mM EDTA pH 8.0). Electrophoresis was carried out in

0.1x TPE buffer which was only allowed to form a meniscus with the gel instead of submerging it. RNA samples (and DNA markers) were prepared in 60% DIF and denatured by heating at 65°C for 5 minutes before loading into the wells of the gel. Electrophoresis was carried out for 3 hours at 40 mA. The gel was stained in 0.1µg mg/ml EtBr for several hours or O/N, destained in H₂O and visualised on a uv illuminator.

2.10 *IN VITRO* TRANSCRIPTIONS

Capped, positive sense RNA was produced from DNA copies of various genes cloned into transcription vectors.

2.10.1 Linearisation of the DNA Template

30µg of plasmid DNA was linearised in a single reaction using either *Sma*I or *Hind*III. Reaction volumes were typically 50µl, and following digestion a 1µl aliquot was analysed on an agarose gel to check for complete linearisation.

Linearised samples were then phenol/chloroform extracted, ethanol precipitated and resuspended in 30µl sterile water.

2.10.2 The Transcription Reaction

In order to quantitate the RNA produced from the *in vitro* transcription a trace amount of radioactive uridine triphosphate (5, 6, ³H uridine 5' triphosphate), was included in the reaction mix.

10µCi (10µl), of tritiated uridine triphosphate was dried under vacuum in a sterile, 1.5ml eppendorf tube and resuspended in 25µl of sterile water. The reaction was then set up at room temperature as shown in Table 2.3 (overleaf), in a final volume of 50µl.

Table 2.3 Composition of *In Vitro* Transcription Reactions

Reagent		Final Concentration
³ H-UTP	25μl (in H ₂ O)	
50mM ATP, UTP, CTP	3μl	1mM
5mM GTP	1μl	0.1mM
DNA template (1mg/ml)	3μl	0.06mg/ml
5x Transcription buffer (200mM Tris-HCl pH 7.5, 30mM MgCl ₂ , 10mM spermidine, 50mM NaCl)	10μl	1x
100mM DTT	2.5μl	5mM
10mM m ⁷ GpppG ^m Cap	2.5μl	0.5mM
RNASIN (1u/μl)	1μl	
T3 or T7 Polymerase (30u/μl)	1μl	

The above reaction mix was incubated at 37°C for 30 minutes after which 1μl of 50mM GTP was added and incubation continued for a further 60 minutes. A 1μl aliquot was removed and the insoluble radioactivity measured after trichloroacetic acid (TCA) precipitation (see below).

RNA produced in this reaction was ethanol precipitated in the following manner. The reaction mix was adjusted to a volume of 200μl with sterile water and 5M ammonium acetate to a final concentration of 0.7M. 2.5 volumes of 100% ethanol were added and the sample transferred to a dry ice/IMS bath for 15 minutes. Following this, the sample

was centrifuged at 10K for 10 minutes to pellet the RNA which was subsequently washed in 80% ethanol, dried under vacuum and resuspended in sterile water to give a final concentration of 1mg/ml.

2.10.3 TCA Precipitation

The amount of RNA produced in the *in vitro* transcription reactions was quantitated by measuring the amount of radioactivity incorporated into TCA insoluble material.

1 μ l of the reaction mix was removed upon completion of the transcription reaction and placed in 400 μ l of 10% TCA. This was incubated on ice for 10 minutes and then spotted onto a 3MM Whatman filter disc under vacuum. The disc was washed twice in 5% TCA, twice in ethanol, dried and placed in 5ml. of Optiphase Safe scintillation fluid. The amount of TCA precipitable radioactivity was then measured in an LKB Wallac 1219 Rackbeta liquid scintillation counter and the resulting figure utilized to calculate the amount of RNA that had been synthesized. This is shown overleaf.

Calculation to Determine the Amount of RNA Synthesized *in vitro*:

10 μ Ci of ³H-UTP was included in the reaction

10 μ Ci = 50nmoles UTP

1 μ Ci = 2.22 x 10⁶ dpm

No. counts x sample volume = total counts

Total counts/2.22x10⁷ = no. moles UTP incorporated (inc.) into RNA

Assume equal amounts of the 4 rNTPs are incorporated into RNA

No. moles UTP inc. x average molecular weight for a rNTP x 4 = yield of
RNA

Yield of RNA x %age efficiency of counting = actual yield of RNA

The efficiency of counting of the scintillation counter was determined by spotting a 1 μ l aliquot of a 1:100 dilution of the ³HUTP onto a Whatman 3MM disc placing it in scintillation fluid and counting in the scintillation counter.

No. counts x 100 (dilution factor) x 100 = % counting efficiency
2.22 x 10⁶

2.11 *IN VITRO* TRANSLATIONS

2.11.1 The Rabbit Reticulocyte Lysate System

In vitro transcribed RNA was translated into protein using the rabbit reticulocyte lysate system. Commercially available lysate (Promega) and lysate prepared in the laboratories of T. Hunt (Cambridge University) and J. Pain (University of Sussex) were used. All, except the Promega lysate, were optimised for the potassium and magnesium ion concentrations (section 2.11.3).

A typical 25 μ l reaction is shown overleaf in Table 2.4. The reaction mix was incubated at 30°C for 1 hour and then utilized immediately or stored at -70°C. 2 μ l aliquots were sampled at various times during the reaction and TCA precipitated (see section 2.11.2).

The Promega lysate was used according to the manufacturer's instructions.

2.11.2 TCA Precipitation

2 μ l samples of the translation mix were taken after 0, 30 and 60 minutes and spotted onto Whatman 3MM 25mm filter discs. These were placed immediately into 5% TCA and when all samples had been taken, they were transferred to 5% TCA/3% cas-amino acids, boiled for 5 minutes, washed twice in 5% TCA, twice in 100% ethanol and dried by washing twice in diethyl ether. The discs were then placed in Optiphase Safe scintillation fluid and radioactivity counted in an LKB Wallac 1219 Rackbeta scintillation counter.

Table 2.4 Composition of *In Vitro* Translation Reactions

Reagent	Volume added	Final concentration
K/M	2 μ l	~80mM K+/ ~0.8mM Mg ²⁺
Creatine Phosphate (CP)/ Creatine Kinase (CK) mix	1.25 μ l	5mg/ml CP ml / 0.2mg/ml CK
tRNA (1.125mg/ml)	1 μ l	45 μ g/ml
³⁵ S methionine	1.25 μ l (~ 20 μ Ci)	
mRNA (1mg/ml)	1 μ l	40 μ g/ml
Lysate	17.5 μ l	

K/M = Potassium acetate/Magnesium acetate

2.11.3 Optimisation of the Translation Reactions

Each fresh source of lysate was optimised with respect to the magnesium and potassium ion concentration and also to the amount of *in vitro* transcribed RNA necessary to achieve maximum stimulation of the system.

Reactions were set up as shown in Table 2.4 with final potassium acetate concentrations ranging from 20mM - 120mM whilst the magnesium ion concentration was kept constant at 0.6mM. The magnesium ion final concentration was titrated between 0 - 1mM with a constant potassium ion concentration of 50mM. Finally, the amount of exogenous RNA needed to achieve maximum stimulation was also determined over a twofold dilution series, at constant potassium and magnesium ion concentrations of 0.6 and 50mM respectively.

Promega lysate was not optimised in this manner as it was supplied with the exogenous magnesium and potassium already added to the lysate.

2.11.4 Analysis of Translation Products on SDS-Polyacrylamide Gels

Aliquots of *in vitro* translation mixes were analysed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) to ascertain the size of the resulting protein products.

10% polyacrylamide gels (made with a 5% stacking gel) were run according to the method of Laemmli (1970). Stacking gel and separating gel mixes are given in Table 2.5; stock 30% acrylamide solution used in their preparation contained acrylamide and bisacrylamide in the ratio 29:1. One fifth of the final volume of 5x sample buffer (2% SDS, 25% β -mercaptoethanol, 250mM Tris-HCl pH 6.8, 50% glycerol and 0.02%

bromophenol blue) was added to 5-6 μ l of *in vitro* translation mix, the remainder of the volume made up with water and the sample boiled for 5 minutes. Samples were then pipetted into the wells of the gel and electrophoresed at 40mA for 2.5 hours in 1x running buffer (52mM Tris-base, 53mM glycine, 0.1%SDS, pH adjusted to 8.3). Following electrophoresis, gels were fixed in 10% acetic acid/25% methanol for 1 hour before being fluorographed and dried (section 2.11.5).

2.11.5 Fluorography of Polyacrylamide Gels

After fixing, gels were incubated in Amplify (Amersham) for 15 minutes and dried for 1-2 hours at 60°C. They were then exposed to Fuji RX X-Ray film overnight at room temperature or at -70°C after pre-flashing the film, when the bands on the gel were to be quantified by scanning laser densitometry (see section 2.11.6).

Table 2.5 Composition of SDS-Polyacrylamide Gel Mixes

Separating Gel Mix

8.3ml 30% acrylamide (29:1
acrylamide : bisacrylamide)
6.25ml 4x separating gel buffer
(4x = 1.5M Tris-HCl pH 8.8, 0.4% SDS)
2.5ml glycerol
15 μ l TEMED
200 μ l 10% ammonium persulphate (APS)
7.8ml water

Stacking Gel Mix

2ml 30% acrylamide
3ml 4x stacking gel buffer (0.5M Tris-HCl pH 6.8,
0.4% SDS)
3ml APS (70mg/25ml H₂O)
4ml water

2.11.6 Scanning Laser Densitometry

2.11.6.1 Calibration of the X-Ray Film

X-ray film was pre-flashed with a Hanimex flashgun to enable a linear response of the film to the radioactivity and hence quantification of bands by densitometry. The exposure of the film to the pre-flash was calibrated according to the Amersham Autoradiography Handbook (1987). Briefly, strips of film were exposed to the light source at various distances from the flashgun and their A_{595} values compared to that of non pre-flashed film. An A_{595} of 0.1-0.2 indicated the correct distance at which to pre-flash the film. After pre-flash treatment, the film was exposed to the dried, processed gel overnight at -70°C .

2.11.6.2 Quantifying the Bands

Gels were scanned and bands quantified on a Molecular Dynamics scanning laser densitometer according to the manufacturers instructions.

2.12 L-CELL BINDING ASSAYS

Two binding assay protocols were used; one in which the amount of radioactive protein (produced in the *in vitro* translation reaction) bound to the cells was assayed by a direct counting method and a second in which bound protein was visualised by SDS-PAGE and fluorography. These are described overleaf.

2.12.1 Direct Counting Method

Monolayers of L-cells in 24 well dishes were washed once with cold PBS and placed on ice. 4-5 μ l of reticulocyte translation mixture were diluted into 100 μ l of ice-cold PBS and overlaid on the monolayers of cells. Binding was allowed to occur for one hour with intermittent rocking, after which the binding mix was removed and the monolayers washed three times with cold PBS and once with cold sterile distilled water. They were then allowed to dry before being solubilized in 100 μ l 1M NaOH. This was neutralized with 100 μ l of 1M HCl before being added to 10ml of Optiphase Safe scintillant and counted in an LKB Wallac scintillation counter.

2.12.2 Visualisation of Bound Protein

Monolayers of L-cells in 12-well dishes (5 x 10⁵ cells) were washed once with cold PBS and placed on ice. Reticulocyte translation mix was diluted 6-8 times in ice-cold PBS and 100 μ l applied to the monolayers for one hour with intermittent rocking. After this time the binding mix was removed, the cells washed 3x in cold PBS, scraped into 0.5ml of cold PBS and pelleted by a brief spin in a microfuge. The PBS was decanted and the cells resuspended in solubilization mix (0.5% NP40 in PBS) and then left on ice for 15 minutes. Following this, nuclei were pelleted by centrifugation for 1 minute in a microfuge and 50 μ l of the supernatant analyzed by SDS-PAGE and fluorography, as in sections 2.11.4 and 2.11.5 with the exception that the incubation step in sample buffer was carried out at either 37°C for 30 minutes or 100°C for 5 minutes prior to gel loading.

CHAPTER 3

Cloning Reovirus S1 Type 1 and Type 3 cDNA Sequences into a Transcription Vector

The production of reovirus mRNA *in vitro* from purified virions was first possible over twenty years ago (Skehel and Joklik, 1969; Levin *et al.*, 1970 and Banerjee and Shatkin, 1970). Relatively large amounts of mRNA could be obtained owing to the stability of the core associated RNA dependent RNA polymerase and this obviated the need for purifying viral mRNA from infected cells. A disadvantage of this method is that a heterogeneous mix of all 10 viral transcripts is obtained. A route for studying individual species of message sense RNA was devised by McCrae and Joklik (1978) who demonstrated that the ds RNA genome was a source of mRNA. The resolution of the ds RNA segments of reovirus on polyacrylamide gels is far superior to that of the ss RNA species (Schuerch *et al.*, 1975) and hence individual segments could generally be isolated in a reasonably pure state. Denaturation of these isolated segments followed by *in vitro* translation produced polypeptides that were similar in mobility to that of native reovirus proteins. The major disadvantages of this approach are however, that it is time consuming and carries the inherent risk of the presence of contaminating species in any single gel purified population. Also, it was designed to specifically solve the problem of the coding potential of the reovirus genome and is of limited use when a more detailed, molecular analysis of a single gene and its products is required.

The commercial availability of transcription vectors (e.g., Promega: pGEM-Z vectors and Stratagene: Bluescribe and pBluescript vectors) has greatly facilitated the study of individual gene products *in vitro*. Problems associated with isolating rare species of mRNA or of producing sufficient quantities as a relatively pure product are largely overcome if the gene of interest, or its cDNA counterpart, can be cloned into a transcription vector, allowing microgramme quantities of RNA to be produced *in vitro*.

A large selection of such transcription vectors is now available; many combine several of the following features to make them particularly useful as cloning vehicles:

- 1) A polylinker containing a wide number of restriction enzyme sites, enabling flexibility in cloning strategies.
- 2) A selectable marker, often the β -lactamase gene, conferring antibiotic resistance.
- 3) Location of the polylinker within a portion of the *lac Z* gene allowing blue/white colour selection of bacterial colonies.
- 4) Different bacteriophage promoters situated at either end of the polylinker. This facilitates the production of plus and minus sense RNA. Transcription also begins at a defined, known point and terminates either under the direction of the inserted fragment or at a chosen site in the vector via a template run off process.
- 5) An M13 type origin of replication which enables ss DNA to be rescued from the vector via helper phage. This facilitates sequencing and/or site-directed mutagenesis of the cloned gene.

Creating mutants of a wild type gene provides one means of studying the expression or function of that gene. However, techniques to manipulate either ss or ds RNA with the same precision as DNA do not exist at present. Thus, by generating a cDNA clone of the appropriate reovirus gene, an opportunity is provided to create genetically altered variants. These can be subsequently expressed *in vitro* to determine their effect on the function of the wild type polypeptide.

Expression of a cloned gene in a eukaryotic system is an alternative to the *in vitro* approach. Vectors based on eukaryotic viruses, such as vaccinia virus, can confer the advantage of high yields of protein from a cloned gene, but a rigorous and stringent isolation procedure is required for its purification. A further disadvantage is that manipulations and

preparations involving these vectors are generally more time consuming than those utilizing bacterial vectors.

3.2 STRATEGY

It was decided to clone cDNA copies of the reovirus S1-1 and S1-3 genes into a transcription vector which incorporates all of the above listed features. The resulting clones could then be used to direct the synthesis of what should be a homogeneous population of message sense RNAs via a simple *in vitro* reaction.

The mRNA thus synthesized would be used to produce protein via *in vitro* translation reactions. Again, this ensures that an individual protein species derived from a specific mRNA is investigated.

The transcription vectors used in this study were Bluescribe (Stratagene) and pGEM-3Z (Promega). Bluescribe contains the T7 and T3 bacteriophage promoters and pGEM-3Z the SP6 and T7 phage promoters. Maps of each vector illustrating their salient features are given in appendix A and B respectively. The use of pGEM-3Z was more indirect as it is the basis of clone pG3T3, a gift from Dr. P. Lee (University of Calgary Health Sciences Centre, Canada). Plasmid pG3T3 is a transcription plasmid containing the S1-3 coding sequence (see later chapters).

3.3 RESULTS

3.3.1 Cloning the Reovirus S1-1 Gene into Bluescribe

A cDNA copy of the reovirus S1-1 gene (S1 gene from reovirus type1) had been cloned by M. A. McCrae (University of Warwick) and placed into the bacterial vector pAT153 by the method of GC tailing to create pATS1T1. During the cloning process, oligonucleotides containing various restriction sites had been added to the 5' and 3' ends of the cDNA sequence to facilitate further cloning manipulations. These will be referred to as the 5' and 3' gene oligonucleotides and their sequences and restriction sites are shown in Figure 3.3.1.

The strategy adopted for placing the S1-1 gene into Bluescribe to create clone pBSS1-1 is shown in Figure 3.3.2. Plasmid pATS1T1 was digested with *Bgl*III and *Bam*HI and the ends filled as described in sections 2.6.1 and 2.6.2 respectively. The approximately 1.5 Kb S1-1 band was separated on an agarose gel, purified and ligated into *Hinc*II digested Bluescribe. Recombinant clones producing white colonies on indicator plates were screened by digestion with a range of restriction enzymes. If oriented such that the 5' end of the gene is adjacent to the T3 promoter, a *Hind*III fragment of approximately 450 base pairs (bp) and a *Sph*I fragment of about 1Kb are predicted. The restriction digests of Figure 3.3.3 confirm these and other similar predictions. A full-length clone was identified in this way and designated pBSS1-1.

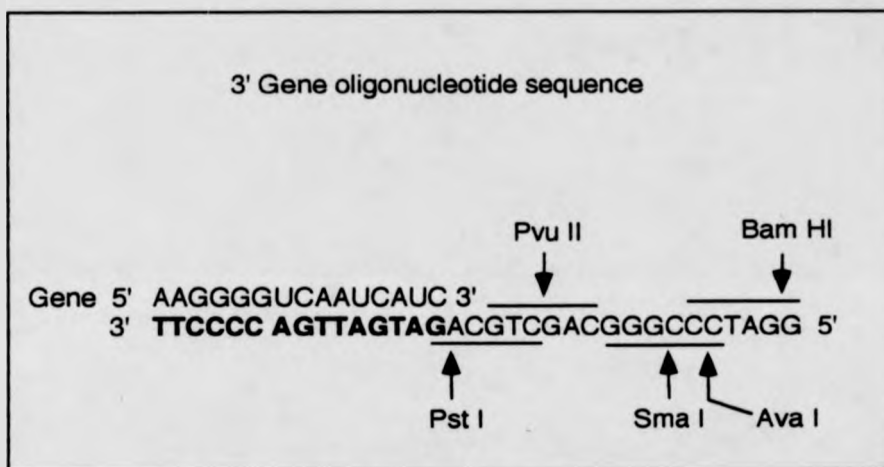
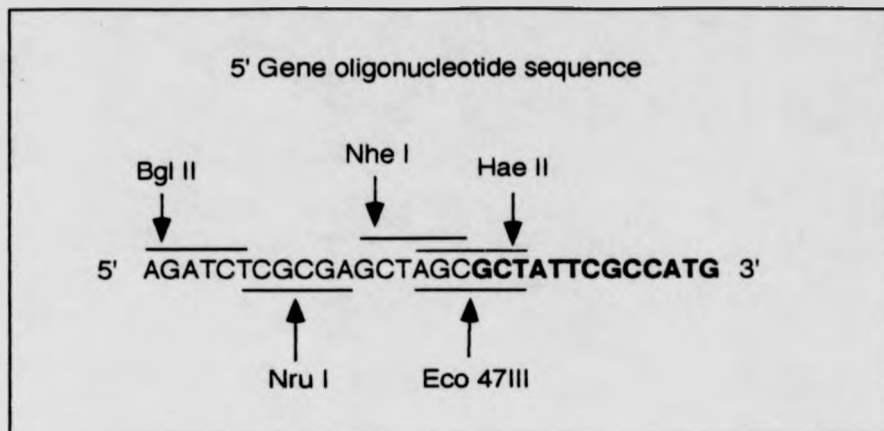


Figure 3.3.1 Sequences of the Reovirus S1-1 Gene Oligonucleotides

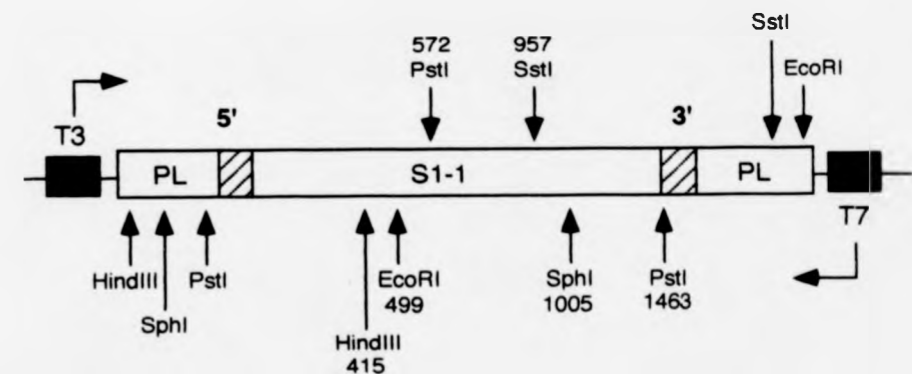
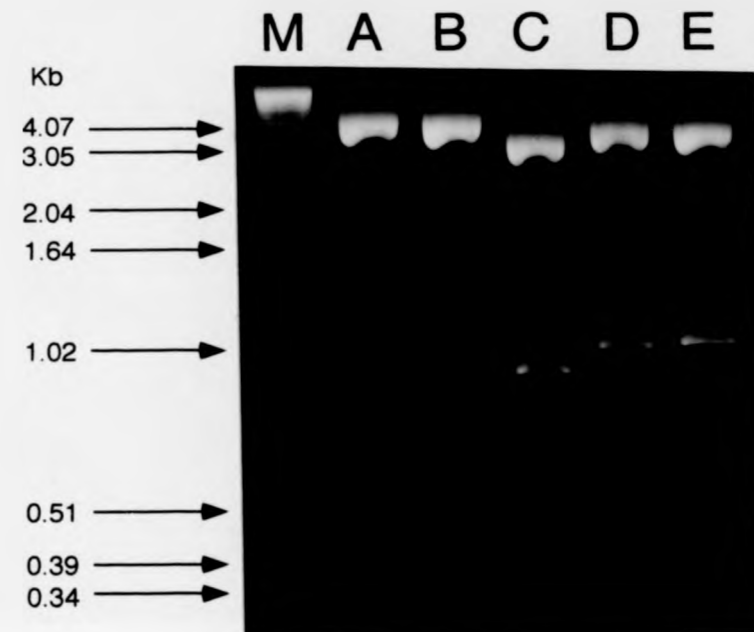
The sequence of the 5' and 3' gene oligonucleotides used in the cloning of the reovirus S1-1 gene are shown in this diagram. For clarity, the sequence of the 3' end of the plus strand of the gene is written above the 3' gene oligonucleotide sequence. Restriction enzyme sites are underlined and the bases marked in bold type represent sequence derived from the respective ends of the S1-1 gene.

Figure 3.3.3 Restriction Enzyme Digest Analysis of pBSS1-1

Shown opposite is a 1.5% agarose gel of the restriction enzyme digests used to characterise pBSS1-1. 1µg of plasmid DNA was digested with the appropriate enzymes (see key below), before electrophoresis as described in section 2.9. A ladder of DNA fragments (BRL) was used as size markers. A map of pBSS1-1 based on interpreting the restriction enzyme digests performed is shown below the photograph of the agarose gel. The S1-1 cDNA restriction enzyme sites are based on sequence data reported by Munemitsu *et al.* (1986) and Duncan *et al.* (1990).

Key

- Lane M = Ladder
- Lane A = *Hind*III
- Lane B = *Sst* I
- Lane C = *Pst* I
- Lane D = *Eco*RI
- Lane E = *Sph*I



- PL = Polylinker
- = Gene oligonucleotide sequences

3.3.2 Cloning a cDNA Sequence of the S1-3 Gene into Bluescribe

A cDNA sequence of the S1-3 gene was cloned into Bluescribe by M. A. McCrae essentially as described above for the S1-1 gene, to generate clone pBReo3S1. As for the type 1 gene, a unique set of 5' and 3' gene oligonucleotides were also placed at the respective ends of the gene as an aid to further cloning procedures (Figure 3.3.4). The *SphI* site of Bluescribe had been tailed with cytidine residues to recreate the site and complement the guanosine tailed S1-3 cDNA clones; restriction enzyme analysis revealed that the S1-3 gene was full length and oriented with its 5' end adjacent to the T7 promoter. The G/C tails flanking the cDNA insert were undesirable for future *in vitro* syntheses of s1-3 transcripts and so another Bluescribe/S1-3 recombinant was made.

The subcloning strategy used to place the S1-3 gene (minus the G/C tails) in Bluescribe is shown in Figure 3.3.5. Plasmid pBReo3S1 was digested with *NruI* and *SmaI* and the approximately 1.5 Kb fragment containing the S1-3 gene separated on and purified from an agarose gel. This fragment was ligated into the *HincII* site of Bluescribe to create clone pBSS1-3B in which the 5' end of the gene is adjacent to the T3 promoter. In this orientation a *BamHI* fragment of approximately 1.4 Kb is predicted. This fragment should be cut by *BglII* to generate two fragments of approximately 1.1 Kb and 0.3 Kb. The restriction enzyme digest analysis shown in Figure 3.3.6 confirmed this, and allowed the full-length nature and unambiguous orientation of the gene to be determined. This analysis also revealed that contrary to expectation, the *EcoRI* recognition sequence was not present in the 5' gene oligonucleotide sequence (see lanes G and H in Figure 3.3.6).

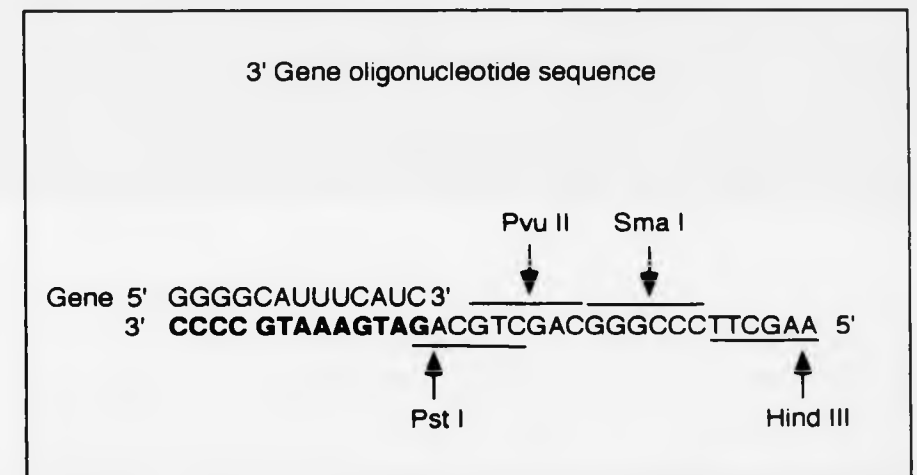
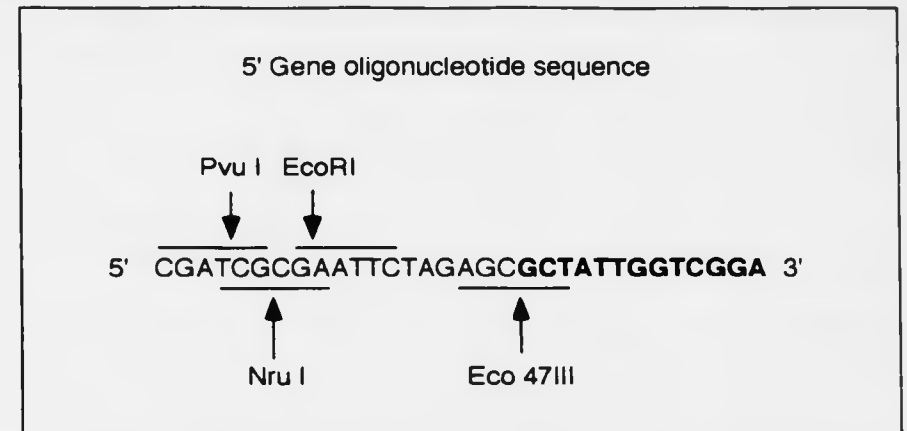


Figure 3.3.4 Sequences of the Reovirus S1-3 Gene Oligonucleotides

The sequence of the 5' and 3' gene oligonucleotides used in the cloning of the reovirus S1-3 gene are shown in this diagram. For clarity, the sequence of the 3' end of the plus strand of the gene is written above the 3' gene oligonucleotide sequence. Restriction enzyme sites are underlined and the bases marked in bold type represent sequence derived from the respective ends of the S1-3 gene.

Figure 3.3.5 Strategy Adopted to Create Plasmid pBSS1-3B

The cDNA sequence for the S1-3 gene was released from plasmid pBReo3S1 by digestion with restriction enzymes *NruI* and *SmaI* which cut in the 5' and 3' gene oligonucleotide sequences respectively. The ~ 1.5 Kb S1-3 "gene fragment" was purified from a 1% agarose gel (section 2.9.1) and ligated into *HincII* digested and phosphatased Bluescribe. The ligation and phosphatase treatment were carried out as described in sections 2.6.5 and 2.6.4 respectively.

Key : This is the same as given in Figure legend 3.3.2

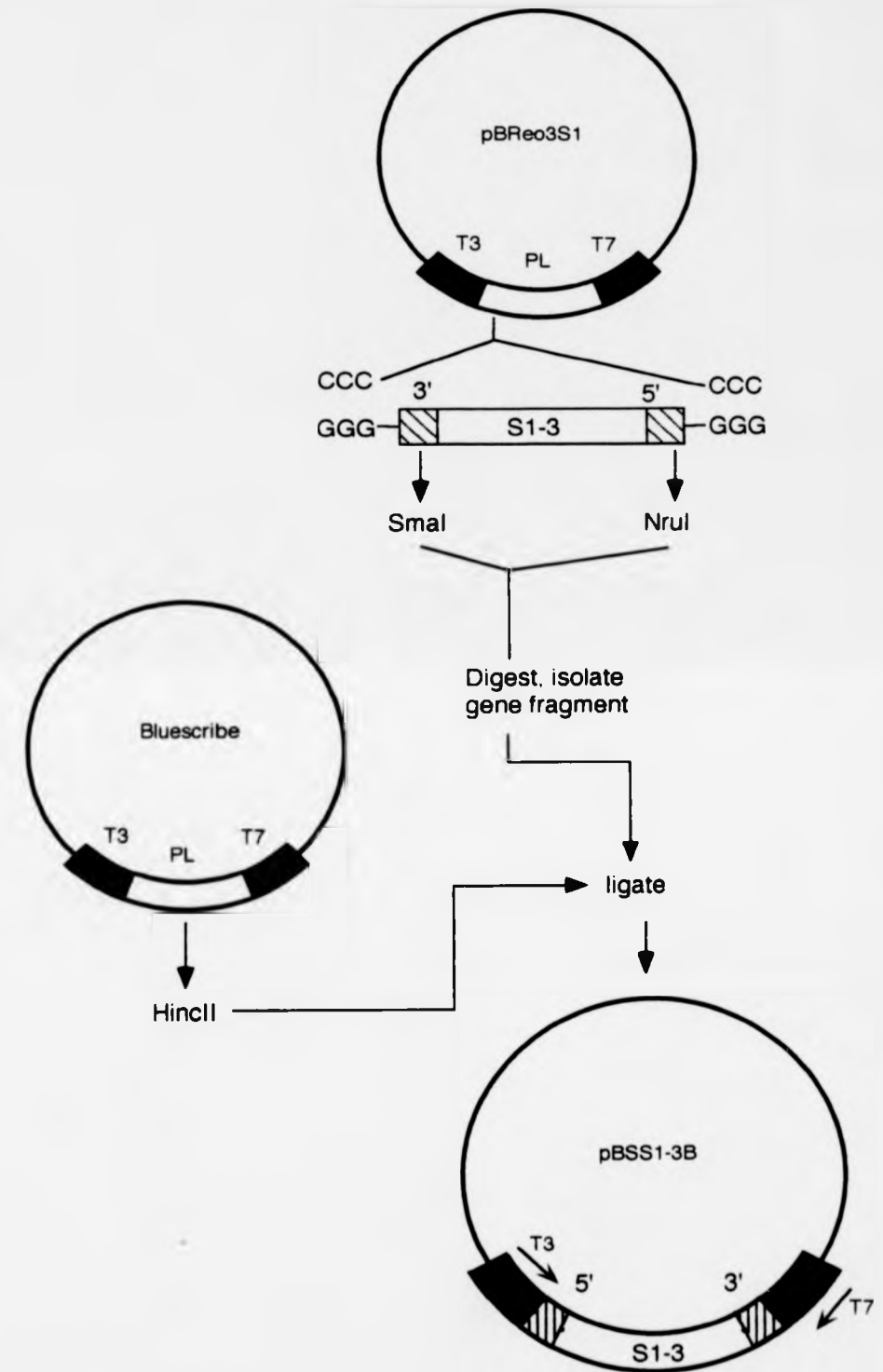


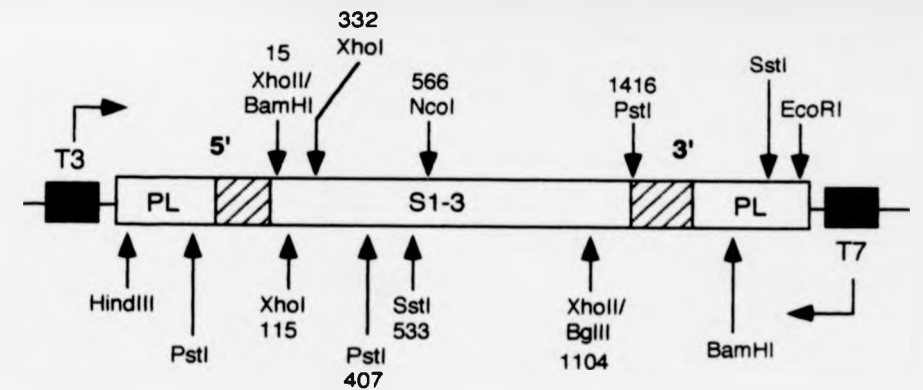
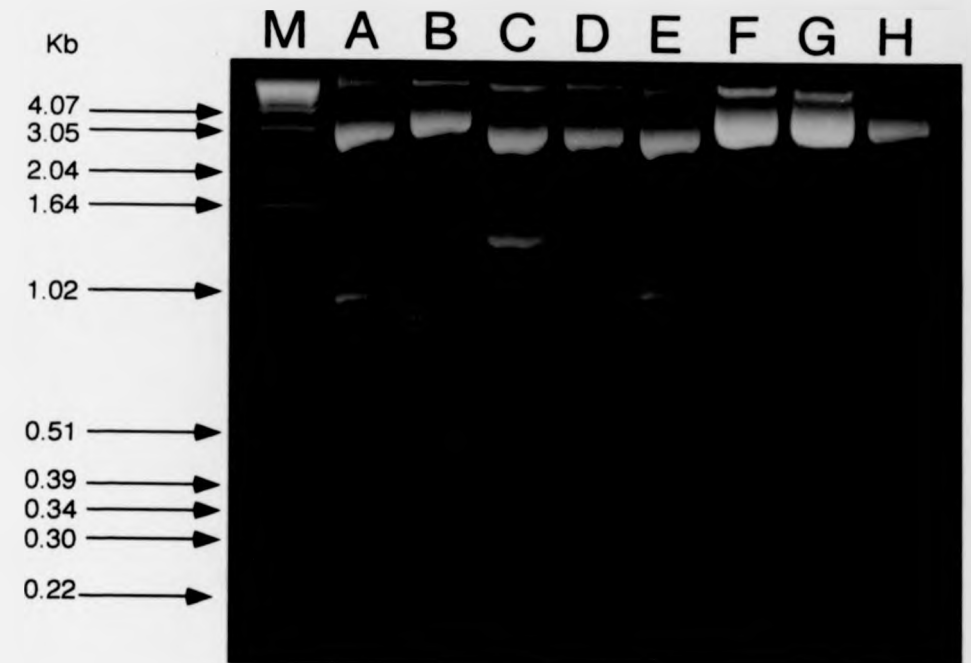
Figure 3.3.6 Restriction Enzyme Digest Analysis of pBSS1-3B

Shown opposite is a 1.5% agarose gel of the restriction enzyme digests used to characterise pBSS1-3B. 1µg of plasmid DNA was digested with the enzymes indicated in the key below before electrophoresis on the gel (section 2.9).

A map of pBSS1-3B based on the interpretation of restriction enzyme digests performed is given below the photograph of the agarose gel. The positions of the restriction enzyme recognition sites within the S1-3 cDNA are derived from analysis of the sequence data of Cashdollar *et al.* (1985).

Key

- Lane M = Ladder
- Lane A = *Pst* I
- Lane B = *Sst* I
- Lane C = *Bam*HI
- Lane D = *Hind*III/*Sst* I
- Lane E = *Bam*HI/*Bg*III
- Lane F = *Xho*I
- Lane G = *Eco*RI
- Lane H = *Eco*RI/*Nco*I



- PL = Polylinker
- = Gene oligonucleotide sequences

3.4 SUMMARY

Both the S1-1 and S1-3 cDNAs were therefore oriented within their respective vectors such that plus sense RNA could be produced from them by using T3 RNA polymerase.

CHAPTER 4

***In Vitro* Transcription of the Reovirus S1-1
and S1-3 cDNA Sequences**

4.1 INTRODUCTION

The cDNA sequences for the reovirus S1-1 and S1-3 genes had been cloned into Bluescribe such that T3 RNA polymerase could be used to produce message sense RNA *in vitro*. A cap analogue was included in the transcription reaction for two main reasons:

1) To give the RNA a 5' terminal structure analogous to that found *in vivo*.

2) The presence of a 5' terminal cap can increase the translational activity of *in vitro* transcribed mRNA in cell-free translation systems (Paterson and Rosenberg, 1979 and Krieg and Melton 1984).

The transcription clones used in this study were linearised at a restriction enzyme site immediately downstream of the 3' end of the gene in the vector polylinker. This site determines the 3' end of the transcript and so each round of RNA synthesis is terminated via a "run-off" step where the polymerase literally falls off the end of the DNA template.

Throughout the study, restriction enzymes producing blunt or 5' overhanging ends were used to linearise templates in order to prevent the possibility of "snapback" (Vector Cloning Systems manual 1985) which can result from DNA with a 3' overhanging end. Snapback creates the potential for the production of spurious transcripts as the 3' overhanging end of the DNA can fold back on itself and allow the RNA polymerase to non-specifically initiate RNA synthesis.

In this chapter the quantitation and integrity of RNA synthesized from clones pBSS1-1 and pBSS1-3B are described.

4.2 RESULTS

4.2.1 Quantitation of the RNA Transcribed *In Vitro*

A trace amount of ^3H -UTP was included in the *in vitro* transcriptions to enable the yield of RNA to be determined from the number of TCA precipitable counts present upon completion of the reaction (section 2.10 and Figure 4.2.1). The chemical amount of ^3H -UTP in the reaction is negligible in comparison to the cold UTP and so is not considered in the calculation.

Typically, approximately 8,000cpm/ μl were obtained from the transcription reactions, which is equal to approximately 20 μg of RNA or an incorporation of approximately 28% of the ^3H -UTP. This level of incorporation is not optimal but was not improved by increasing the amount of linearised DNA template. Addition of a further aliquot of RNA polymerase after the first 60 minutes of reaction time followed by incubation at 37°C for a further hour also had no effect. Hence, neither template or enzyme concentration appeared to be limiting factors. Impurities associated with the DNA can decrease the yield of RNA (Sambrook *et al.*, 1989), but template was always phenol extracted after being linearised for use in subsequent transcription reactions. PEG precipitation of the DNA template can act as a further purification step (Sambrook *et al.*, 1989) but its effect was not assessed in this study. Several commercial transcription buffers plus the buffer recommended in Sambrook *et al.* (1989) were employed during this investigation. These buffers varied slightly with respect to the concentration of some of their components (such as magnesium chloride and sodium chloride); however, none of the buffers were found to markedly affect the polymerase enzyme activity.

Figure 4.2.1 Quantitation of RNA Transcribed from the S1 cDNA Transcription Plasmids

Worked example of a calculation to determine the yield of RNA from a transcription reaction. The number of TCA precipitable counts in 1 μ l of the completed reaction mix was determined as in section 2.10.3 and a typical value (8,384) is used in this example. The calculation is based on that given in section 2.10.3.

No. counts/ μ l reaction = 8,384 cpm

Reaction volume = 50 μ l

Total no. counts = 4.19×10^5

10 μ Ci of ^3H -UTP are included in the reaction. 10 μ Ci = 2.22×10^7 dpm

Specific activity of ^3H -UTP = 52Ci/mmol.

10 μ Ci = 0.19nmol ^3H -UTP

No. moles cold (non-radioactive) UTP in the reaction = 1 μ l of 50mM UTP
= 50 nmol

Thus, the chemical amount of ^3H -UTP (0.19 nmol) is negligible in comparison to the amount of cold UTP (50 nmol)

Therefore, effectively 10 μ Ci of ^3H -UTP = 50 nmol cold UTP

OR 2.22×10^7 dpm = 50 nmol UTP

No. mol UTP incorporated into RNA = $\frac{50 \times (4.19 \times 10^5)}{2.22 \times 10^7}$

Average M.wt. of a ribonucleotide = 360

Amount RNA = $\frac{50 \times (4.19 \times 10^5) \times 360 \times 4}{2.22 \times 10^7}$ ng = 1.36 μ g

Efficiency of counting = 7.3% (determined by counting 1 μ l of a 1:100 dilution of ^3H -UTP as described in section 2.10.3)

Actual yield of RNA = 18.6 μ g

4.2.2 Integrity of the RNA

The integrity of the *in vitro* transcribed RNA was analysed to ensure it was both full length and not degraded. Such an analysis was important as the proteins encoded by the s1 transcripts were to be used in functional assays. Figure 4.2.2 shows the migration of the s1 transcripts when electrophoresed through a 50% formamide/agarose gel. This method allows direct visualisation of the RNA bands by uv illumination.

The 1.5 Kb size of the s1-1 and s1-3 transcripts is as expected from their cDNA sequences (lanes A and B respectively). No degradation of the s1 RNAs was evident (see Figure 4.2.2).

Digestion of the DNA template with DNase and subsequent phenol extraction are often recommended as part of the *in vitro* transcription procedure in protocols in the literature (for example, Promega "Transcription *in vitro*" Technical Manual 1989). These steps were omitted in the protocol used in this study in order to speed the process and so reduce the points at which the RNA can be degraded. Neither of these factors were found to have any effect on the *in vitro* translation of the s1 transcripts.

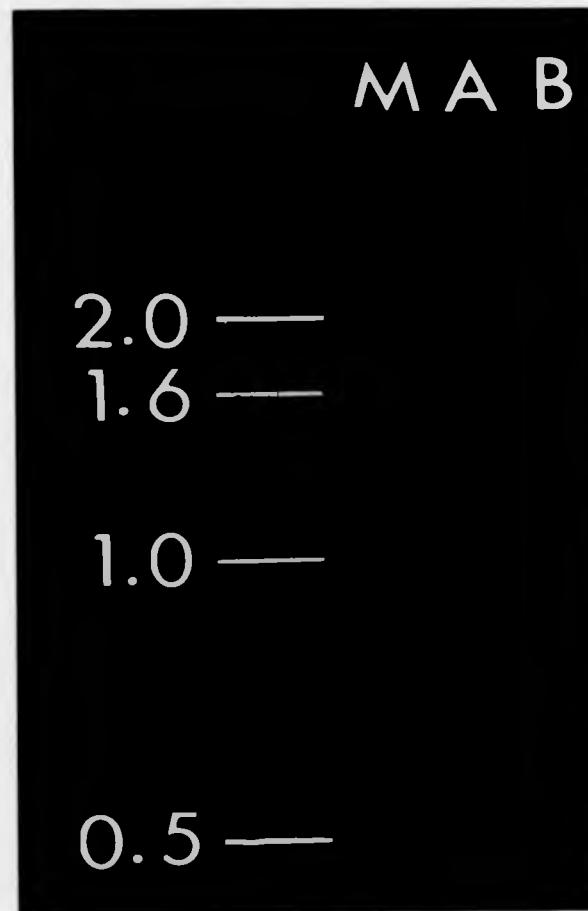
Figure 4.2.2 Agarose Gel Electrophoresis of *In Vitro* Transcribed s1-1 and s1-3 mRNA

Transcription reactions were performed as described in Materials and Methods, section 2.10.2. Approximately 1 μ g of the RNA was then denatured in 60% de-ionised formamide, electrophoresed in a 1.2% agarose gel containing 50% formamide and stained as in section 2.9.2.

Lane M = DNA Ladder

Lane A = s1-1 RNA

Lane B = s1-3 RNA



4.3 SUMMARY

A homogeneous population of intact, full-length s1-1 and s1-3 RNA was consistently synthesized from their respective transcription clones using the phage T3 RNA polymerase.

CHAPTER 5

***In Vitro* Translation of the S1-1 and S1-3 Constructs**

5.1 INTRODUCTION

Pelham and Jackson (1976) produced a highly efficient rabbit reticulocyte lysate *in vitro* translation system that for the first time was dependent upon the addition of exogenous mRNA to provide the major stimulus for protein synthesis. It is now a widely used laboratory technique having the advantage of a very low level of endogenous activity plus the ability to efficiently translate mRNAs into full-length products of up to 200 kDa. Additionally, some cotranslational processing events can be studied by including microsomal membranes in the reaction mix (Walter and Blobel, 1983).

A second system used for the *in vitro* production of protein is the wheat germ lysate, originally devised by Roberts and Paterson (1973). A disadvantage of this system however, was that incomplete products often resulted from the reaction, especially where high molecular weight proteins were involved (Pelham and Jackson, 1976). This problem has since been overcome by the incorporation of spermidine in the reaction mix to stimulate efficient chain elongation (Anderson *et al.*, 1983).

It is therefore largely a matter of choice as to which method is employed. The rabbit reticulocyte lysate was chosen for this study as a source was readily available in the laboratory. Messenger RNA derived from the cloned S1-1 and S1-3 genes was translated and analysed by SDS-PAGE and the lysate optimised with respect to salt and RNA concentration in order to maximize the translational capability of the system.

5.2 RESULTS

5.2.1 Optimisation of the Reticulocyte Lysate System

To achieve maximum stimulation of translation with exogenous mRNAs over the background level of synthesis seen in the absence of added mRNA, the lysate system must be optimised with respect to potassium and magnesium ion concentration and the concentration of the exogenous RNA. The optimum values for each of these variables is dependent upon the particular RNA employed in the translation reaction. The lysate was optimised using *in vitro* transcribed s1-1 mRNA. Reactions were set up as described in section 2.11.1 (with the exception that 20 μ l volumes were used) and the level of protein synthesis measured by TCA precipitation of the radioactive protein products as in section 2.11.2.

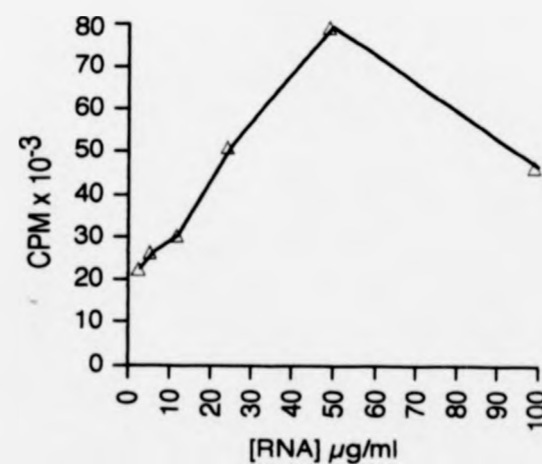
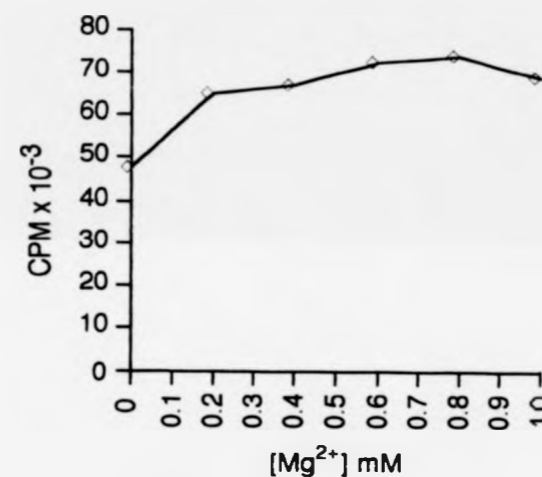
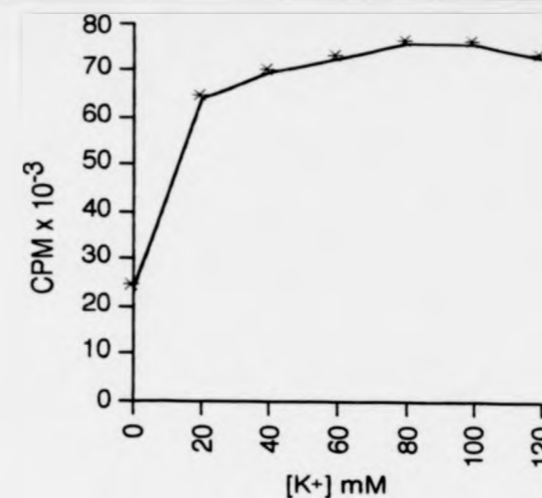
The results of these assays are shown graphically in Figure 5.2.1. The optimum values for potassium (K^+), magnesium (Mg^{2+}) and mRNA concentrations were 80mM, 0.8mM and 50 μ g/ml respectively. These optimum concentrations were used in subsequent experiments and did not vary significantly between the different non-commercial sources of lysate (those from Cambridge University and Sussex University) utilised in this study. The lysate obtained from Promega was already supplemented with potassium and magnesium (to final concentrations of 113mM and 1mM respectively).

Typical lysate stimulation values obtained with the two types of s1 RNA and two control RNA species (tobacco mosaic virus [TMV] RNA and *in vitro* transcribed s4-1 mRNA, the latter supplied by P. Martin, University of Warwick - see also next section) are given in Table 5.2.1. As expected, TMV RNA produced the largest stimulation when added to the lysate, the high value confirming the quality of the lysate. On first inspection, the s4-1

Figure 5.2.1 Optimisation of the Reticulocyte Lysate Translation System

The lysate system was optimised for maximum translation efficiency with respect to potassium and magnesium ion concentration and *in vitro* transcribed RNA concentration; the graphs depicting the results of such titrations are shown opposite. All reactions were performed at 30°C for 1 hour in a final volume of 20µl. 2µl aliquots were TCA precipitated at 0, 30 and 60 minutes and the associated radioactivity counted as described in 2.11.2. The graphs opposite were constructed using the 60 min. data, the time when the highest count (cpm) values were obtained.

A) For the optimum potassium ion determination, reactions were set up as shown in Table 2.4, with the exception that potassium acetate and magnesium acetate were added individually. In all the reactions, the Mg²⁺ and s1-1 RNA concentrations were constant at 0.6mM and 30µg/ml respectively, whilst that of K⁺ was varied between 0-120mM (final concentration) in 20mM increments. B) The Mg²⁺ concentration was titrated between 0-1mM, as described above with a constant K⁺ concentration of 50mM and s1-1 RNA of 30µg/ml. C) The optimum concentration of s1-1 RNA was determined using a twofold dilution series whilst K⁺ and Mg²⁺ concentrations were maintained at 50mM and 0.6mM respectively.



TRANSCRIPT	CPM	STIMULATION
Endogenous	13,840	
s1-1	57,926	4.19
s1-3	38,859	2.8
s4-1	139,027	10.04
TMV	637,897	46.1

Table 5.2.1 Typical Values Obtained when Cloned Reovirus Genes were Translated in a Reticulocyte Lysate

Various *in vitro* transcribed RNAs were translated in the Cambridge lysate and the reactions followed by TCA precipitation as described in sections 2.11.1 and 2.11.2. The values in the table represent the number of counts determined after 60 minutes of reaction time.

control mRNA appeared to be translated more efficiently than both s1-1 and s1-3 mRNA. However, the *in vitro* translations carried out in this study utilized radiolabelled methionine. When the differences in methionine content of these proteins are taken into account, the relative translation efficiencies are almost equivalent, although two major protein products do contribute (approximately equally) to the TCA precipitable count value in the case of σ 1-1. When this is accounted for, polypeptide σ 3-1 (derived from s4-1 mRNA) was synthesized approximately 2.5 times more efficiently than either of the σ 1-1 doublet proteins.

5.2.2 SDS-PAGE Analysis of the S1-1 and S1-3 Translation Products

As the proteins derived from the *in vitro* expression of the two reovirus S1 cDNA clones were to be functionally assayed, it was necessary to determine that full-length proteins could be produced. Accordingly, the two reovirus S1 transcription plasmids, pBSS1-1 and pBSS1-3B, were transcribed and translated *in vitro* and the translation products analysed by SDS-PAGE. A further transcription plasmid containing the cDNA sequence of the reovirus S4-1 gene (pBS4-1) was used as a positive control for the translation reaction. This was utilized because *in vitro* transcribed s4-1 RNA derived from this clone was known to translate well in reticulocyte lysate (P. Martin, personal communication). The protein product (σ 3-1) could also be used as a size marker as it has a known molecular weight of approximately 34 kDa. A map of pBS4-1 is given in appendix C. Tobacco mosaic virus RNA or brome mosaic virus (BMV) RNA were also used as control substrates to check the translational activity of the lysate. These RNAs are known to stimulate reticulocyte

lysate 50-100 times over the endogenous level (Pelham and Jackson, 1976 and Promega "Translation *in vitro*" Manual, 1989).

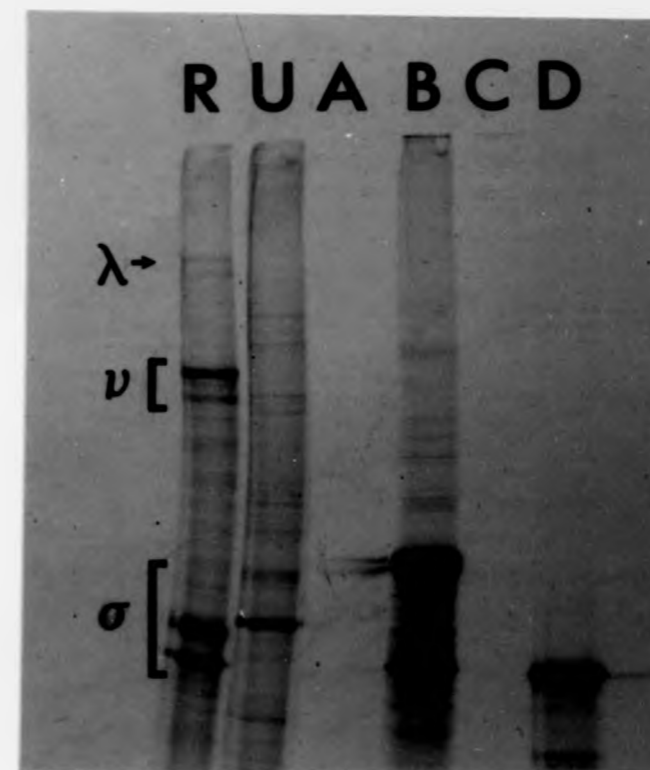
Figure 5.2.2 shows a photograph of these translation reactions after SDS-PAGE and fluorography. (Neither the TMV or BMV profiles are shown). Translation of the s1-1 mRNA produced a doublet of bands, at the size expected for protein $\sigma 1$ type 1 ($\sigma 1-1$) of approximately 51 kDa. The molecular weight of $\sigma 1-1$ is that predicted from the sequence data of Duncan *et al.* (1990) and Nibert *et al.* (1990). It is not always possible to visualise a band representing $\sigma 1-1$ in the reovirus infected cell profile (lane R) as it only represents 1-2% of the total viral protein and is synthesized in small quantities *in vivo* (Smith *et al.*, 1969; Skehel and Joklik, 1969; Joklik, 1981 and Gaillard and Joklik, 1985). In addition to the doublet at the size expected for $\sigma 1$, several lower molecular weight bands are also evident on the gel and probably represent premature termination or internal initiation events. Similar putative incomplete $\sigma 1$ products have also been reported by others carrying out *in vitro* transcription/ translation analyses of the reovirus S1-3 gene (Roner *et al.*, 1989 and Banerjea and Joklik, 1990).

No band of the correct size is present for the $\sigma 1$ -type 3 protein ($\sigma 1-3$); this is discussed in section 5.2.4. Translation of *in vitro* transcribed s4-1 mRNA gave the expected $\sigma 3$ -type 1 protein ($\sigma 3-1$) of approximately 34 kDa which co-migrated with the authentic $\sigma 3$ in the reovirus marker track (lane R).

Figure 5.2.2 SDS-PAGE Analysis of *In Vitro* Translated σ 1 Proteins

Reovirus mRNAs were transcribed *in vitro* and then translated in a rabbit reticulocyte lysate as in section 2.11.1. The plus sense s4-1 RNA was derived from plasmid pBS4-1, a gift from P. Martin (University of Warwick). Protein products were fractionated on a polyacrylamide gel (section 2.11.4) which was processed as described in 2.11.5. A photograph of the resulting autoradiograph is shown opposite. Radiolabelled reovirus T3 infected cells were prepared by the method described in section 2.4.5 and the labelled reovirus polypeptides served as markers; an uninfected cell preparation was electrophoresed for comparison. In this and subsequent appropriate Figures, the positions of the reovirus λ , μ and σ proteins are indicated alongside the "reovirus infected cell" track.

Lane R	Reovirus infected cells
Lane U	Uninfected cells
Lane A	Endogenous lysate reaction
Lane B	<i>In vitro</i> translated s1-1 RNA from pBSS1-1
Lane C	<i>In vitro</i> translated s1-3 RNA from pBSS1-3B
Lane D	<i>In vitro</i> translated s4-1 RNA from pBS4-1



5.2.3 Analysis of the Appearance of $\sigma 1-1$ as a Doublet of Bands

Several possible explanations can be put forward to account for the appearance of a doublet of protein products as opposed to the expected single band; these include :

a) Translational readthrough of the normal stop codon. A doublet of bands could be produced if termination at two sites was occurring. That is, a proportion of the translating ribosomes pass through the first termination codon and terminate on reaching a second downstream stop codon. However, there is a gap of just one triplet of bases between the first and second termination codons in the published $\sigma 1-1$ sequence (Duncan *et al.*, 1990 and Nibert *et al.*, 1990), which would not be sufficient to account for the sizes of the proteins constituting the doublet. Readthrough of two such closely juxtaposed stop codons to terminate at a third site seems unlikely.

b) Initiation at a second initiation codon. Readthrough of the normal $\sigma 1$ start codon by some of the translating ribosomes and subsequent initiation of protein synthesis at a downstream AUG site could also result in a doublet of bands. Inspection of the published sequence for the S1-1 gene revealed that there are no in frame AUG codons downstream of the authentic $\sigma 1$ start codon in close enough proximity to produce the observed doublet of bands. However, there is an upstream AUG codon in frame with the authentic $\sigma 1-1$ initiation codon as a result of the cloning strategy adopted for pBSS1-1. The sequence containing the putative initiation codon is shown in Figure 5.2.3. This codon is derived from the recognition sequence for the restriction enzyme *Sph* I, which constitutes part of the Bluescribe polylinker. Initiation at this upstream site would yield a protein with an N-terminal 14 amino acid extension.

T3 ⁺¹ $\xrightarrow{\hspace{1.5cm}}$ G GGA ACA AAA GCT ⁻³ TGC
ATG ⁺⁴ CCT GCA GGT CGA
TCT CGC GAG CTA GCG
CTA TTC GCG ⁻³ CCT ATG
⁺⁴ GAT GCA TCT CTC

Figure 5.2.3 5' Untranslated Leader Sequence of Clone pBSS1-1

The Bluescribe sequence is shown in plain type and the +1 nucleotide of the T3 promoter is indicated. cDNA derived S1-1 gene sequence is shown in bold type. The authentic σ 1-1 ATG codon is boxed and the potential upstream initiation codon is underlined. The nucleotides in positions -3 and +4 with respect to both of these initiation codons are appropriately marked.

The putative extra 14 amino acids would be sufficient to account for the larger of the two proteins. This number of amino acids would result in a polypeptide approximately 1.5 kDa larger than the expected σ 1-1 protein (~ 51 kDa). Proteins of these sizes (51 kDa and 52.5 kDa) can be resolved in 10% polyacrylamide gels (Sambrook *et al.*, 1989) and would therefore be apparent as distinct entities under the SDS-PAGE conditions used in this study.

The rules proposed by Kozak (1981; 1983; 1984 and 1986) provide a mechanism by which ribosomes can read through upstream initiation codons. In accordance with this model, the putative upstream codon (in the S1-1 cDNA) lies in a poor context for initiation, with pyrimidines in the -3 and +4 positions (U and C respectively). Translational readthrough of this weak initiation sequence, would allow initiation at the authentic σ 1-1 codon in addition to the upstream AUG. The two bands in the doublet are of roughly equal intensity, which could be explained by the fact that the authentic σ 1-1 codon is also in a poor context for initiation, having a C and G present in positions -3 and +4 respectively.

That the upstream codon was responsible for the second band of the doublet was resolved as a result of work shown in Chapter 7 (section 7.2.2).

c) Initiation at an alternative codon, e.g. GUG. Initiation at a codon other than AUG is possible and has been shown to occur in the Sendai virus P/C gene at an ACG site in an excellent context for initiation (Curran and Kolakofsky, 1988 and Gupta and Patwardhan, 1988). However, this phenomenon has mainly been observed in bacteria at GUG sites and occasionally CUG (Ghosh *et al.*, 1967 and reviewed in Kozak, 1983) and was therefore deemed unlikely to have occurred here.

d) Proteolytic cleavage. Proteolysis of the full-length σ 1 protein was another possible cause for the production of two bands. To

investigate this possibility, translation was carried out in the presence of the protease inhibitors aprotinin and soybean trypsin inhibitor. However, no difference was observed in the banding patterns obtained after SDS-PAGE and fluorography (results not shown).

e) **A lysate specific effect.** The doublet may have been due to a lysate specific effect in that a factor or factors could cause premature termination of translation or degradation of RNA. To test this possibility, the s1-1 mRNA was translated in a wheat germ lysate. However, no difference was seen in the resulting translation products compared to those produced in the reticulocyte lysate system (results not shown).

5.2.4 Analysis of the Unsuccessful Translation of σ 1-3

Translation of the s1-3 transcript did not give a full-length protein product of the expected 49 kDa size, a phenomenon which could not be attributed to the integrity of the RNA as it was undegraded and of the correct size. This RNA also stimulated the lysate less than the s1-1 mRNA (Table 5.2.1).

Several experiments were performed in an effort to successfully translate σ 1-3 *in vitro*. Translation in wheat germ lysate was carried out to ascertain if the failure in the reticulocyte system was a lysate specific effect. For this experiment, *in vitro* transcribed preproricin mRNA (a gift from M. Westby, University of Warwick) was translated as a positive control to give a correctly sized protein product. However, priming the wheat germ system with *in vitro* transcribed s1-3 mRNA failed to give a full-length σ 1-3 molecule (results not shown).

The inclusion of protease inhibitors in the reticulocyte lysate reaction had no effect on the banding pattern of the products on

polyacrylamide gels. Hence, degradation of any putative intact σ 1-3 polypeptides was unlikely.

The above two experiments suggested that the apparent inability to synthesize a full-length σ 1-3 protein *in vitro* was not due to an inherent property of the reticulocyte lysate. The alternative was that the s1-3 mRNA itself was somehow defective. As the *in vitro* transcribed s1-3 mRNA was intact, another feature of the molecule may have rendered it non-functional for translation purposes. One such possibility is that a stop codon may have been introduced into the S1-3 cDNA during the cloning procedure. This is discussed further in section 5.3.

5.3 DISCUSSION

The optimum conditions for the *in vitro* translation of RNA derived from the reovirus S1 transcription clones were determined. The RNA concentration required to maximally stimulate the system is relatively high, being greater than the concentration of endogenous globin RNA necessary for saturation of the system (Pelham and Jackson, 1976 and Jackson and Hunt, 1983). This may be due in part to the fact that the s1 message is an inherently poorly translated species as shown by *in vivo* studies (Skehel and Joklik, 1969; Levin and Samuel, 1980; Gaillard and Joklik, 1985 and Atwater *et al.*, 1987). Therefore, large amounts of RNA may be required for *in vitro* translations. However, optimum concentrations as high as 50 μ g/ml have also been reported for encephalomyocarditis (EMC) virus RNA stimulation of lysates (Jackson, 1986). It has been reported that high concentrations of mRNA can have a detrimental effect on the fidelity of translation, reducing the ratio of full-length to incomplete products (whilst simultaneously enabling the greatest level of stimulation) (Dasso and Jackson, 1989). Although the

s1-1 transcript was inefficiently translated, a poor ratio of complete to truncated products was not evident (Figures 5.2.1 and 6.3.2 for example).

SDS-PAGE analysis of the *in vitro* translation products obtained from the s1-1 and s1-3 mRNAs did not give the expected bands. A doublet of bands of the expected molecular weight was present for σ 1-1 but no correct sized band was present for σ 1-3. In both cases this did not appear to be due to either proteolysis or a reticulocyte lysate specific effect. The respective transcripts were also intact and undegraded.

Initiation at a codon upstream of and in addition to the authentic AUG codon for σ 1-1 was thought to be a likely explanation for the doublet of bands observed following translation of s1-1 mRNA. In the *in vitro* transcribed mRNA, there is a candidate upstream initiation codon that is in-frame with the authentic start site and which lies in a poor context of initiation. Thus, potentially, the ribosomes could sometimes scan through this site and initiate downstream at the correct σ 1 codon. Subsequent experiments (Chapter 7) confirmed this explanation as the likely cause of the doublet.

The reason for the absence of a σ 1-3 sized band was less obvious. One possibility relates to an inherent defect in the s1-3 transcript itself. Thus, incorrect copying by reverse transcriptase during cloning may have resulted in the insertion or deletion of one or two bases in the coding sequence. This would result in translation being thrown out of frame leading to premature termination of translation and the absence of a correct sized protein product. A second but less likely possibility was a mutation in the initiation codon to render it non-functional. As the primers used to clone the S1-3 gene (described in Chapter 3) were already available it was decided to re-clone the gene. It was hoped that the re-cloned cDNA would be capable of expressing a full-length protein *in vitro*. This work is described in the next chapter.

5.4 SUMMARY

The reticulocyte lysate system was optimised with respect to magnesium, potassium and s1 RNA concentration to maximise the efficiency of translation. Translation of s1-1 RNA in this system produced a doublet of bands apparently representing protein σ 1-1, whilst translation of s1-3 RNA did not produce a full-length σ 1-3 polypeptide.

CHAPTER 6

Expression of the S1-3 Gene

6.1 INTRODUCTION

The preceding chapter described the *in vitro* translation of s1 mRNA derived from the S1-1 and S1-3 transcription clones. Whilst the former produced a full-length protein, the latter did not, which was thought to be due to an error being introduced into the cDNA during the original cloning procedure.

A second cDNA clone of the S1-3 gene was therefore isolated using PCR and the same 5' and 3' oligonucleotide primers as before (Chapter 3). The reovirus RNA employed as template in the reaction was from a different virus stock to that used in the original cloning. The PCR, subsequent ligation into pAT153 and transformation into *E. coli* was performed by M. A. McCrae.

6.2 RESULTS

6.2.1 Construction of the Re-cloned S1-3 Gene-Bluescribe Recombinant

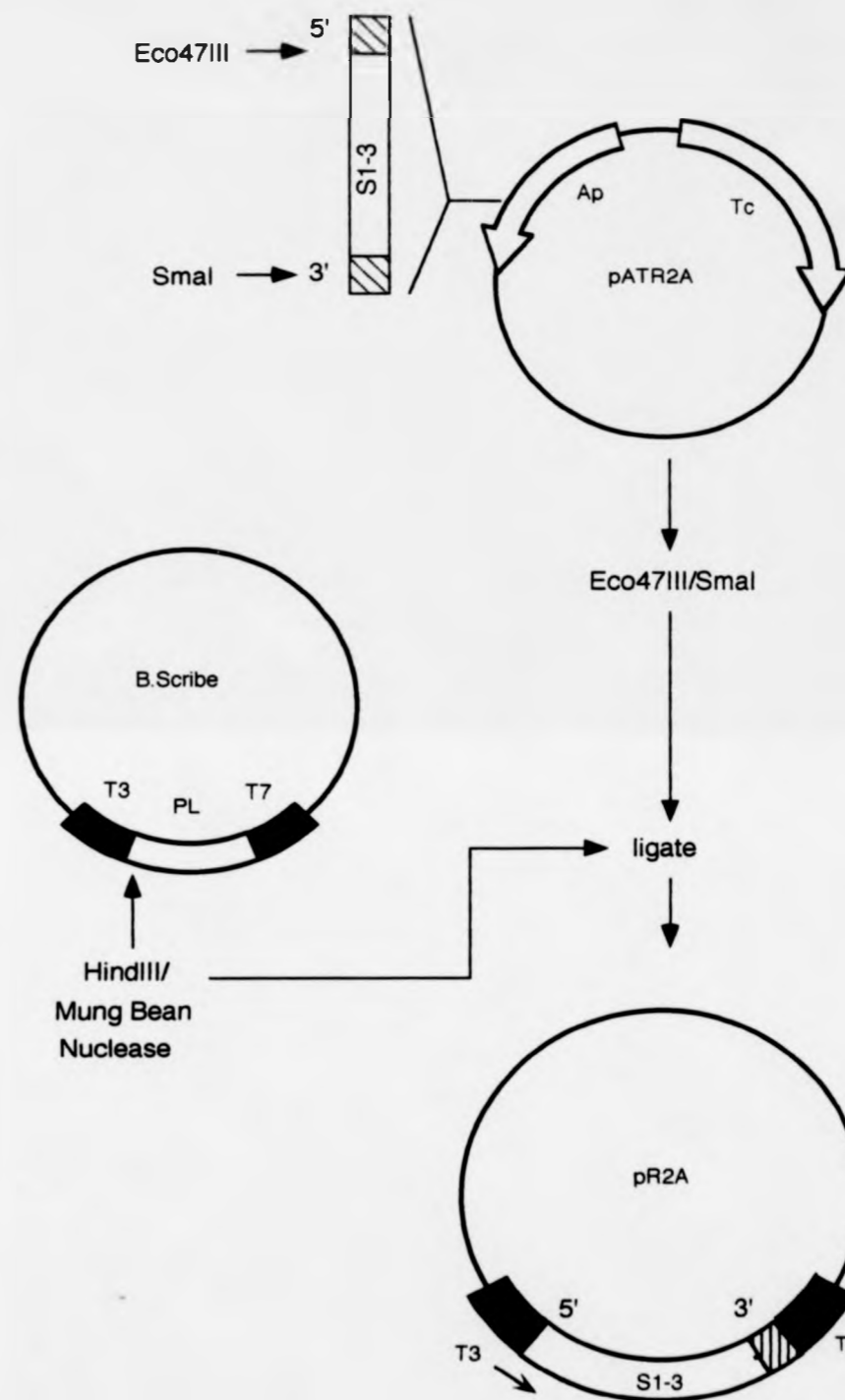
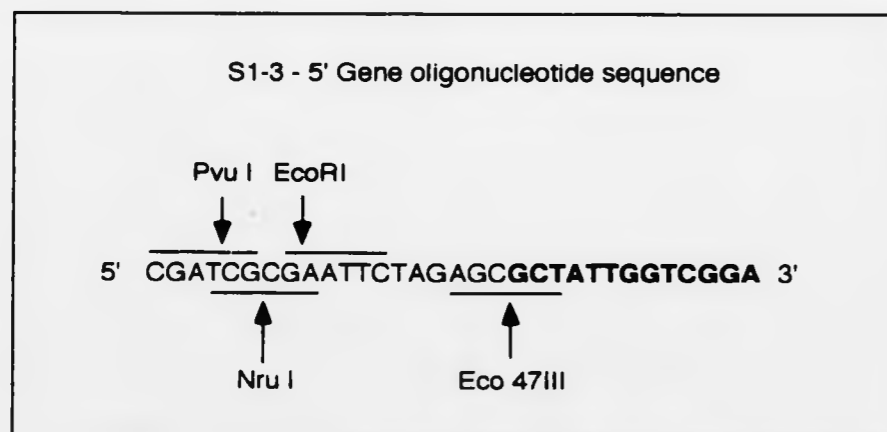
Putative clones containing the re-cloned S1-3 gene in vector pAT153 were screened by restriction enzyme digests in order to select a recombinant plasmid containing a full-length copy of the gene. The S1-3 gene was isolated from an appropriate clone and ligated into Bluescribe to form recombinant pR2A as shown in Figure 6.2.1. The strategy chosen to create pR2A ensured that there were as few nucleotides as possible between the T3 bacteriophage polymerase promoter (which would direct the synthesis of plus sense s1-3 RNA) and the beginning of the S1-3 gene, to minimise the possibility of spurious initiation events in the ensuing *in vitro* expression, as was observed with the S1-1 construct. Consequently,

Figure 6.2.1 Construction of Plasmid pR2A

The reovirus S1-3 gene was cloned for a second time into the vector pAT153 at the *Pst* I site (by M. A. McCrae) to generate pATR2A. Synthetic oligonucleotides containing several restriction enzyme recognition sites were incorporated at the 5' and 3' ends of the cDNA. The 5' end of the cloned S1-3 gene was digested with *Eco*47III to generate an exact 5' end of the gene. The 3' end was then digested with *Sma*I to release the S1-3 gene fragment which was purified from a 1% agarose gel as described in section 2.9.1. Bluescribe vector DNA was digested with *Hind*III, treated with mung bean nuclease (section 2.6.3) to create flush ends and phosphatased by the action of CIP (section 2.6.4). The S1-3 gene fragment was then ligated into Bluescribe.

Key : This is the same as for Figure 3.3.2

Shown below is the sequence of the 5' gene oligonucleotide utilized in the cloning described above. Restriction enzyme sites are marked and the bases shown in bold type denote sequence derived from the 5' end of the S1-3 gene. From this, it can be seen that digestion with *Eco*47III would generate an exact 5' end.



there were no restriction enzyme sites between the T3 promoter and the 5' end of the S1-3 gene.

The integrity of the re-cloned gene was subsequently checked by restriction enzyme digest analysis (Figure 6.2.2). A fragment of approximately 1.4 Kb would be expected after *Bam*HI digestion of pR2A if the gene is full-length and adjacent to the T3 promoter; an *Ss* tI fragment of about 0.9 Kb is similarly predicted. Digestion with the enzyme *Hind*III should leave the DNA uncut as there is no *Hind*III recognition site in the S1-3 gene whilst that in the polylinker sequence should have been removed during the cloning procedure. This is confirmed in the data shown in Figure 6.2.2.

6.2.2 Transcription and Translation of pR2A

Plus sense s1-3 RNA was transcribed *in vitro* from plasmid pR2A and analysed on a formamide-agarose gel as shown in Figure 6.2.3. As can be seen, full-length, undegraded transcripts were synthesized. However, upon *in vitro* translation of the mRNA and SDS-PAGE analysis of the protein products, no band of the size expected for σ 1-3 (~ 49 kDa) was evident. This is shown in Figure 6.2.4 (lane B).

Interestingly, measurement of the radioactivity associated with the TCA precipitable products from the *in vitro* translation reactions (section 2.11.2) revealed that the s1-3 mRNA had stimulated the lysate approximately eightfold more than that observed for the background level from translation of the endogenous globin RNA (see Table 6.2.1 in section 6.2.4). This was equivalent to that routinely observed for the s4-1 message and greater than for s1-1 mRNA, yet no obvious protein band representing this level of translation efficiency was seen on the polyacrylamide gel. A possible explanation is that translation was occurring from the short ORF

Figure 6.2.2 Restriction Enzyme Digest Analysis of Plasmid pR2A

Shown opposite is a 1.5% agarose gel of the restriction enzyme sites used to characterise pR2A. 1µg of plasmid DNA was digested with the appropriate enzymes (see key below) and electrophoresed as described in section 2.9. Undigested pR2A DNA (lane F) was electrophoresed in the lane next to that containing the *Hind*III incubated DNA for comparative purposes as this enzyme was not expected to cut. A ladder of DNA fragments (BRL) was used as size markers.

A map of pR2A based on the interpretation of these restriction enzyme digests is shown below the photograph of the agarose gel. The S1-3 cDNA restriction enzyme sites are based on sequence data reported by Cashdollar *et al.* (1985).

Key:

- Lane M = Ladder
- Lane A = *Sst* I
- Lane B = *Bam*HI
- Lane C = *Bam*HI/*Bgl* II
- Lane D = *Pst* I
- Lane E = *Hind*III
- Lane F = Undigested
- Lane G = *Pvu*II/*Pst* I

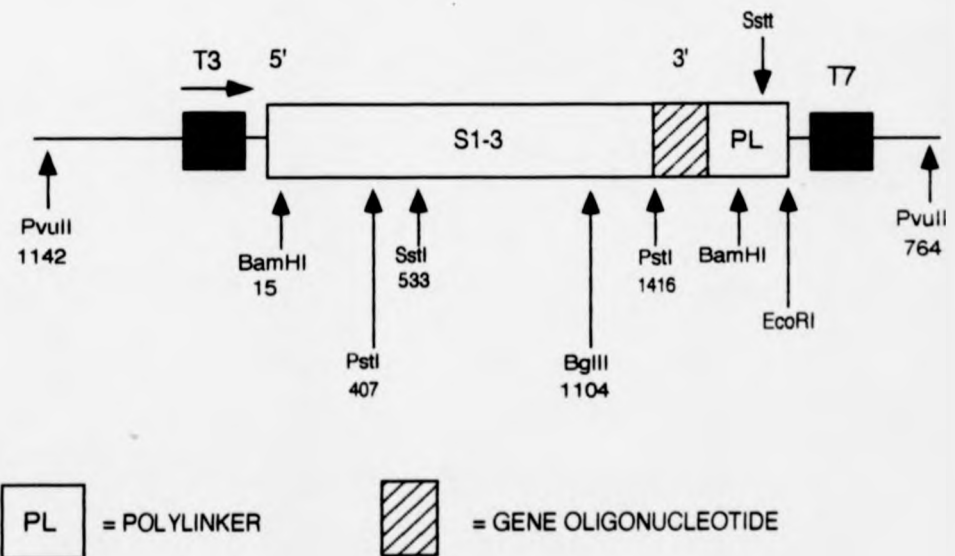
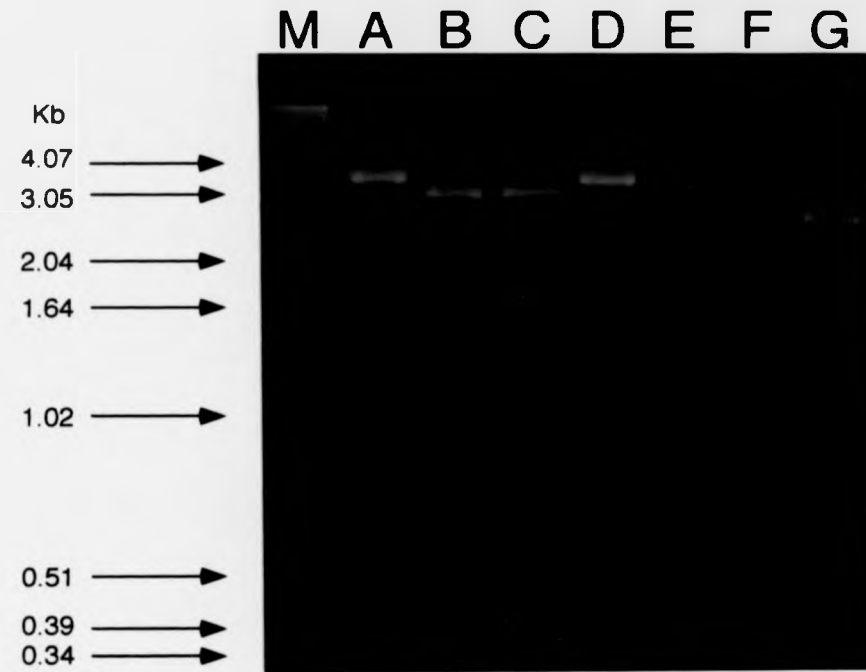


Figure 6.2.3 Agarose Gel Electrophoresis of *In Vitro* Transcribed s1-3 mRNA Derived from Plasmid pR2A

In vitro transcription reactions were performed as described in section 2.10.2 utilizing plasmids pBSS1-1 and pR2A as templates. The integrity of the resulting s1 RNA species was examined by electrophoresis through a 1.2% agarose gel containing 50% DIF as described in section 2.9.2.

- Lane M = Ladder of DNA fragments (BRL)
- Lane A = pBSS1-1 derived RNA
- Lane B = pR2A derived RNA

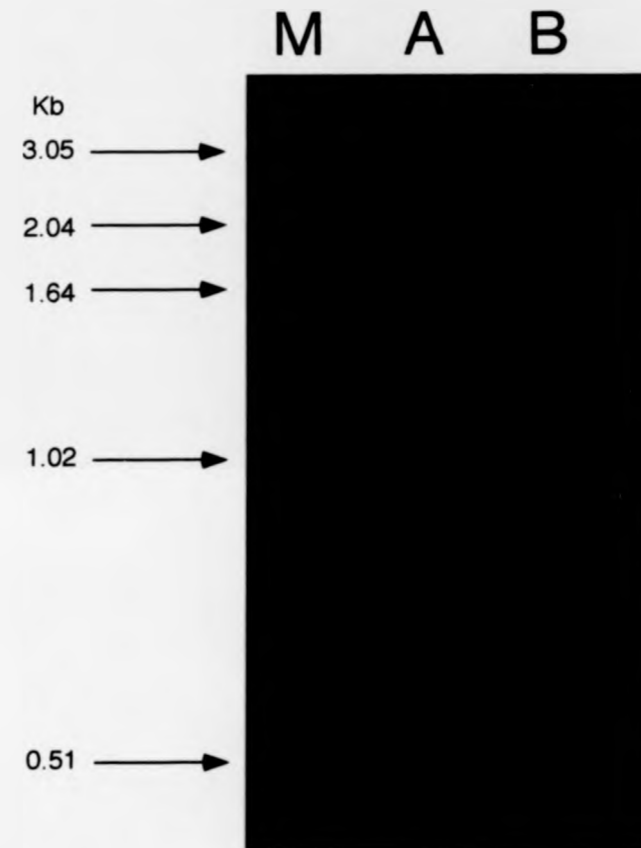


Figure 6.2.4 SDS-PAGE Analysis of the *In Vitro* Translation Products Synthesized from s1-3 mRNA

s1-3 message sense RNA was *in vitro* transcribed from plasmid pR2A and translated in a reticulocyte lysate (section 2.11.1). The protein products were analysed by SDS-PAGE (section 2.11.4) and the gel processed as described in Materials and Methods section 2.11.5. *In vitro* translated σ 1-1 was electrophoresed as a size marker. Radiolabelled reovirus polypeptides (prepared as detailed in section 2.2.5) were also used as size markers.

Lane R = Reovirus infected cell polypeptides

Lane U = Uninfected cell polypeptides

Lane A = *In vitro* translated s1-1 RNA from pBSS1-1

Lane B = *In vitro* translated s1-3 RNA from pR2A



within the S1-3 gene, which is downstream of the start codon for $\sigma 1$ and would lead to the synthesis of $\sigma 1s$ (see section 1.1.3). As $\sigma 1s$ is only about 14 kDa in size, it would not be visualized under the conditions of analysis, migrating with the dye front to the bottom or even off the gel. The $\sigma 1s$ AUG start codon lies in a relatively good context for initiation having an A and a G in the -3 and +4 positions respectively. If the translating ribosomes were to read through the initiation codon for $\sigma 1$ synthesis, then the next AUG they would encounter would be that for initiating $\sigma 1s$. Roner *et al.* (1989) did find an inverse relationship between $\sigma 1$ and $\sigma 1s$ in that if the former was given a strong initiation sequence then its synthesis increased whilst that of the latter decreased; weakening the initiation sequence surrounding the AUG of $\sigma 1$ had the opposite effect.

The reason(s) for the apparent readthrough of the $\sigma 1$ initiation codon observed in this study are unclear. Further discussion concerning the inability of pR2A to yield a $\sigma 1$ -3 protein product is given in sections 6.2.4 and 6.3.

6.2.3 Clone pG3T3

As $\sigma 1$ -3 could not be expressed *in vitro* from either of the two separate cloned copies of the S1-3 gene, it was decided to obtain an S1-3 cDNA clone from another source. Clone pG3T3 was a gift from P. Lee (University of Calgary Health Sciences Centre, Canada) and contains a cDNA of the S1-3 coding sequence downstream of the T7 phage promoter of plasmid pGEM-3Z (Promega). The essential features of this clone are shown in Figure 6.2.5. Advantage had been taken of the fact that there is a *Bam*HI restriction enzyme site immediately after the initiating ATG codon for $\sigma 1$ in the S1-3 cDNA. A DNA copy of the gene was therefore digested at the 5' end with the enzyme *Bam*HI and ligated into pGEM-3Z by means

Figure 6.2.5 Construction of plasmid pG3T3

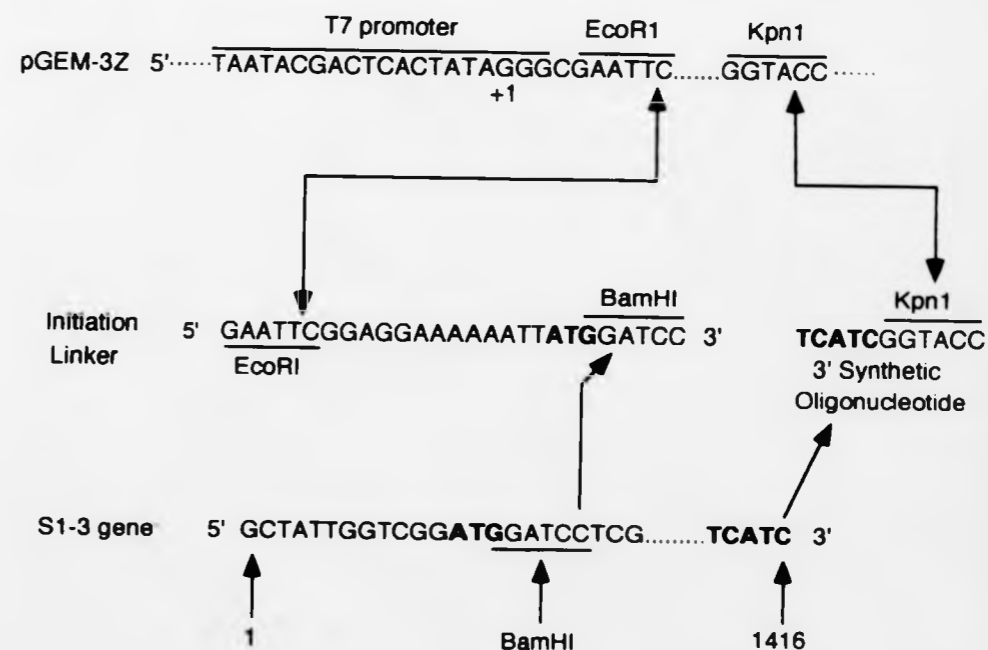
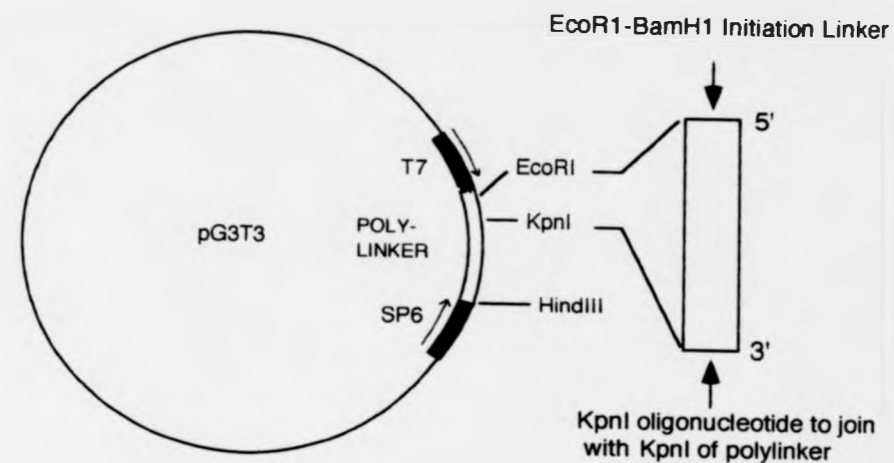
Plasmid pG3T3 was a gift from P. Lee (University of Calgary Health Sciences Centre) and had been constructed as shown opposite. A cDNA clone of the reovirus S1-3 gene was digested at the 5' end with *Bam*HI and ligated to an *Eco*RI - *Bam*HI initiation linker. The 3' end of the gene was joined to a synthetic oligonucleotide containing the recognition sequence for *Kpn*I. This construct was then ligated into *Eco*RI - *Kpn*I digested pGEM-3Z.

The circular plasmid map opposite shows the orientation of the S1-3 gene within pG3T3.

The scheme outlined below the map represents the cloning strategy and shows the relevant restriction enzyme sites and initiation codons of the S1-3 gene and initiation linker.

Key

- SP6 = Promoter sequence for SP6 RNA polymerase
- T7 = Promoter sequence for T7 RNA polymerase



of a *Bam*HI initiation linker. This regenerated the σ 1-3 initiation codon and placed it in a strong consensus sequence. A synthetic oligonucleotide was employed in the ligation of the 3' end of the gene to the vector (P. Lee, personal communication). The main difference between this clone and the two S1-3 clones previously described - namely pBSS1-3B and pR2A - is that the latter two contain the 5' untranslated region of S1-3 whereas pG3T3 does not.

6.2.3.1 Expression of pG3T3

Plus sense s1-3 RNA produced by the *in vitro* transcription of pG3T3 was translated *in vitro* and the proteins thus synthesized size-fractionated by SDS-PAGE. The resulting autoradiograph of the gel is shown in Figure 6.2.6. A full-length σ 1-3 product of the expected size of approximately 49 kDa was evident (lane B) that migrated just below the doublet of bands representing the *in vitro* translated σ 1-1 protein. An approximate fivefold stimulation over the endogenous level of the lysate was achieved with s1-3 mRNA derived from plasmid pG3T3, which is slightly greater than was routinely observed with the type 1 s1 mRNA (see Table 6.2.1, which is shown below Figure 6.2.6).

6.2.4 Expression of a pG3T3/pR2A Hybrid

Protein σ 1-3 could be successfully expressed *in vitro* from plasmid pG3T3 but not pR2A. The most significant difference between the cDNA copies of the S1-3 genes within these two plasmids was that the 5' untranslated leader sequence in pG3T3 had been replaced with an initiation linker. It was therefore decided to substitute the S1-3 coding sequence of pG3T3 with that from pR2A. To this end, each of the plasmids

Figure 6.2.6 *In Vitro* Translation of σ 1-3

RNA was transcribed *in vitro* from the plasmids indicated in the key below and translated in a reticulocyte lysate (as in section 2.11.1). The resulting proteins were subjected to SDS-PAGE (section 2.11.4) and the polyacrylamide gel processed as described in section 2.11.5. A photograph of the resulting autoradiograph is shown opposite.

- Lane M = Radiolabelled protein markers (Amersham)
- Lane A = *In vitro* translated σ 1-1 proteins from pBSS1-1 derived RNA
- Lane B = *In vitro* translated σ 1-3 from pG3T3 derived RNA
- Lane C = *In vitro* translated products from pBSS1-3B derived RNA
- Lane D = *In vitro* translated products from pR2A derived RNA

The molecular weights (in kDa) of some of the radiolabelled markers are marked on the left hand side of the photograph.

Table 6.2.1 Stimulation of a Rabbit Reticulocyte Lysate by Reovirus s1 Transcripts

The incorporation of ^{35}S -methionine into protein products from the *in vitro* translation reactions described above was measured by TCA precipitation and subsequent counting in scintillation fluid as described in section 2.11.2; the results are shown in the table opposite. The value for pBSS1-1 in the final column has been corrected to account for the difference in methionine content between σ 1-1 and the σ 1-3 product. The values for pBSS1-3B and pR2A are not similarly corrected as the identity of the protein products derived from the *in vitro* expression of these plasmids is uncertain.

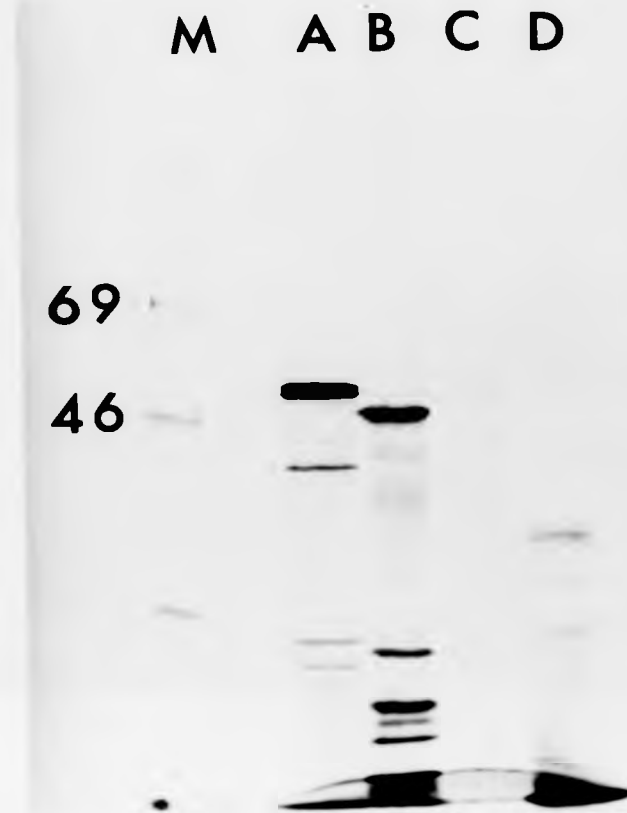


Table 6.2.1 Stimulation of a Rabbit Reticulocyte Lysate by Reovirus s1 Transcripts

PLASMID	TRANSCRIPT	COUNTS	RELATIVE STIMULATION
	Endogenous	15,961	
pBSS1-1	s1-1	70,900	3.23
pG3T3	s1-3	74,972	4.70
pBSS1-3B	s1-3	21,476	1.35
pR2A	s1-3	197,609	12.38

was digested with the restriction enzyme *Bam*HI (which cuts the S1-3 gene immediately after the σ 1 initiation codon) to release both S1-3 gene fragments. The gene fragment from pR2A (minus the untranslated leader sequence) was subsequently ligated into the vector fragment of pG3T3, thereby generating clone pG3S1-3. This strategy is shown schematically in Figure 6.2.7.

Restriction enzyme sequences contained within the 3' gene oligonucleotide sequence of the pR2A derived S1-3 sequence enabled the hybrid clone to be distinguished from pG3T3 when screening bacterial colonies for the putative hybrid. Analysis of twenty potential pG3S1-3 clones by digestion with the restriction enzyme *Pvu*II is shown in Figure 6.2.8. Putative positives (i.e. pG3S1-3 clones) were expected to produce bands of approximately 1.5 Kb and 0.3 Kb in comparison to a 1.8 Kb band from pG3T3 DNA. The former can be seen in lanes 6, 7 and 9 and the latter in lane 1 respectively. Further digests were performed to confirm the integrity of the chosen pG3S1-3 clone (results not shown).

The autoradiograph obtained after SDS-PAGE of the *in vitro* translation products directed by s1-3 mRNA derived from this clone is shown in Figure 6.2.9. A band of the correct size for σ 1-3 can be seen as shown by its co-migration with σ 1-3 protein similarly derived from clone pG3T3 (lanes C and D respectively). This indicated that the coding sequence for the S1-3 gene cloned in our laboratory was functional and that the sequence upstream of the initiation codon appeared to be abrogating the expression of a full-length protein *in vitro*.

Figure 6.2.7 Construction of pG3S1-3

The S1-3 gene from plasmid pR2A was used to replace that of pG3T3. The S1-3 gene within pR2A was released by *Bam*HI digestion and purified from an agarose gel (section 2.9.1). Plasmid pG3T3 was similarly digested (i.e. with *Bam*HI to remove its copy of the S1-3 gene) and the resulting vector fragment purified on an agarose gel. The pR2A S1-3 gene was then ligated into the pG3T3 vector fragment to generate clone pG3S1-3. The *Pvu*II recognition site in the 3' gene oligonucleotide of the pR2A S1-3 gene is shown, as it enabled selection of the appropriate recombinant clones in the screening process.

- Key**
- SP6 = SP6 phage promoter sequence
 - T7 = T7 phage promoter sequence
 - = pGEM-3Z polylinker sequence
 - ▨ = 3' gene oligonucleotide sequence

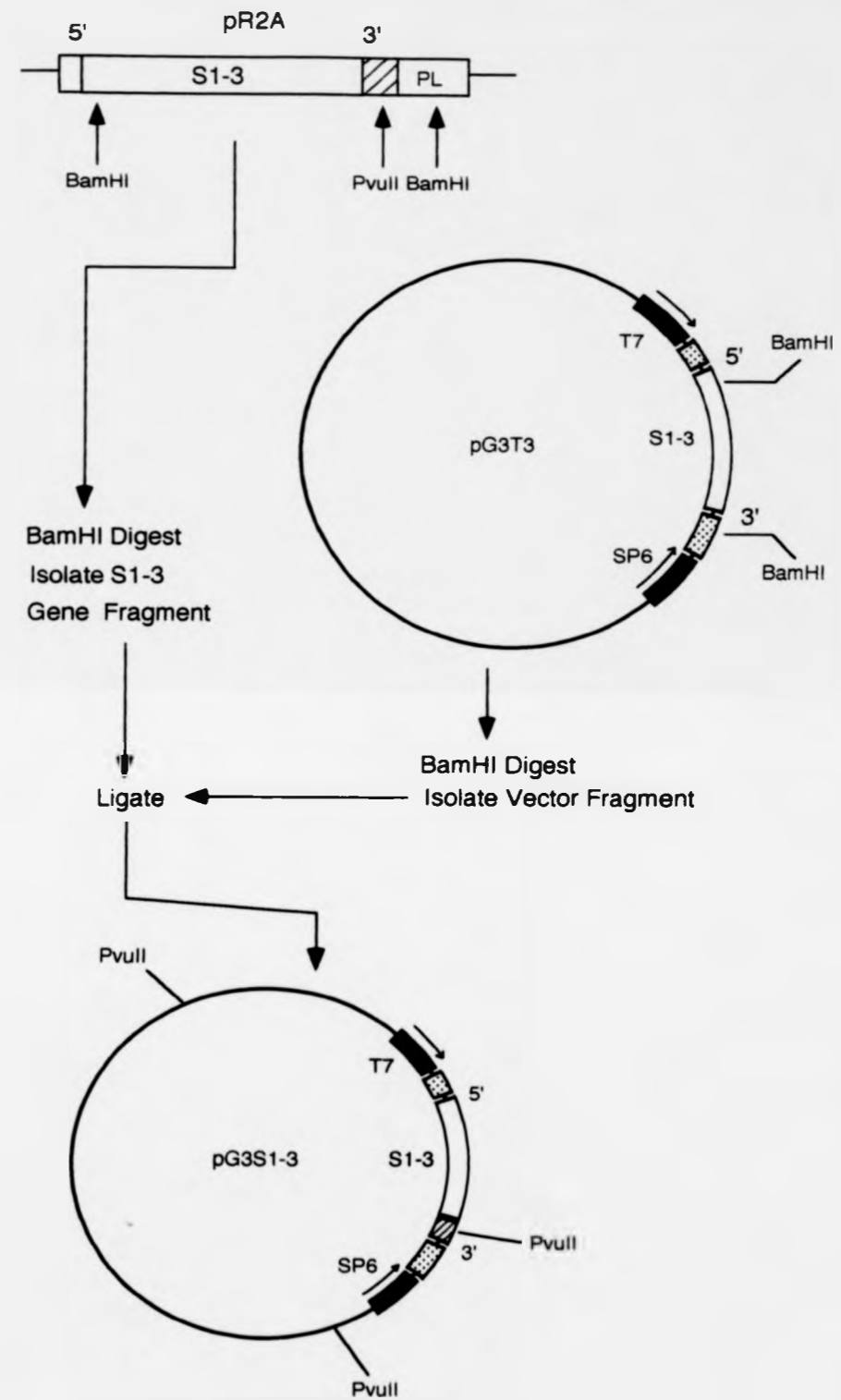


Figure 6.2.8 Selection of pG3S1-3 Recombinant Clones

Twenty putative pG3S1-3 clones were screened by *Pvu*II digestion of plasmid DNA prepared from white bacterial colonies as described in section 2.7.3. Digests were electrophoresed on a 1.2% agarose gel as described in section 2.9 and DNA bands visualised by uv illumination. pG3T3 DNA digested with *Pvu*II was also electrophoresed to illustrate the different banding pattern of pG3T3 and pG3S1-3.

A photograph of the gel is shown opposite, below which is a map of pG3S1-3 with the positions of the *Pvu*II sites marked. The asterisked *Pvu*II site is not present in pG3T3.

Lanes 6, 7 and 9 of the photograph show the correct sized fragments of ~ 1.5 Kb and 0.3 Kb expected for the substituted S1-3 cDNA in the correct orientation.

- Lane M = Ladder
- Lane A = pG3T3 DNA digested with *Pvu*II
- Lanes 1-20 = Mini-plasmid preparations of putative pG3S1-3 clones.

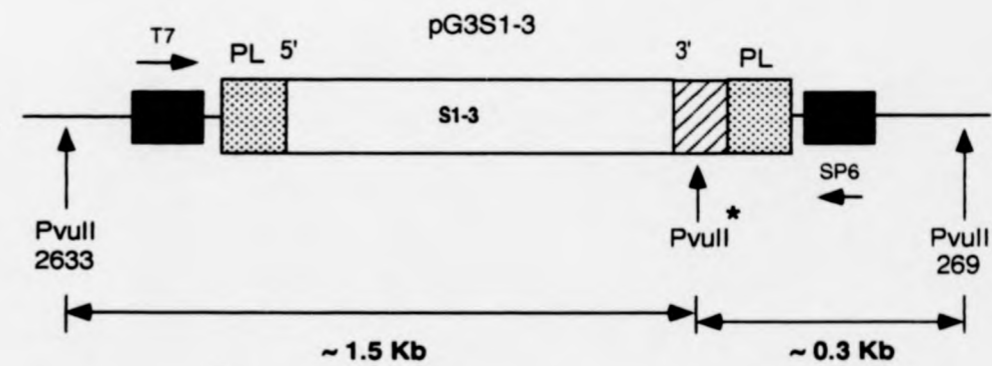
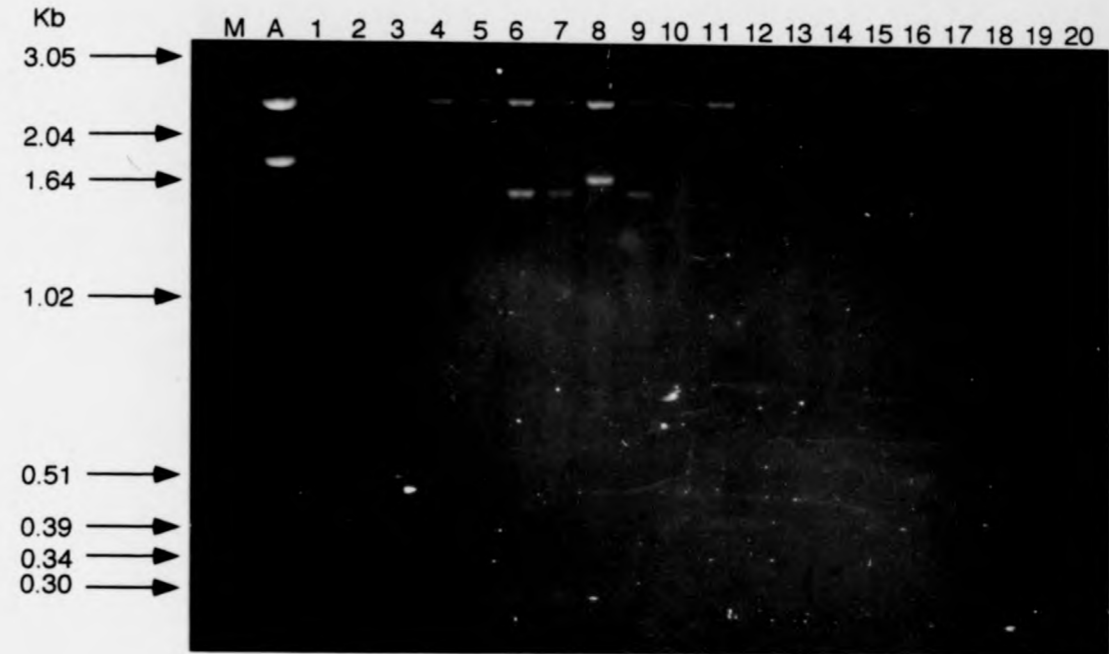
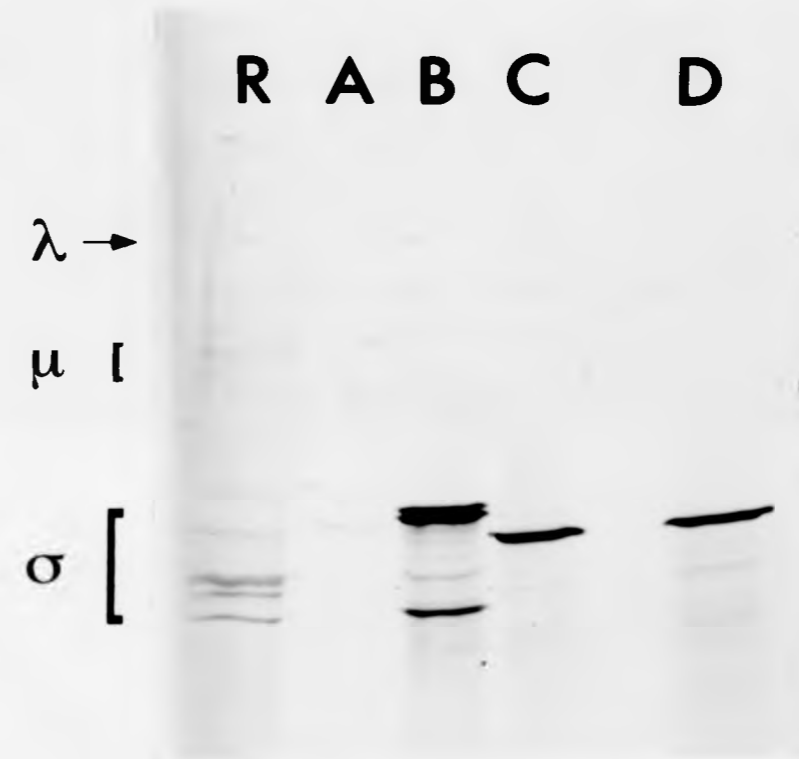


Figure 6.2.9 *In Vitro* Translation of RNA Derived from Plasmid pG3S1-3

Plasmid pG3S1-3 was employed in an *in vitro* transcription reaction to produce s1-3 mRNA. This RNA was used as template in the reticulocyte lysate translation system. Protein products were analysed by SDS-PAGE and the gel processed by fluorography. The result is shown opposite. Lane R shows many host cell polypeptides as well as the reovirus proteins that act as size markers. This was probably due to an insufficiently high moi being used in the preparation of the cells (section 2.2.5) resulting in an incomplete inhibition of host cell protein synthesis.

- Lane R = Reovirus infected cell lysate
- Lane A = Endogenous lysate mix
- Lane B = pBSS1-1 RNA translation mix
- Lane C = pG3T3 RNA translation mix
- Lane D = pG3S1-3 RNA translation mix



6.3 DISCUSSION

The S1-3 gene was cloned for a second time as the original cDNA sequence had not produced a full-length protein when expressed in *in vitro* transcription/translation reactions. However, when the second clone, pR2A, was similarly expressed no σ 1-3 protein was produced despite attaining an eightfold stimulation over that observed with the residual, endogenous RNA of the lysate. This was proposed to be due to translation occurring from the short ORF of the s1-3 mRNA, the initiating AUG of which lies in a fairly strong initiation context sequence approximately 70 nucleotides downstream of the initiation codon for σ 1. The protein encoded by this short ORF, σ 1s, was not visualised on the SDS polyacrylamide gels utilized in the analysis of the *in vitro* translation products as it is only approximately 14 kDa and would have co-migrated with the dye front to the bottom or off the gel. Roner *et al.* (1989) found it necessary to immunoprecipitate this protein from reticulocyte lysate reactions in order to visualise it as a sharp band after SDS-PAGE. The diffuse band otherwise observed was thought to be due to the presence of endogenous globin that is co-synthesized in the reaction mix.

The ribosomes therefore appeared to be scanning through the AUG for σ 1 in the s1-3 mRNA derived from pR2A. Evidence consistent with such "leaky" scanning of *in vitro* transcribed s1-3 mRNA has been obtained by Roner *et al.* (1989) who reported that in general, the strength of the sequence around the σ 1 initiation codon had a direct effect on the translation efficiency of the downstream initiated σ 1s. Thus, a weak Kozak consensus sequence for σ 1 resulted in a poor yield of this protein but a high yield of σ 1s, whereas a better consensus sequence for the former had the opposite effect.

Similar observations have been reported for other virus systems. A mutational analysis of the Sendai virus P/C gene, where the P and C proteins are encoded in different, overlapping reading frames gave results that were consistent with leaky scanning (Curran and Kolakofsky, 1988). More recently, Lin and Lo (1992) reported *in vitro* and *in vivo* data that the hepatitis B virus *pol* gene product resulted from partial readthrough of the C (core) gene AUG. Expression of the HIV-1 *env* gene is also dependent upon a weak consensus sequence for the upstream situated *vpu* AUG (Schwartz *et al.*, 1990; 1992).

Further investigation of the putative readthrough phenomenon that was apparently taking place with the pR2A derived s1-3 mRNA expressed *in vitro* in this study was not pursued as the priority was to achieve synthesis of σ 1-3.

Another S1-3 transcription clone, pG3T3, did yield the desired σ 1-3 protein product upon *in vitro* transcription and translation. Hence, the previous failures to express σ 1-3 from plasmids pBSS1-3B and pR2A was not owing to an s1-3 mRNA-specific inhibitory effect of the lysate on s1-3 mRNA. Equally unlikely was the possibility that an adverse mutation had been incorporated into the coding sequence of two independent cDNA clones of S1-3.

Replacing the coding sequence for S1-3 from pG3T3 with that from pR2A, created plasmid pG3S1-3 that also gave a full-length σ 1-3 *in vitro* expression product. The main difference between clones pG3T3 and pG3S1-3 and pR2A and pBSS1-3B was that only the latter two contain the 5' untranslated leader sequence (i.e. the region upstream of the σ 1-3 initiation codon) of the S1-3 gene (Figure 6.3.1). The former two clones contain an initiation linker sequence in place of the leader sequence. This implied that the 5' leader region present on s1-3 mRNA transcribed from pR2A (and pBSS1-3B) was in some way preventing the synthesis of σ 1-3

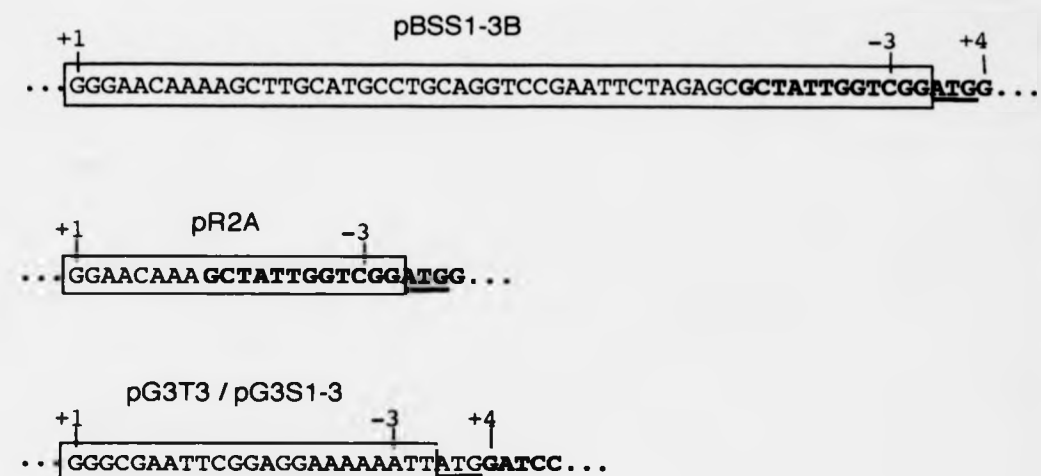


Figure 6.3.1 Comparison of the 5' Untranslated Sequences of the Four S1-3 Transcription Plasmids

The 5' untranslated regions derived from the S1-3 cDNA's in plasmids pBSS1-3B, pR2A and pG3T3 / pG3S1-3 are shown above as boxed residues. The first base transcribed is indicated by a "+1" and the bases in the "-3" and "+4" positions with respect to the σ 1-3 initiation codons in each of the plasmids are marked. Sequence in bold type represents S1-3 gene sequence. In the pG3T3 / pGS1-3 sequence, the ATG initiation codon is not shown in bold type as it is derived from an initiation linker utilized in the cloning procedure.

in the *in vitro* translation reactions. Clone pR2A had been created in such a manner that the polylinker sequence of the vector contributed as few nucleotides as possible to this untranslated region. Native σ 1-3 mRNA produced by the virus would not have a very different sequence upstream of the initiation codon for σ 1 compared to the mRNA transcribed *in vitro* from plasmid pR2A. Since the type 3 virus appeared to replicate as expected in tissue culture and was therefore presumably synthesizing σ 1-3, the effect was apparently specific to the *in vitro* situation.

The precise reason for the inability to express σ 1-3 from the plasmid pR2A is unclear. The 5' untranslated region is only short, so the ribosomes do not have to scan very far. One other possibility is that it was an inherent feature of the 5' untranslated sequence that was responsible for the putative inhibition of translation. A particularly stable secondary structure could have an inhibitory effect but a previous analysis of this region in viral σ 1-3 mRNA, did not predict such a feature (Antczack *et al.*, 1982). Although the σ 1-3 AUG codon has a fairly weak initiation sequence - CGGAUGG - compared to the Kozak consensus sequence of -ACCAUGG - (Kozak, 1981; 1983 and 1986), it is not the poorest and so some synthesis of σ 1 would be expected. Other individual bases or sequences lying upstream (or even downstream) of the AUG may have been effecting the apparent inhibition. Investigation of this possibility would have required a time consuming mutagenesis approach and was not within the scope of my objectives.

6.4 SUMMARY

The re-cloned S1-3 gene did not produce a full-length σ 1-3 protein when expressed *in vitro* from clone pR2A. However, σ 1-3 was expressed from an alternative S1-3 recombinant clone, pG3T3, provided by another

laboratory. Substituting the coding frame of the re-cloned S1-3 gene for that within pG3T3, revealed that the former was functional. The precise reason(s) for the non-expression of σ 1-3 from pR2A is unclear, but may have involved translation-inhibitory effects mediated by the 5' untranslated region of the S1-3 cDNA.

CHAPTER 7

Increasing the Translational Efficiency of the s1 Messenger RNAs

7.1 INTRODUCTION

The primary aim of translating $\sigma 1$ in the reticulocyte lysate system was to enable an investigation of the cell binding properties of the protein. Small quantities of protein are produced by this method, a yield of 1-10ng being typically obtained from a 30 μ l reaction volume (Ausubel *et al.*, 1990). The s1 type 1 and type 3 *in vitro* synthesized mRNAs only stimulated the lysate poorly (see sections 5.2 and 6.2.3.1) and consequently yields were probably less than the average value quoted above. It was therefore considered desirable to increase the amount of protein synthesized in this system. The lysate reaction conditions had already been optimised (Chapter 5) and so two further methods for increasing the amount of $\sigma 1$ produced in the translation system were decided upon, one "genetic" and the other biochemical.

The genetic approach involved oligonucleotide directed mutagenesis of plasmid pBSS1-1 to effect both a point mutation and a deletion. The point mutation was aimed at improving the poor context of initiation of the $\sigma 1$ ATG in the cloned S1-1 gene by altering the -3 nucleotide from a "weak" C to a "strong" A. The deletion would remove all the intervening nucleotides between the T3 phage promoter of pBSS1-1 and the first base of the S1-1 gene (Figure 7.1.1). This would enable the production of transcripts with 5' untranslated (leader) sequences identical (apart from the -3 nucleotide) to that of s1 mRNA synthesized *in vivo*. Such a deletion would also address the question of whether the putative upstream initiation codon present in the leader region was in fact functional and initiating the synthesis of one of the bands of the $\sigma 1$ doublet (see section 5.2.1).


The biochemical method employed to improve translation efficiency was to include 2-aminopurine (2AP), an inhibitor of ds RNA activated

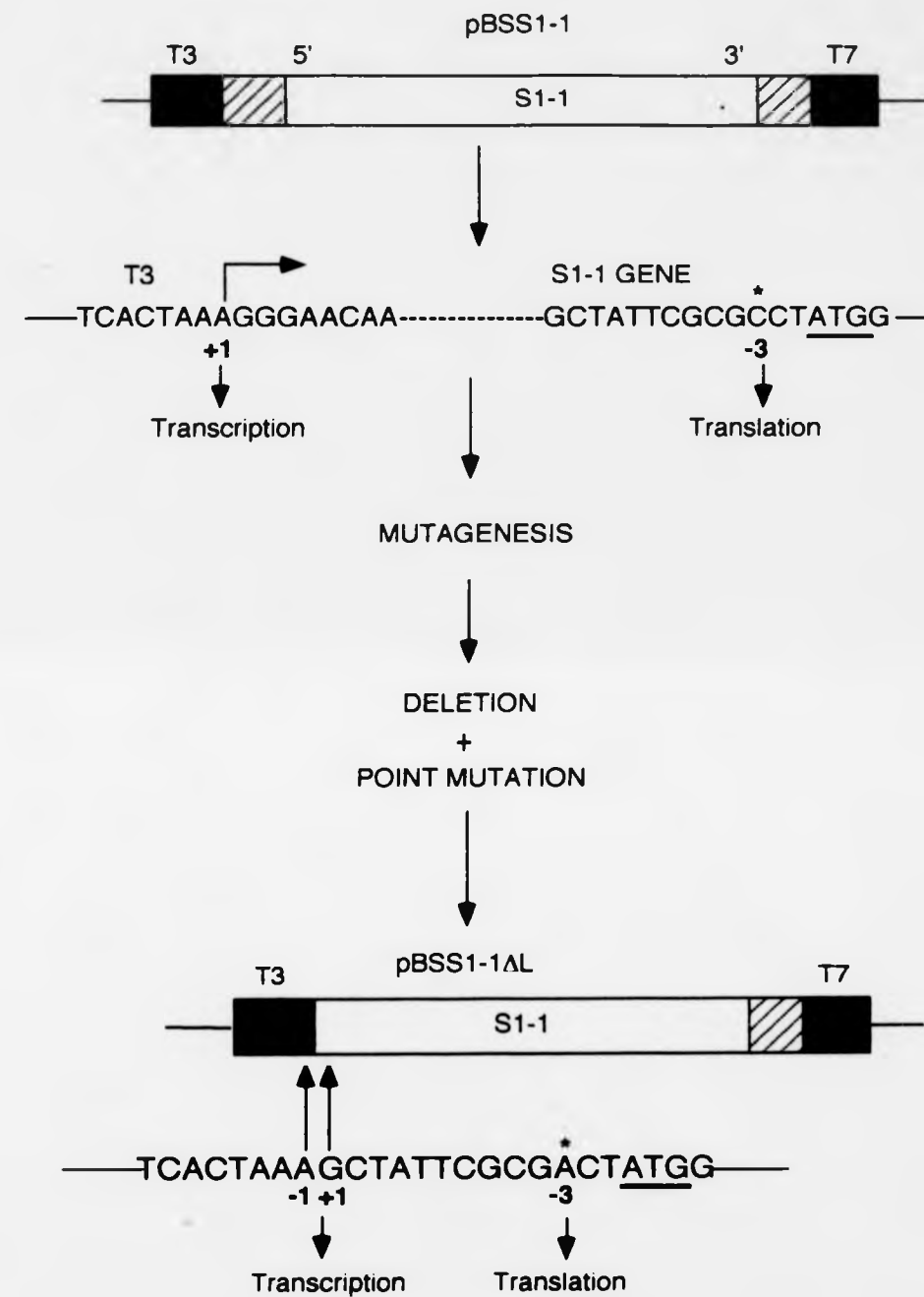
Figure 7.1.1 Schematic Representation of the Mutagenesis of Plasmid pBSS1-1

The diagram opposite shows the point mutation of the nucleotide in position -3 with respect to the $\sigma 1$ initiation codon and the region of DNA between the T3 promoter and first base of the cloned S1-1 gene to be deleted. The $\sigma 1$ initiation codon is underlined and the -3 nucleotide marked with an asterisk. +1 and -1 refer to the first base transcribed from the T3 promoter and the nucleotide immediately preceding it respectively. The bent arrow at the top of the diagram represents the direction of transcription from the T3 promoter.

T3 = T3 phage promoter sequence

T7 = T7 phage promoter sequence

 = Polylinker + gene oligonucleotide sequence



protein kinase P1, in the translation reaction mix. A brief summary of P1 function will be given to more clearly define the reason for the inclusion of this inhibitor.

One of the factors involved in the initiation of translation is eukaryotic initiation factor-2 (eIF-2), a multi-subunit protein that binds methionyl-tRNA_i and GTP to form a ternary complex, this then binds 40S ribosomal subunits (Safer, 1983 and Ochoa, 1983). The activity of eIF-2 is down-regulated by phosphorylation of its α subunit, a reaction catalysed by an RNA dependent kinase known as the P1/eIF-2 α kinase (Samuel, 1979; Berry *et al.*, 1985 and Pestka *et al.*, 1987). This kinase is induced physiologically by interferon (Samuel, 1979 and Pestka *et al.*, 1987) and has two distinct activities: first it activates protein P1 by autophosphorylation (Berry *et al.*, 1985 and Galabru and Hovanessian, 1987) and second, it phosphorylates eIF-2 α . Protein kinase P1/eIF-2 α can be activated *in vivo* by virus infection, for example with reovirus, or by ds RNA treatment of cell cultures (Gupta, 1979; Samuel *et al.*, 1984 and Rice *et al.*, 1985). Activation *in vitro* is achieved with either synthetic or natural ds RNA (Baglioni, 1979; Samuel, 1979; Lasky *et al.*, 1982 and Samuel *et al.*, 1984). However, reovirus ss RNA, as a mixture of all ten transcripts synthesized by the core associated polymerase can similarly activate the kinase (Samuel, 1979). These interactions are shown in Figure 7.1.2.

The adenosine analogue 2-aminopurine is a known inhibitor of P1/eIF-2 α kinase activity (Legon *et al.*, 1974 and De Benedetti and Baglioni, 1983). It was therefore decided to include 2AP in the *in vitro* translations to alleviate possible inhibitory effects induced by s1 mRNA activation of the kinase.

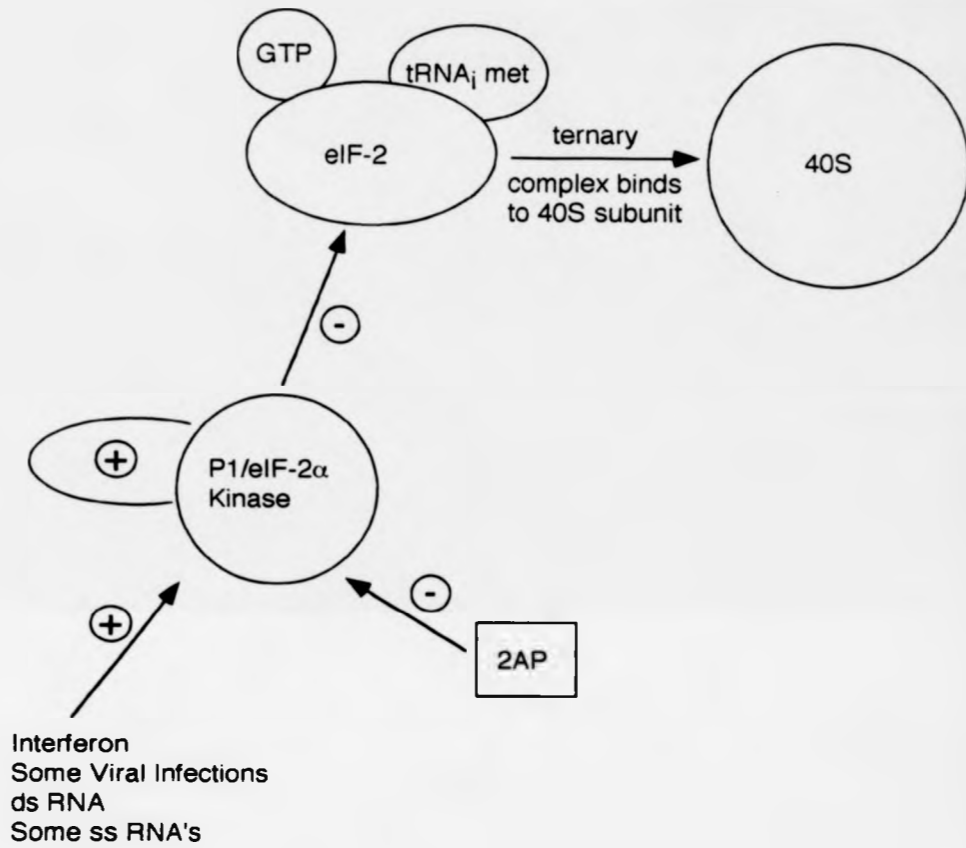


Figure 7.1.2 Interactions of Protein Kinase P1/eIF-2 α

The above figure summarises the effects of various agents upon protein kinase P1/eIF-2 α . An encircled + indicates a stimulatory effect and an encircled - indicates an inhibitory effect. See text for details.

7.2 RESULTS

7.2.1 Mutagenesis of pBSS1-1

The strategy adopted for the oligonucleotide directed mutagenesis of pBSS1-1 to both place the S1-1 gene exactly adjacent to the T3 promoter and change the $\sigma 1$ -3 initiation nucleotide from a C to an A is shown in Figure 7.2.1. Thus, a fragment encompassing the T3 promoter and the S1-1 gene was subcloned from pBSS1-1 into M13mp19 to create clone mp19S1-1. The sequence of the oligonucleotide used in the pBSS1-1 mutagenesis is also shown in Figure 7.2.1. One oligonucleotide was used to effect both mutational changes.

The method of Vandeyar *et al.* (1988) was used to create the mutation as it results in a high proportion of mutant clones (~ 90%) unlike the original method for oligonucleotide directed mutagenesis of Zoller and Smith (1983). An efficiency of 80% was achieved in this particular instance (data not shown). This method of mutagenesis was also used at a later stage in this study and is described in more detail in Chapter 10.

Putative mutant clones were cultured and replicative form (RF) DNA prepared as described in section 2.8.2. Mutant clones were initially identified by digestion of RF DNA with the appropriate restriction enzyme (*HindIII*) as shown schematically in Figure 7.2.2. Mutated and non-mutated DNA produced different banding patterns upon agarose gel electrophoresis of the digested DNA as a consequence of the deletion in the former. A mutant was selected and a fragment of DNA encompassing the mutated region was subcloned back into pBSS1-1 (from which the equivalent piece of DNA had been excised), to create pBSS1-1 Δ L. Sequencing was then performed to confirm that both the deletion and the point mutation had been generated and that no other mutations were

Figure 7.2.1a Strategy for the Mutagenesis of pBSS1-1

Plasmid pBSS1-1 was digested with the restriction enzymes *PvuII* and *BamHI* and the ~ 1.8 Kb fragment encompassing the S1-1 gene and T3 promoter sequence was purified from a 1% agarose gel (section 2.9.1). This fragment was ligated into M13mp19 vector DNA that had been previously digested with *HincII* and *BamHI*, to generate clone mp19S1-1.

Mutagenesis was carried out as described in section 2.8.5 using oligonucleotide S1del, whose sequence is shown overleaf (Figure 7.2.1b). DNA from half of the resulting reaction mix was then transformed into *E. coli* Mcr A⁻B⁻ cells (section 2.8.6). RF DNA was prepared (as described in section 2.8.2) from an appropriate mutant and digested with *HindIII*. The ends of the DNA were then made flush by Klenow filling (section 2.6.2) before further digestion with *XhoI* to release a small fragment containing the mutated sequence. Plasmid pBSS1-1 was partially digested with *PvuII*, exactly as described in section 2.6.1.1 followed by digestion with *XhoI* to release a small fragment (encompassing the T3 promoter and ~ 250 nucleotides of the 5' end of the S1-1 gene), and a large, vector fragment; the latter was purified from an agarose gel (section 2.9.1). The small mutated fragment was then ligated into the large pBSS1-1 vector fragment to create pBSS1-1ΔL.

Key

T3 = T3 phage promoter sequence

T7 = T7 phage promoter sequence

▨ = Polylinker sequence of M13 and/or pBSS1-1

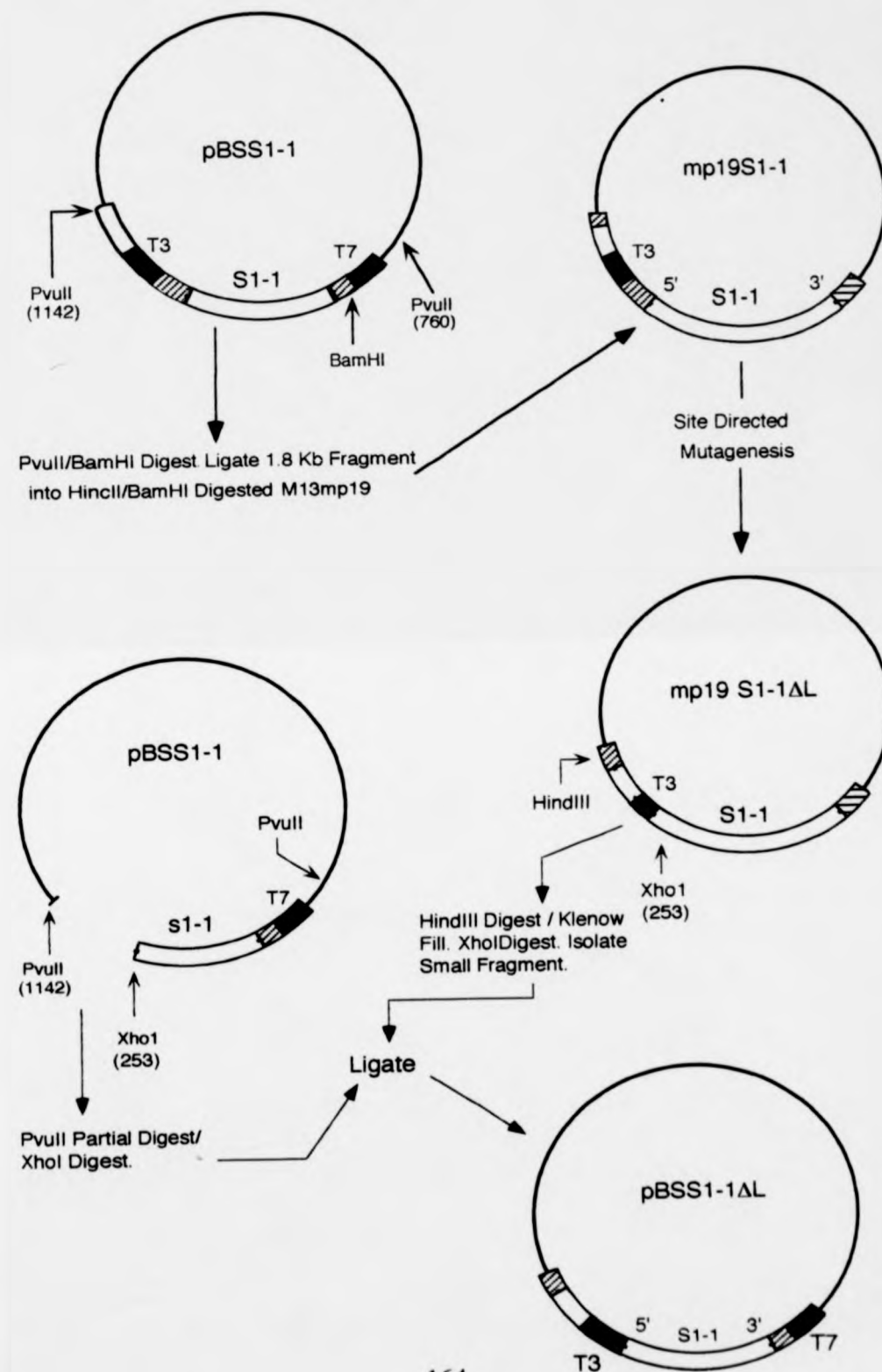
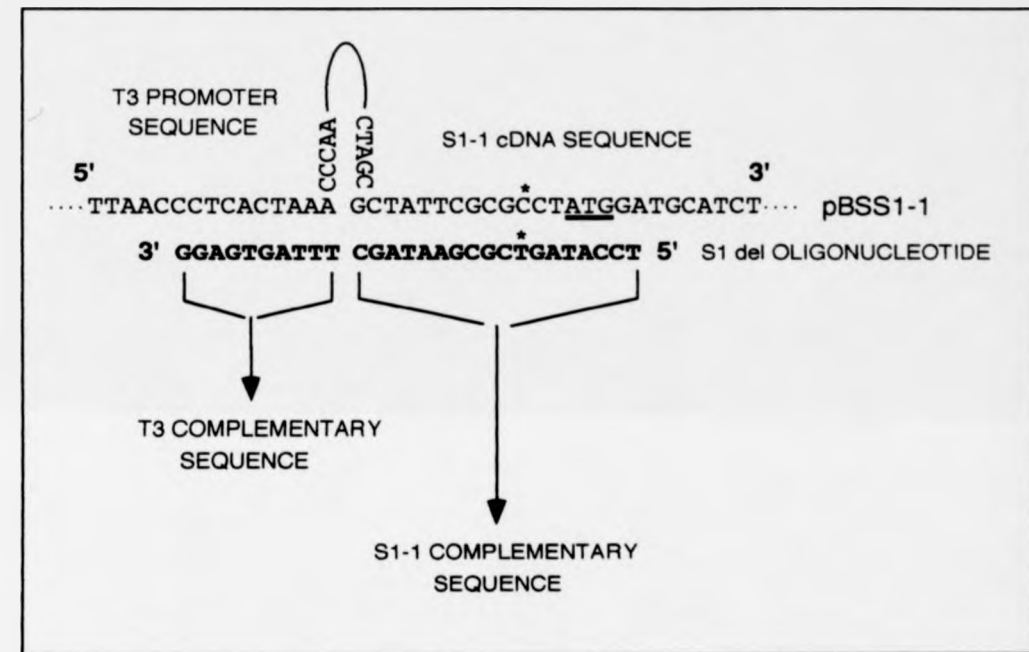


Figure 7.2.1b Sequence of Oligonucleotide S1del



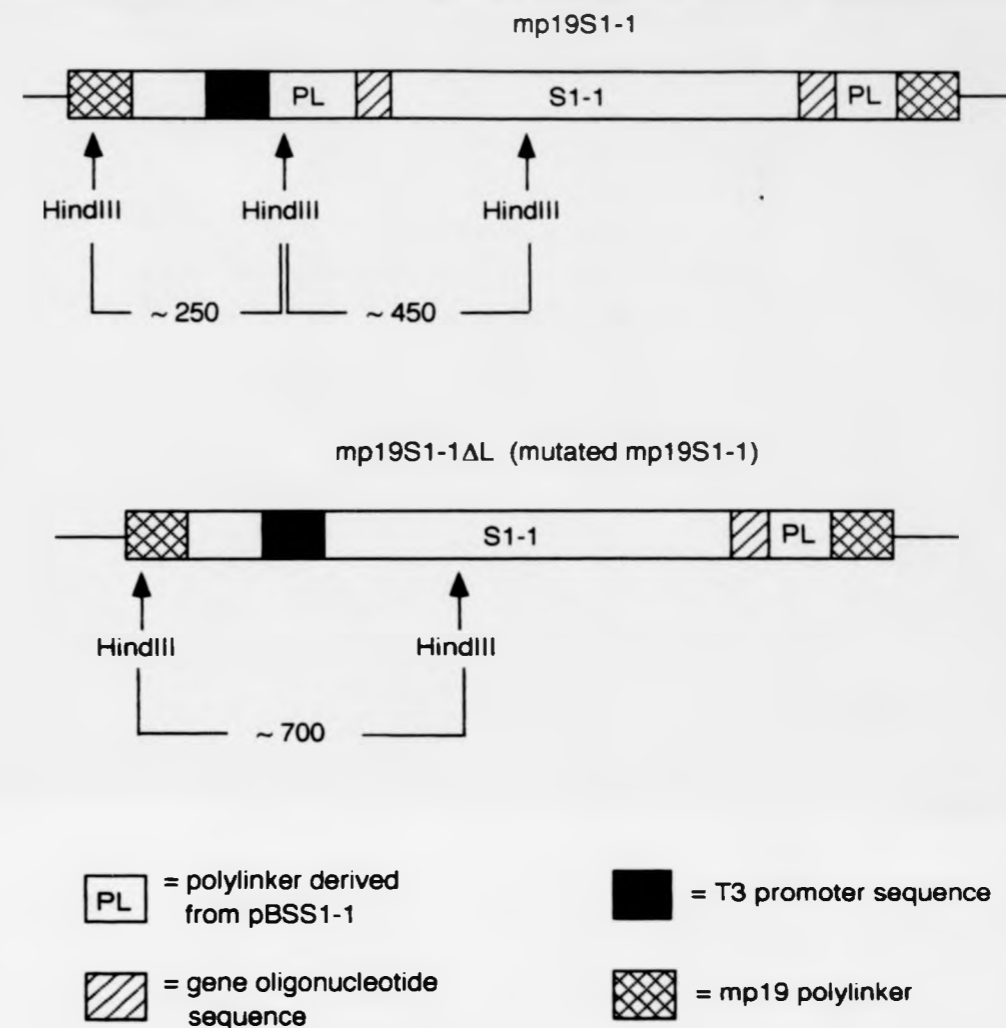


Figure 7.2.2 Schematic Representation of the Selection of mp19S1-1 Mutants

The maps above show the number and positions of the *HindIII* recognition sites present in mp19S1-1 before and after the mutagenesis described in the text. The sizes of the fragments that enabled differentiation of the mutated and non-mutated clones after digestion with *HindIII* are indicated by the numbers between the restriction enzyme recognition sites.

present in this fragment. Figure 7.2.3 shows that in the selected clone the S1-1 gene is positioned immediately adjacent to the T3 promoter such that the first base transcribed is the first base of the S1-1 gene and that the -3 initiation nucleotide has been mutated to an A.

7.2.2 Effect of the Mutation on the Synthesis of σ 1-1

Plasmids pBSS1-1 Δ L and pBSS1-1 were transcribed and translated *in vitro* and equal volumes of reaction mix were analysed by SDS-PAGE. A photograph of the fluorographed gel is shown in Figure 7.2.4; lane B represents the expression of pBSS1-1 and lane C that of pBSS1-1 Δ L. Two features are immediately obvious: the mutated clone directs the synthesis of a single band of the expected size for σ 1, instead of a doublet and the efficiency of translation is increased as shown by a more intense band in comparison to either of the bands belonging to the doublet. The single σ 1 protein band derived from the mutated S1-1 clone comigrated with the lower of the doublet bands resulting from expression of pBSS1-1. Thus, it would appear that the putative upstream initiation codon present in pBSS1-1 (see Chapter 5) was responsible for initiating the synthesis of the larger of the doublet bands.

Aliquots of the two different σ 1 translation mixes described above were TCA precipitated and the associated radioactivity measured by scintillation counting (section 2.11.2) to enable an estimation of the efficiency of translation of the respective s1 mRNAs to be made (see Table 7.2.1 shown below Figure 7.2.4).

The figures for the TCA precipitated counts revealed that RNA synthesized from pBSS1-1 Δ L was translated approximately twice as efficiently as that from pBSS1-1. However, the latter RNA directs the translation of two σ 1 proteins, only one of which can be considered as an

Figure 7.2.3 Sequencing of pBSS1-1 and pBSS1-1ΔL

DNA from plasmid pBSS1-1ΔL was sequenced as described in section 2.8.8.2. A photograph of the resulting autoradiograph is shown opposite. The tracks on the left hand side of the picture represent analysis of pBSS1-1ΔL and those on the right hand side pBSS1-1 for comparison. The asterisks denote the -3 nucleotide of the σ 1 initiation sequence and confirms that in the mutant it has been altered to an A from a C. The arrows denote the first base of the S1-1 gene. In the mutant sequence this base is immediately preceded by an A which is the -1 nucleotide of the T3 promoter confirming that the intended deletion had taken place. Compare this with the pBSS1-1 sequence where the nucleotides preceding the first base of the S1-1 gene are derived from the 5' gene oligonucleotide used in its cloning.

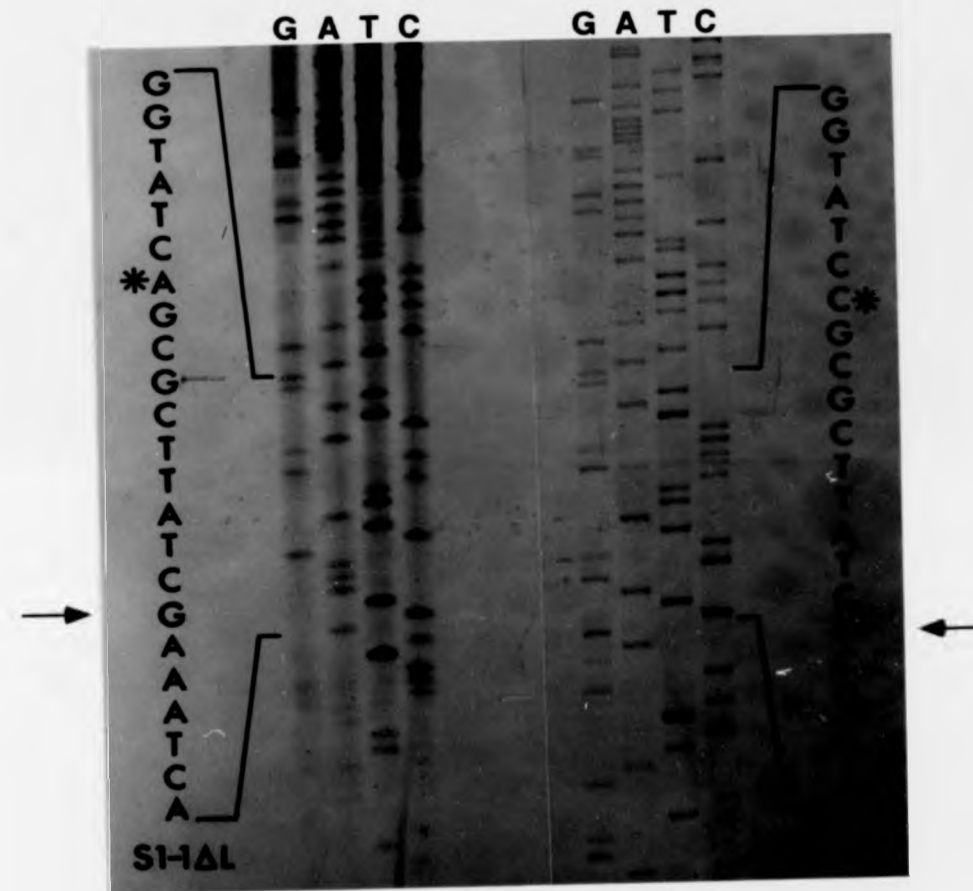


Figure 7.2.4 SDS-PAGE Analysis of $\sigma 1$ Proteins Encoded by Transcripts Synthesized from pBSS1-1 and pBSS1-1 Δ L

s1-1 mRNA was transcribed *in vitro* from plasmids pBSS1-1 and pBSS1-1 Δ L. Each mRNA was used to prime *in vitro* translation reactions carried out as described in section 2.11.1 for the non-commercial sources of lysate. Equal volumes of the reaction mixes were subjected to SDS-PAGE (2.11.4) and the polyacrylamide gel processed as described in section 2.11.5. A photograph of the resulting autoradiograph is shown opposite. The radiolabelled reovirus polypeptides (lane R) were prepared as described in section 2.2.5.

- Lane R Reovirus infected cells
- Lane A Endogenous lysate reaction
- Lane B $\sigma 1$ protein from pBSS1-1 transcripts
- Lane C $\sigma 1$ protein from pBSS1-1 Δ L transcripts

Table 7.2.1 Comparison of $\sigma 1$ -1 Synthesized from pBSS1-1 and pBSS1-1 Δ L mRNAs

2 μ l aliquots of the *in vitro* translation reactions described above were TCA precipitated and the radiolabel incorporated into protein products measured (section 2.11.2). The resulting values are shown in the Table opposite. The column headed "Ratio $\sigma 1$ -1 Δ L : $\sigma 1$ -1" represents the amount of $\sigma 1$ synthesized from pBSS1-1 Δ L derived RNA compared to that from pBSS1-1 RNA. In the "cpm value" for this comparison, a value equal to half the number of counts given for the pBSS1-1 transcript is used to calculate the ratio since two $\sigma 1$ proteins are produced from this RNA in approximately equal quantities. The densitometer value was derived by laser scanning densitometry (as described in section 2.11.6) of the autoradiograph shown opposite.

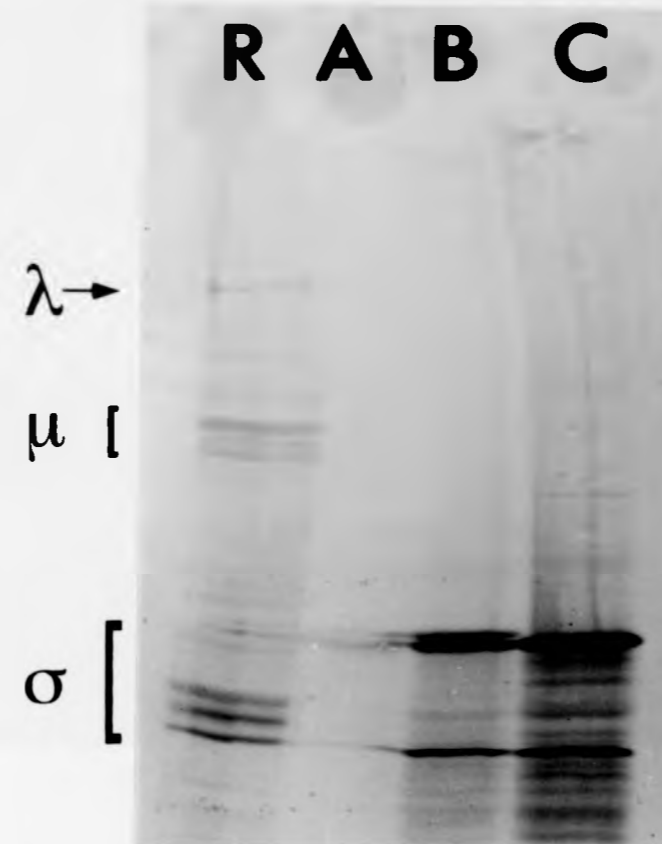


Table 7.2.1 Comparison of $\sigma 1$ -1 Synthesized from pBSS1-1 and pBSS1-1 Δ L mRNAs (Legend is opposite).

TRANSCRIPT	COUNTS	Ratio $\sigma 1$ -1" Δ L" : $\sigma 1$ -1	
		From cpm values	From densitometer values
Endogenous	17.853		
s1-1	103.869	3.5	3.8
s1-1 Δ L	182,098		

authentic product (the lower molecular weight polypeptide). Each of these proteins are synthesized in approximately equal amounts and thus contribute equally to the TCA counts value. Therefore the mutations performed in generating pBSS1-1 Δ L result in a fourfold increase in the amount of σ 1-1 expressed. A more accurate comparison would have been possible if only the lower band of the σ 1 doublet had been synthesized. Quantitation of the autoradiograph shown in Figure 7.2.4 by laser scanning densitometry also revealed a fourfold difference between the single σ 1 band in lane B derived from the mutant clone and the lower band of the doublet in lane C (Table 7.2.1).

One other effect of the mutation in pBSS1-1 Δ L was to decrease the amount of s1-1 mRNA produced in *in vitro* transcription reactions to about 1/3 that of the non-mutated type1 clone (results not shown). This effect was probably due to the T3 promoter nucleotides deleted in the process of placing the S1-1 gene exactly adjacent to this promoter. This is discussed further in the final section of this chapter (7.2.4).

7.2.3 The Effect of 2AP on Translation

Plus sense s1 RNA derived from plasmids pBSS1-1, pBSS1-1 Δ L and pG3T3 was translated in reticulocyte lysate in the presence and absence of 2AP and the protein products subjected to SDS-PAGE. The gel was processed by fluorography and the result is shown in Figure 7.2.5. Quantitation of the representative σ 1 bands by scanning laser densitometry revealed that all three mRNAs were translated 3-4 times more efficiently in the presence of 2AP (Table 7.2.2).

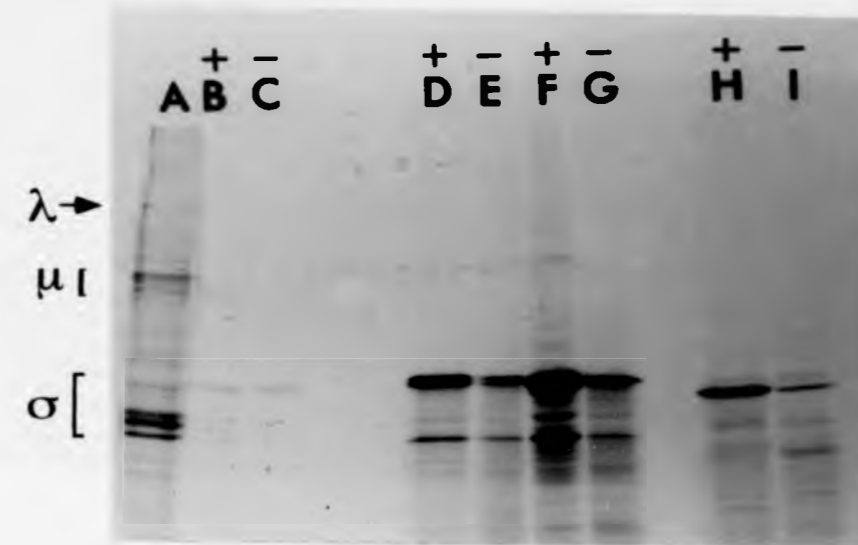
To ascertain if this effect was specific to the s1 mRNA, further reactions were conducted to include an analysis of the effect of 2AP on the translation efficiency of s4-1 mRNA (transcribed *in vitro* from plasmid

Figure 7.2.5 **The Effect of 2AP on the *In Vitro* Translation of Reovirus Messenger RNAs**

Reovirus s1 mRNA was transcribed *in vitro* from plasmids pBSS1-1, pBSS1-1ΔL and pG3T3 . These transcripts were translated in a non-commercial reticulocyte lysate as described in section 2.11.1 in the presence or absence of 15mM 2AP. Equal volumes of the individual reaction mixes were electrophoresed on an SDS-polyacrylamide gel as described in section 2.11.4 and the gel processed by fluorography as in section 2.11.5. The results shown opposite. Radiolabelled reovirus proteins were prepared from infected cells as described in section 2.2.5.

Lane A	Reovirus infected cells
Lanes B and C	Endogenous lysate
Lanes D and E	σ1-1 translated from pBSS1-1 RNA
Lanes F and G	σ1-1 translated from pBSS1-1ΔL RNA
Lanes H and I	σ1-3 translated from pG3T3 RNA

The + and - symbols represent the presence or absence of 2AP in the *in vitro* translations.



TRANSCRIPT	CPM			PROTEIN	DENSITOMETER VALUES :	
	-2AP	+2AP	$\frac{+2AP}{-2AP}$		+ 2AP/-2AP	Relative Synthesis cf to $\sigma 1-1\Delta L(-2AP)$
$\sigma 1-1$	64,512	203,894	3.23	$\sigma 1-1^*$	3.7	0.22
$\sigma 1-1\Delta L$	253,218	744,060	3.07	$\sigma 1-1$	3.6	1
$\sigma 1-3$	166,582	773,049	3.80	$\sigma 1-3$	4.2	0.8
$\sigma 4-1$	375,372	990,686	2.57	$\sigma 3-1$	2.2	0.7

Table 7.2.2 Effect of 2AP on the Translation Efficiencies of Reovirus mRNAs

In vitro translations carried out in the presence and absence of 2AP and the subsequent SDS-PAGE were performed as described in the legend to Figure 7.2.6 with the exception that s4-1 mRNA transcribed from plasmid pBS4-1 was included in the analysis and both commercial and non-commercial sources of reticulocyte lysate were utilized. The appropriate bands on the resulting autoradiographs (corresponding to the relevant species of σ protein) were analysed by laser scanning densitometry (section 2.11.6) and the results are presented in the Table opposite. * denotes that only the lower band of the $\sigma 1$ doublet was analysed. The final column in the table is the amount of each protein synthesized in the absence of 2AP and normalized with respect to $\sigma 1$ translated from s1-1 Δ L RNA; differences in the methionine content of each protein are accounted for. The cpm values were determined essentially as described in the legend for Table 7.2.1. The densitometer values represent the average of three experiments and the cpm values are typical of one experiment; actual values varied between experiments (and the source of the lysate) but the calculated ratios were similar in each case.

pBS4-1). This RNA was routinely used as a control in the *in vitro* translation reactions and the encoded protein product ($\sigma 3$) was intended for use as a control in future cell binding assays (Chapter 8). Thus, *in vitro* translations of s1 and s4 mRNAs were carried out in the presence and absence of 2AP and the amounts of respective σ proteins quantitated by densitometry scanning as described above. These results are presented in Table 7.2.2. Including 2AP in the reaction mix increased the production of $\sigma 3$ twofold and $\sigma 1$ approximately threefold (as initially observed). The amount of TCA precipitable radioactivity incorporated into the products of these translations was measured by scintillation counting (Table 7.2.2) and gave values close to those obtained by the scanning densitometry analysis. Therefore, the effect of 2AP did not appear to be specific to the translation of *in vitro* transcribed s1 mRNAs. The increase in translation efficiency of the s1 and s4 mRNA induced by 2AP also implies that these transcripts are activating the P1/eIF-2 α kinase in the reticulocyte lysate. Further discussion concerning this issue is given below.

7.3 DISCUSSION

Oligonucleotide directed mutagenesis was performed to alter the weak $\sigma 1$ initiation codon sequence in the S1-1 cloned gene to a stronger context and to delete the nucleotides between the T3 phage promoter and the S1 gene of plasmid pBSS1-1, such that *in vitro* transcripts would be devoid of vector derived nucleotides at their 5' ends. The purpose of the mutagenesis was to effect an increase in the *in vitro* translation of $\sigma 1$ -1. This was observed, as transcripts from the mutated plasmid, pBSS1-1 Δ L, directed the *in vitro* synthesis of $\sigma 1$ -1 more efficiently than their pBSS1-1 counterparts.

Several other groups of workers have used a similar approach in an attempt to explain the poor translation of $\sigma 1$. Munemitsu and Samuel (1988) mutated DNA copies of the S1 type 1 gene that had been cloned into an SV40 based expression vector. Quantitation of expressed $\sigma 1$ was achieved after transfection of the plasmids into COS cells. These authors found that changing the nucleotide in position -3 relative to the $\sigma 1$ AUG codon, from a C to a G in line with the strong initiation sequence proposed by Kozak (Kozak, 1981; 1983 and 1986), had no significant effect on $\sigma 1$ synthesis compared to a wild type clone. In an *in vitro* study, Roner *et al.* (1989) mutated the -3 to -1 positions of the $\sigma 1$ -3 initiation sequence to the strongest Kozak consensus sequence (ACC respectively) which resulted in a tenfold increase in the amount of $\sigma 1$ translated. The result obtained in this study, namely a 4-6 fold increase in $\sigma 1$ synthesis effected by the mutant clone is more in agreement with the latter study and suggests that the nature of the -3 nucleotide is an important factor in controlling the translation frequency of s1 mRNA. The expression vectors of Munemitsu and Samuel (1988) produced s1-1 transcripts with a 5' untranslated sequence longer than authentic s1-1 mRNA as a consequence of the method of expression. It is possible that these sequences were exerting a dominant influence over the introduced mutation.

Another effect of the mutation was that $\sigma 1$ was resolved as a single band on SDS polyacrylamide gels unlike protein expressed from the original type1 plasmid which produced a doublet of bands. A putative, weak initiation codon upstream of the normal $\sigma 1$ AUG was one of the possibilities suggested in explanation of the doublet and was deleted in pBSS1-1 Δ L. This resulted in the *in vitro* translation of a $\sigma 1$ polypeptide equivalent in size to the lower of the doublet bands. Hence, the role of the putative initiation codon in producing the doublet was confirmed. The reduction in the length of the 5' untranslated sequence of s1-1 mRNA

transcribed from this clone to the same as that found in s1-1 mRNA *in vivo* may also have contributed to the increase in translation efficiency, as Roner *et al.* (1989) determined an optimum 5' untranslated length of approximately 14 nucleotides in effecting maximum stimulation of $\sigma 1$ synthesis.

The deletion of some T3 phage promoter nucleotides in pBSS1-1 Δ L affected the transcriptional activity of the clone, reducing RNA yields from *in vitro* transcriptions by 1/2 - 1/3 that of the non-mutated pBSS1-1, which contained an intact T3 promoter. Despite this decrease, 8-10 μ g of s1-1 mRNA were still obtained upon transcription *in vitro* (results not shown). An SP6 based vector designed to allow the production of transcripts with authentic 5' ends, and therefore containing no vector derived bases, has been constructed and found to have no effect on RNA yields (Jobling *et al.*, 1988). However, only crude estimates were performed and comparison with a "wild type" clone containing a complete SP6 promoter sequence was not made.

Inclusion of 2-aminopurine in the reticulocyte lysate translation reactions resulted in an increase in the synthesis of $\sigma 1$ (of both serotypes) and $\sigma 3$ -1 (Figure 7.2.5 and Table 7.2.2). The observed increase was marginally greater for $\sigma 1$ than for $\sigma 3$. The effect was presumably due to inhibition by 2AP of P1/eIF-2 α kinase activation, as has been reported by others (Legon *et al.*, 1974 and De Benedetti and Baglioni, 1983). This in turn implies that both *in vitro* transcribed s1 and s4 messages were activating the kinase in the reticulocyte lysate system. Reovirus ss RNA, produced by transcription from viral cores and the genomic ds RNA are potent activators of this kinase (Samuel, 1979). Later studies revealed that s1 mRNA, but not s4, is an activator of protein kinase P1/eIF-2 α *in vitro* (Bischoff and Samuel, 1989) and that 2AP increases the synthesis of $\sigma 1$ but not $\sigma 3$ when expressed *in vivo* by means of plasmid based

transfections (Samuel and Brody, 1990). The latter, *in vivo* study, is clearly contradictory to the results presented above where a 2AP mediated increase of $\sigma 1$ and $\sigma 3$ was noted. The discrepancy may be a consequence of the two systems utilised to examine the influence of 2AP on translation. In the investigation of Samuel and Brody (1990) cells were transfected with plasmids containing the appropriate reovirus cDNA and the cell culture treated with 2AP. Expression of the reovirus gene product was examined 48 hours after transfection. Between 4-12 hours of exposure to 2AP was necessary to increase $\sigma 1$ synthesis; no concomitant effect upon host cell protein synthesis was noted. Steady state levels of s1 mRNA were also comparable to untreated control cells. Obviously, the action of 2AP is more immediate in the reticulocyte lysate system. The opportunity for 2AP to potentially interact with factors that may be present in the *in vivo* situation but not *in vitro* is a possibility and could contribute to the increase in $\sigma 3$ translation observed here but not in the aforementioned *in vivo* study. A higher order structure of s1-1 mRNA comprising single and double stranded components not possessed by s4 mRNA (Bischoff and Samuel, 1989) and /or the possibility that $\sigma 3$ acts as an antagonist of the kinase by virtue of its ability to bind RNA (Imani and Jacobs, 1988) was proposed by Samuel and Brody (1990) to account for the differential effect of 2AP on $\sigma 1$ and $\sigma 3$ synthesis. However, neither of these explanations appear to be applicable to the observations made in this study as 2AP did effect an increase in the synthesis of $\sigma 3$.

A fivefold increase in s1-1 mRNA translation efficiency was noted by Samuel and Brody (1990) in the presence of 2AP which is slightly greater than the threefold increase noted with the reticulocyte translations performed in this study. This difference may be due to the relatively high concentrations of RNA employed in the *in vitro* translations (40 μ g/ml). Bischoff and Samuel (1989) found that the optimum concentration of s1-1

mRNA required for *in vitro* activation of the kinase was approximately 1 μ g/ml and that activity decreased with increasing RNA concentration.

7.4 SUMMARY

An increase in σ 1-1 expression was effected by the use of plasmid pBSS1-1 Δ L in comparison to pBSS1-1. Therefore, the former plasmid was used to direct the expression of σ 1-1 in all the appropriate experiments described in subsequent chapters. The biochemical 2AP increased the efficiencies with which the σ 1 (and σ 3) proteins were synthesized in the reticulocyte lysate system,. Consequently, it was included in all subsequent *in vitro* translation reactions described in this study unless otherwise stated.

CHAPTER 8

Development of a σ_1 - L-Cell Binding Assay

8.1 INTRODUCTION

Weiner *et al.* (1978) utilized reassortant viruses to identify $\sigma 1$ as the viral haemagglutinin and thus as the protein that interacted with erythrocytes. A more biologically significant finding regarding the virus-host interaction was made by Lee *et al.* (1981b), who showed that it is the virus cell-attachment protein. Here, infected L-cell cytoplasm was used as a source of viral proteins from which $\sigma 1$ (of all three serotypes) was the only species to bind specifically to monolayers of L-cells. This criterion has been used to confirm the identity of $\sigma 1$ when expressed from a variety of sources including bacteria (Masri *et al.*, 1986 and Nagata *et al.*, 1987) and vaccinia virus recombinants (Banerjea *et al.*, 1988) as well as when purified from whole virions (Yeung *et al.*, 1987). All used the method of assay originally employed by Lee *et al.* (1981b) where $\sigma 1$ was applied to cell monolayers and the bound product analysed by SDS-PAGE and autoradiography.

In this study it was initially decided to assess the binding activity of *in vitro* expressed $\sigma 1$ to L-cells by means of a "radioactive counts" method. Reticulocyte translation products are labelled with ^{35}S -methionine. Therefore, appropriate protein products could be bound to cells and the relative amounts of bound protein measured by scintillation counting. The cell binding assay of Lee *et al.* (1981b) (described above) was also used and became the favoured of the two methods. The development of the former method and reasons for switching to the latter are described in this chapter.

8.2 RESULTS

8.2.1 Development of a Direct, Radioactive Counts Assay of the $\sigma 1$ - L-Cell Interaction

Binding assays described in this section were carried out essentially as described in section 2.12.1. The number of radioactive, TCA precipitable counts incorporated into each of the *in vitro* translated proteins to be used in the binding assays was known. An equivalent number of counts for each protein, that is $\sigma 1$ -1, $\sigma 1$ -3 and $\sigma 3$ -1, were diluted into PBS/1% BSA and applied to monolayers of L-cells. The BSA was added to prevent non-specific binding. Two negative controls were used: one was protein $\sigma 3$ -1 and the other a sample of endogenous lysate where no exogenous RNA had been added to the translation reaction mix. For the latter, a volume of lysate equivalent to that for the $\sigma 1$ -1 sample was applied to the monolayers as a control for the number of counts bound due to the translation mix itself. The amount of radioactive material bound to the cell monolayers was then measured for each sample.

In these assays, although more $\sigma 1$ than $\sigma 3$ counts had seemingly bound to the cells, the value for the endogenous control was equal to that for $\sigma 1$ (data not shown). This was thought to be due to the non-specific binding of unincorporated ^{35}S -methionine from the translation reaction. An assay was therefore performed in which aliquots of the appropriate translation mixes were bound in the presence of increasing amounts of "cold" (i.e. non-radiolabelled) methionine. A concentration of 10mM methionine was found to reduce the endogenous control value to a background level; a concomitant reduction in the values for $\sigma 1$ and $\sigma 3$ was also noticed. Typical binding values in the presence of 10mM methionine are shown in Figure 8.2.1. As can be seen, $\sigma 1$ -3 bound

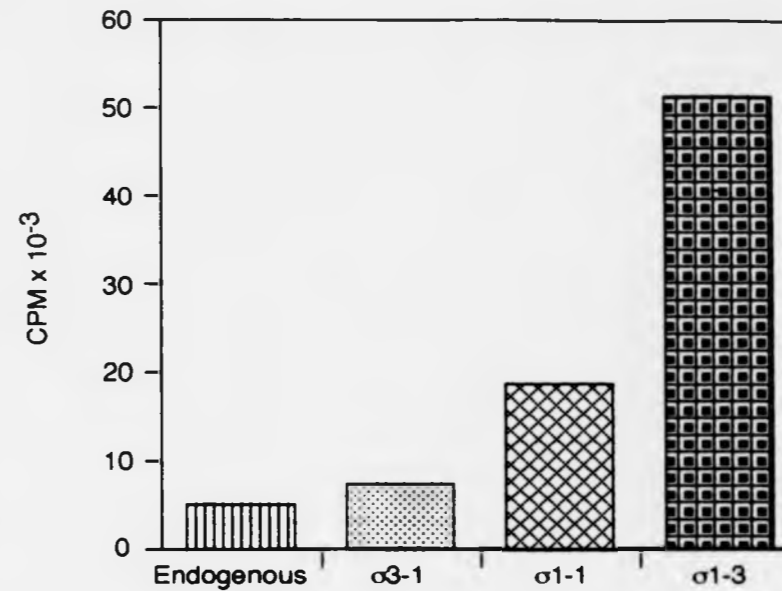


Figure 8.2.1 Cell-Binding Assay of the *In Vitro* Translated σ 1 Proteins

σ 1-1, σ 1-3 and σ 3-1 were *in vitro* translated in commercial lysate at 30°C as described in section 2.11.1 and the radioactivity associated with the TCA precipitable products measured as in section 2.11.2. Volumes of each of the lysates containing ~ 200,000 cpm of TCA precipitable protein product were diluted into 100 μ l of ice-cold PBS containing 1% BSA and 10mM methionine. These were overlayed on PBS washed monolayers of L-cells in 24-well dishes and allowed to bind for 1 hour on ice, with intermittent rocking. The binding mixes were then removed, the monolayers washed 3 times with cold PBS, once with cold sterile distilled water and solubilized in 1M NaOH. This was then neutralized with 1M HCl, placed in scintillant and counted in a scintillation counter. Duplicate samples of each protein were assayed and the data represents the average of two experiments.

approximately 2.5x as well as σ 1-1, which in turn bound twice as well as the σ 3 control. Similar data was obtained when the translation reactions were subjected to gel filtration to separate protein from the unincorporated, labelled methionine (data not shown).

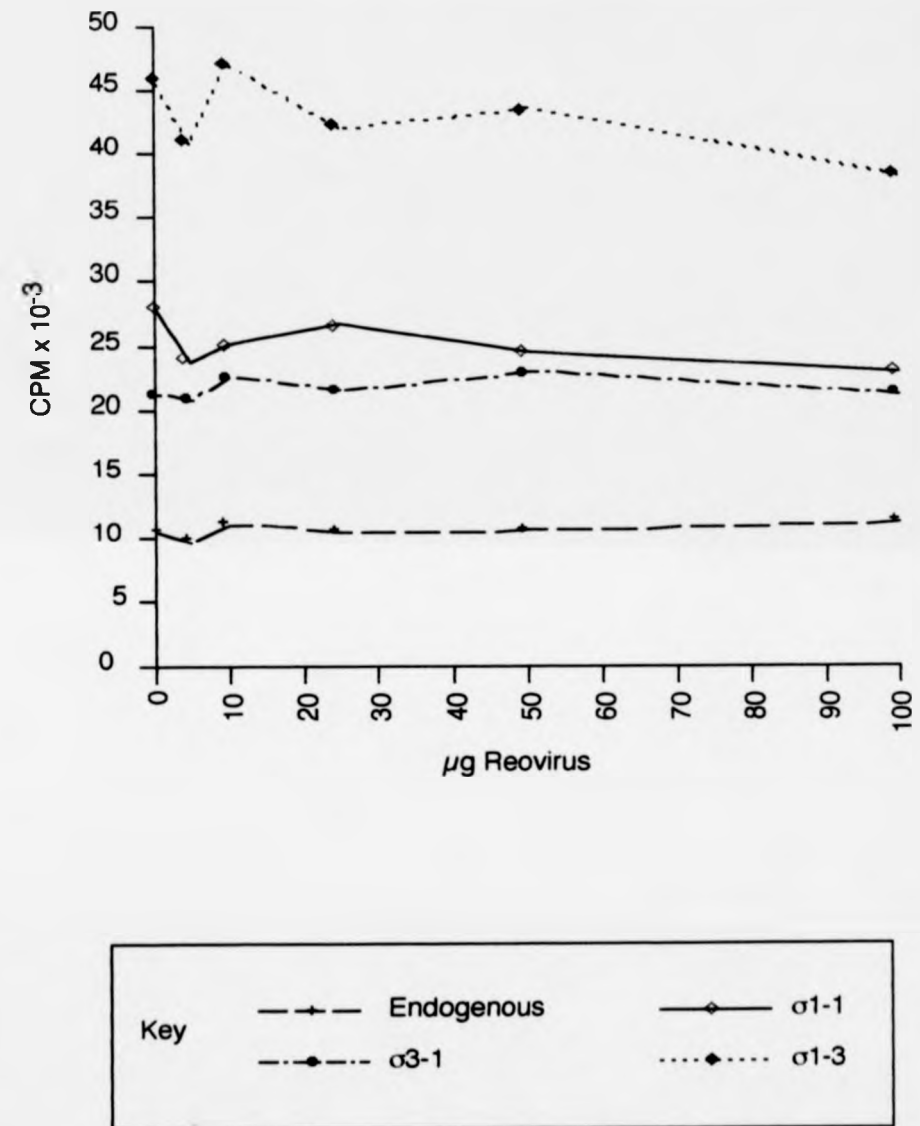
The main points evident from this assay were (i) The apparent level of σ 1 L-cell binding activity was low; (ii) Both type 1 and type 3 σ 1 were more capable of binding to cells than the control protein σ 3-1; (iii) The σ 3-1 negative control showed a relatively high level of apparent binding in comparison to σ 1-1 (approximately 1/2 that of σ 1-1). Since σ 3-1 does not appear to be involved in the virus-cell-attachment process, this was thought to represent non-specific binding of this protein.

8.2.2 Competition Assays

To demonstrate the specificity of the attachment process, assays were carried out with increasing amounts of reovirus in the binding mix, the results from which are shown in Figure 8.2.2. The homologous serotype virus for each of the species of σ 1 protein was used owing to the uncertainty over whether all three serotypes of reovirus utilize the same receptor on L-cells. Conflicting evidence concerning this matter has been presented by Lee *et al.* (1981b), Epstein *et al.* (1984) and Weiner *et al.* (1988); see also Chapter 9 for further relevant data. Several experiments were carried out using both T1 and T3 virus as the competing species; however, at amounts of up to 300 μ g of added virus, no competition was evident for either σ 1-1 or σ 1-3 (data not shown). This was unexpected considering the observations of Lee *et al.* (1981b), Masri *et al.* (1986) and Banerjea *et al.* (1988) who demonstrated that virus was able to inhibit binding of homologous type σ 1 protein.

Figure 8.2.2 Competition Assay Involving *In Vitro* Translated Reovirus Proteins and Reovirions

Proteins σ 1-1, σ 1-3 and σ 3-1 were translated *in vitro* as described in the legend to Figure 8.2.1. Aliquots of each of the reaction mixes equivalent to ~ 200,000 cpm of TCA precipitable protein product were diluted into binding buffer (PBS/1%BSA/10mM methionine) containing increasing amounts of homologous type reovirus (0, 5, 10, 25, 50 and 100 μ g). These mixes were bound to monolayers of L-cells and processed as described in section 2.12.1. The endogenous lysate control reaction was not primed with exogenous RNA and a volume of this lysate equal to that for the σ 1-1 translation mix was assayed; type 3 reovirus was the competing species. The graph opposite represents the average of duplicate samples. The pattern of results seen in replicates of this experiment was the same as shown here.



8.2.3 Oligomerization of Protein $\sigma 1$

Two reports, originating from the same laboratory concerning $\sigma 1$ appeared relevant to the results presented above. One of the studies had investigated the oligomeric nature of $\sigma 1$ (Leone *et al.*, 1991b) and the other its function as a cell-binding protein (Duncan *et al.*, 1991). Importantly, both had expressed $\sigma 1$ -3 in a similar fashion to this study, that is, in reticulocyte lysate utilizing RNA transcribed from a cloned copy of the S1-3 gene. However, both reported an incubation temperature of 37°C for the $\sigma 1$ *in vitro* translation reactions, not the 30°C recommended in the accepted protocol (Pelham and Jackson, 1976 and Jackson and Hunt, 1983); thus far, a temperature of 30°C had been employed in this study. Both studies reported SDS-PAGE analysis of the $\sigma 1$ proteins under non-dissociating conditions, i.e. incubation in sample buffer at 37°C prior to electrophoresis as originally defined by Bassel-Duby *et al.* (1987). These analyses revealed the presence of monomeric and oligomeric forms of the protein. Also, unpublished data referred to in these reports (later published in Leone *et al.*, 1991a) stated that only oligomers were capable of binding to L-cells. As specific attachment to cells had not thus far been observed in this study, it seemed pertinent to examine the oligomeric nature of $\sigma 1$ synthesized at 30°C and 37°C and to determine its significance in relation to the protein's cell-binding ability. This is described in the remainder of this chapter.

8.2.3.1 Comparison of $\sigma 1$ Oligomerization at 30°C and 37°C

The quaternary structure of $\sigma 1$ was examined at the two temperatures of 30°C and 37°C, (essentially as described by Leone *et al.*, 1991b and Duncan *et al.*, 1991) and the results are shown in Figure 8.2.3.

Figure 8.2.3 Oligomerization of σ 1 Proteins at 30°C and 37°C

Plasmids pBSS1-1 Δ L and PG3T3 were transcribed *in vitro* to produce σ 1-1 and σ 1-3 mRNA respectively. These RNAs were translated *in vitro* at 30°C or 37°C for 60 minutes in a commercial reticulocyte lysate as described in 2.11.1; each reaction mix was then diluted 10x in PBS. Duplicate samples from each of the 30°C reactions were added to sample buffer and incubated at either 30°C or 37°C for 30 minutes. Aliquots of the 37°C reactions were mixed with sample buffer and incubated for 30 mins. at 37°C only. All samples were then electrophoresed on a 10% SDS-polyacrylamide gel as described in section 2.11.4. Fluorography of the gel was carried out as detailed in section 2.11.5. A photograph of the processed gel is shown opposite. Radiolabelled reovirus polypeptides (prepared as described in section 2.2.5) were electrophoresed as size markers, lane R; lane U represents an uninfected cell lysate for comparison. Endogenous lysate mixes (i.e. reactions primed only with the endogenous lysate RNA) were treated as described above for the σ 1 translations and acted as controls.

Lanes A and B = *in vitro* translated σ 1-1 and σ 1-3 respectively, boiled in sample buffer before SDS-PAGE

Lanes D and J = σ 1-1 synthesized at 30°C and incubated in sample buffer at 30°C and 37°C respectively

Lanes E and K = σ 1-3 synthesized at 30°C and incubated in sample buffer at 30°C and 37°C respectively

Lane G = σ 1-1 synthesized at 37°C and incubated in sample buffer at 37°C

Lane H = σ 1-3 synthesized at 37°C and incubated in sample buffer at 37°C

Lanes C, F and I = endogenous lysate treated as samples in lanes D, G and J respectively.

The major oligomeric species are arrowed.



Lanes E and K represent σ 1-3 protein that was translated in reticulocyte lysate at 30°C and incubated in sample buffer at 30°C or 37°C respectively. Two major oligomeric species are present in both tracks, although the latter temperature appears to have induced the formation of a slightly greater number of oligomers. Lane H shows the result of carrying out both the translation reaction and subsequent incubation at 37°C. The highest degree of oligomerization is evident with this sample and two major species of σ 1-3 oligomer are also evident. The significance of these different oligomeric species became apparent later when their ability to bind to cells was investigated (section 8.2.4). Oligomers of σ 1-1 were not observed under any of the conditions used (lanes D, G and J).

8.2.3.2 Effect of Sample Buffer on Oligomerization

From the results in section 8.2.3.1, it was seen that oligomers of σ 1-3 were formed at 30°C although not as efficiently as when the translation and subsequent incubation in sample buffer were both carried out at 37°C. Thus, oligomerization appeared to be influenced by temperature. The oligomers of σ 1-3 were the putative cell-binding species but despite their presence in the 30°C translation reactions, specific binding to L-cells had not been observed (section 8.2.2). It was considered possible that the sample buffer was not only stabilizing those molecules from the lysate that possessed quaternary structure but was also promoting their formation. Hence, the relative amounts of oligomers observed in the SDS-PAGE analysis above (Figure 8.2.3) may not have been representative of the amounts in the lysate. This in turn would imply that only small amounts were present in the 30°C reactions. Therefore, the effect of sample buffer on the oligomerization process was determined in the experiments described overleaf.

In vitro translations of σ 1-3 were performed at 30°C and 37°C. Aliquots of these reaction mixes were analysed by SDS-PAGE either directly or following incubation in sample buffer at their respective temperatures; the former conditions precluded a sample buffer step. The autoradiograph resulting from this analysis is shown in Figure 8.2.4. Very little oligomerization of σ 1-3 was evident when it was synthesized at the lower temperature with or without subsequent incubation in sample buffer (lanes C and D). However, at an *in vitro* translation temperature of 37°C, significant quantities of oligomers were formed irrespective of incubation in sample buffer, although this did increase the relative amount of the higher order structure, lanes E and F. The samples in lanes E and F originate from another gel which accounts for the different migration patterns of the monomeric and oligomeric species compared to the 30°C samples (lanes C and D).

Hence, the sample buffer appeared to be aiding the formation of quaternary structures for σ 1-3 following its synthesis at 37°C.

The time course of oligomer formation was next followed, which also confirmed the effect of the sample buffer. A translation primed with σ 1-3 mRNA was incubated at 37°C and duplicate samples were taken at various intervals. These were either incubated in sample buffer for 30 minutes or snap frozen in dry ice. All of the samples were then analysed by SDS-PAGE with the frozen samples thawed and mixed with sample buffer immediately prior to loading. This gel is shown in Figure 8.2.5. Only monomeric species were present after 5 minutes of reaction time and thereafter a mixture of monomers and oligomers were evident. The relative amounts of the oligomers were greatly increased by the incubation in buffer; compare lanes F and G and H and I for example. However, the maximum amount of oligomerization was achieved after 30 minutes of

Figure 8.2.4 The Effect of Sample Buffer on the Oligomerization of $\sigma 1$ Polypeptides

Protein $\sigma 1$ -3 was translated *in vitro* at 30°C and 37°C and diluted in PBS as described in the legend to Figure 8.2.3. Duplicate aliquots from each of these reactions were either incubated in sample buffer for 30 minutes at 37°C (non-dissociating conditions) or stored on ice for 30 minutes before the addition of sample buffer. All samples were then analysed by SDS-PAGE (section 2.11.4) and the gel treated as in section 2.11.5. The resulting autoradiograph is shown opposite.

Lane R = Reovirus infected cell lysate

Lane A = $\sigma 1$ -1 (boiled in sample buffer)

Lane B = $\sigma 1$ -3 (boiled in sample buffer)

Lane C = $\sigma 1$ -3 translated at 30°C, stored on ice

Lane D = $\sigma 1$ -3 translated at 30°C, incubated in sample buffer

Lane E = $\sigma 1$ -3 translated at 37°C, stored on ice

Lane F = $\sigma 1$ -3 translated at 37°C, incubated in sample buffer

NOTE : Samples represented in Lanes E and F are derived from a separate gel to the those in the other lanes.

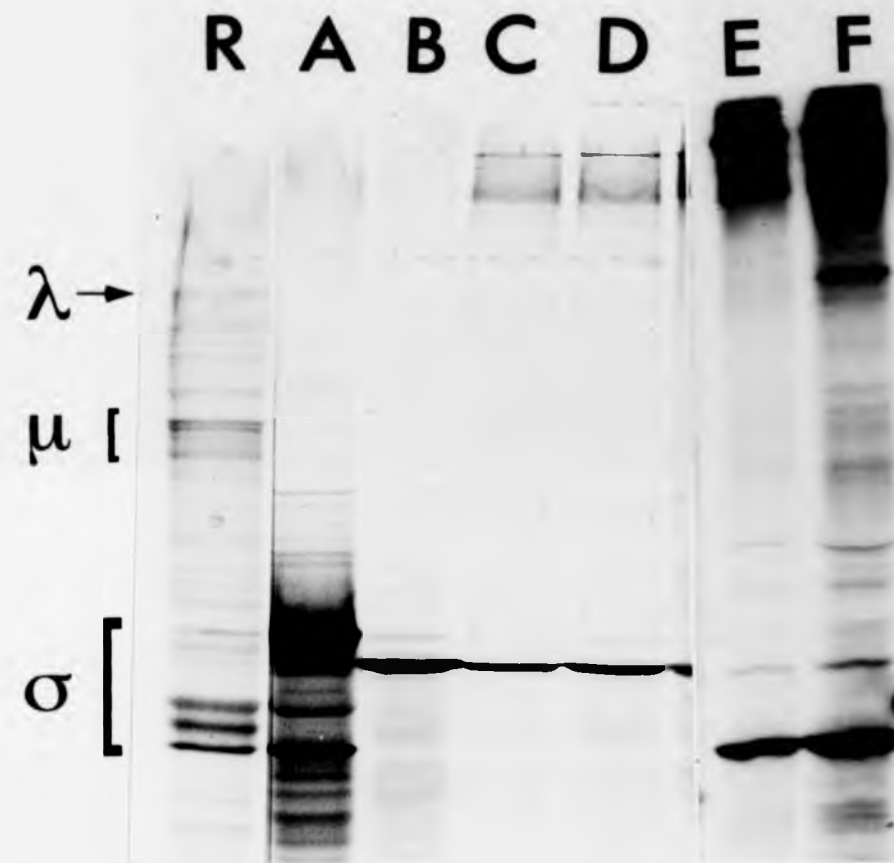


Figure 8.2.5 Time Course of σ 1-3 Oligomerization

An *in vitro* translation reaction mix was primed with σ 1-3 mRNA and incubated at 37°C for 60 minutes. At intervals during the incubation, duplicate aliquots of the mix were removed and either snap frozen on dry ice or diluted 10x in PBS and incubated under non-dissociating conditions (that is, in sample buffer at 37°C) for 30 minutes. After the final incubation period, the frozen aliquots were mixed with sample buffer and all samples were then analysed by SDS-PAGE (section 2.11.4). A sample of the σ 1-3 reaction mix was boiled in sample buffer before electrophoresis to act as a size marker. The autoradiograph of the processed gel (section 2.11.5) is shown opposite.

- Lane R = Reovirus infected cells
- Lane A = Endogenous lysate
- Lane B = 0 minute sample, snap frozen
- Lane C = 0 minute sample, incubated in sample buffer (ISB)
- Lanes D and E = 5 minute sample, snap frozen or ISB respectively
- Lanes F and G = 10 minute samples, snap frozen or ISB respectively
- Lanes H and I = 20 minute samples, snap frozen or ISB respectively
- Lanes J and K = 30 minute samples, snap frozen or ISB respectively
- Lanes L and M = 40 minute samples, snap frozen or ISB respectively
- Lanes N and O = 50 minute samples, snap frozen or ISB respectively
- Lanes P and Q = 60 minute samples, snap frozen or ISB respectively
- Lane S = boiled σ 1-3



synthesis, after which a small decrease was observed. It is important to note that significant amounts of the multimers were formed at 37°C in the lysate reaction mix alone in contrast to the situation at 30°C. In addition to the two previously observed oligomeric species there is another that migrates just below the position of the reovirus λ proteins at approximately 150 kDa, whose presence is abrogated by incubation in sample buffer; compare lanes F and G for example. This was initially thought to be an alternative order of oligomer. Further discussion concerning this issue is presented in section 11.1 of the General Discussion (Chapter 11).

To be certain that the oligomers were composed of the monomeric species, the bands corresponding to the two major, slower migrating multimers were excised from the gel and analysed under dissociating conditions. Both oligomeric species dissociated to give bands that comigrated with σ 1-3 protein that had been *in vitro* translated and denatured in sample buffer before electrophoresis (data not shown). The oligomers were therefore unlikely to be artifacts of the non-dissociating conditions.

8.2.4 Binding of the Monomeric and Oligomeric Species to L-Cells

The result of the time course assay described in section 8.2.3.2 suggested a means by which the cell-binding abilities of the σ 1-3 monomers and oligomers could be ascertained. It was noticed that after 5 minutes of synthesis in the reticulocyte lysate only the former species was present and thereafter a mixture of both; thus, monomers could be produced as a separate species. Accordingly, σ 1-3 *in vitro* translations were carried out for various time intervals between 5-30 minutes and the reaction mixes assayed on monolayers of L-cells at 4°C. Aliquots of the translated protein and the bound and unbound species were subjected to

SDS-PAGE and the result is shown in Figure 8.2.6. Unexpectedly, monomers were not formed after 5 minutes (lane A) and so were not assayed for their cell-binding ability in isolation from the oligomers. However, lanes E-H revealed that only oligomers were able to bind to cells, with the monomers present in the unbound material (lanes J and K). Furthermore, it appeared that it is mainly the slowest migrating oligomeric species that exhibits cell-binding activity, although some activity was apparent for the minor, approximately 150 kDa structure (compare lanes G and H with J and K).

Protein σ 1-1 was similarly assayed with the exception that only a single time of translation was examined. The autoradiograph resulting from this assay is shown in Figure 8.2.7 and revealed that σ 1-1 was unable to bind to L-cells.

8.2.5 Oligomerization of σ 1-1

The results above [and of Leone *et al.*, (1991a) and Duncan *et al.*, (1991)] show that oligomerization of the σ 1-3 protein is necessary for it to bind to L-cells. The type 1 protein was only present as a monomer when translated *in vitro* and exhibited no cell-binding activity. Therefore, it seemed reasonable to believe that σ 1-1 could manifest a cell binding function if the protein was to oligomerize.

Two approaches were taken in an effort to cause the type 1 monomers to attain the relevant quaternary structure; these are described in sections 8.2.5.1 and 8.2.5.2.

Figure 8.2.6 SDS-PAGE Analysis of the Species of σ 1-3 Exhibiting L-cell Binding Activity

Protein σ 1-3 was translated in a commercial reticulocyte lysate at 37°C. 25 μ l aliquots of the reaction mix were removed after 5, 10, 20 and 30 minutes of the incubation period and diluted into 200 μ l of PBS. 100 μ l of these mixes were then assayed on monolayers of L-cells as described in section 2.12.2 and the remainder was stored on ice. After the assay, each of the binding mixes was placed on ice; this represented unbound material. Aliquots of the translated protein (that had been stored on ice), the cell-bound the unbound material were incubated in sample buffer under non-dissociating conditions and analysed by SDS-PAGE as described in sections 2.11.4 and 2.11.5. The resulting autoradiograph is shown opposite.

Lane R =	Reovirus infected cells		
Lane U =	Uninfected cells		
Lane A =	5 minute sample] Translation	
Lane B =	10 minute sample		
Lane C =	20 minute sample		
Lane D =	30 minute sample		
Lane E =	5 minute sample] Cell-bound	
Lane F =	10 minute sample		
Lane G =	20 minute sample		
Lane H =	30 minute sample		
Lane I =	5 minute sample] Unbound	
Lane J =	10 minute sample		
Lane K =	20 minute sample		

There was not sufficient room on the gel for the analysis of the 30 minute unbound sample.

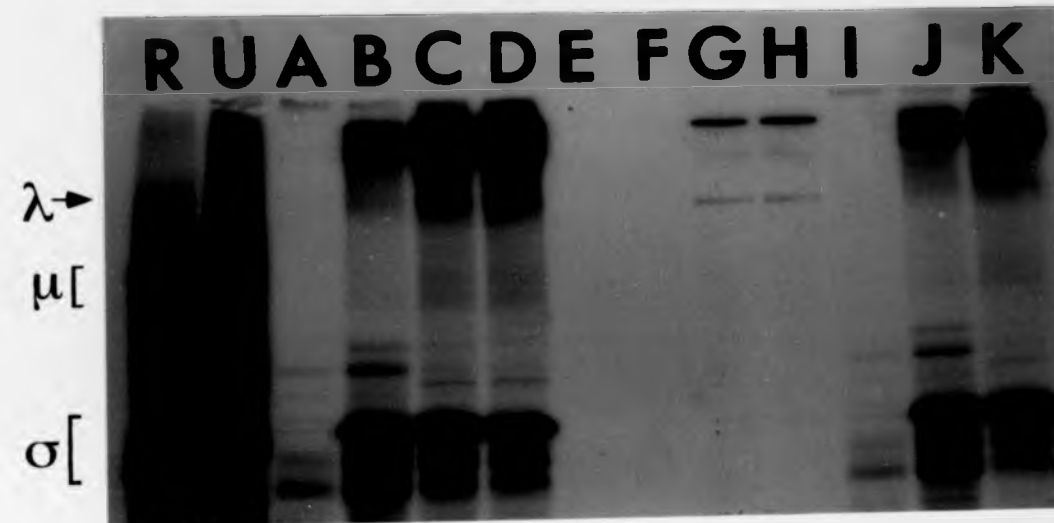
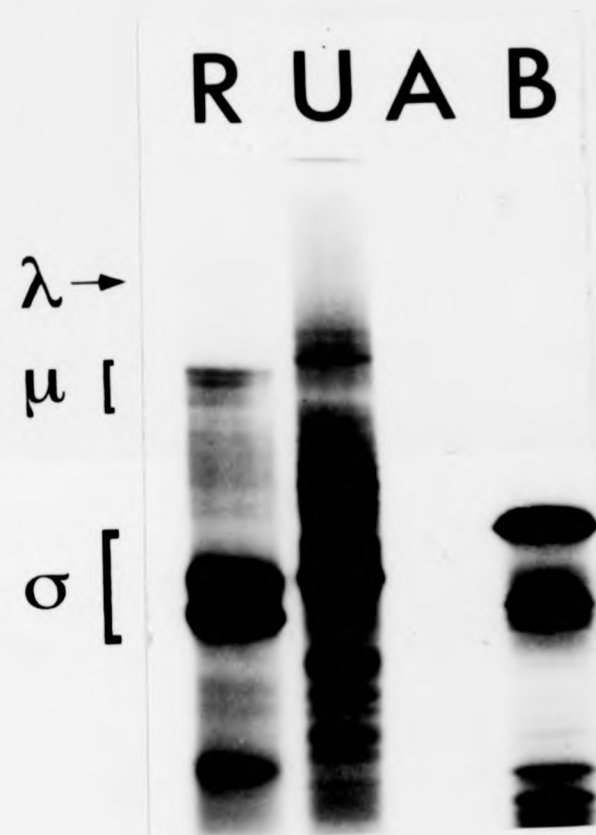


Figure 8.2.7 σ 1-1 : L-cell Binding Assay

Plasmid pBSS1-1 Δ L was *in vitro* transcribed to produce σ 1-1 mRNA. This was utilized to prime a reticulocyte lysate reaction that was incubated at 37°C for 30 minutes. The σ 1-1 protein thus produced was bound to monolayers of L-cells and analysed by SDS-PAGE under non-dissociating conditions as described in section 2.12.2; a sample of the unbound material (i.e. the binding mix resulting from the 1 hour's incubation with the cells) was similarly electrophoresed. Reovirus infected cells were prepared as in section 2.2.5. The autoradiograph resulting from this analysis is shown opposite.

- Lane R = Reovirus infected cells
- Lane U = Uninfected cells
- Lane A = Cell-bound sample
- Lane B = Unbound sample



8.2.5.1 Attempts to Produce Hetero-oligomers

The first approach involved an attempt to form hetero-oligomers between the type 1 and 3 proteins by translating them separately, mixing together aliquots from each reaction and then incubating the mix in sample buffer at 37°C. The σ 1-3 translation reaction was performed with cold methionine and the σ 1-1 with the radiolabelled amino acid. However, no oligomeric species were visible after SDS-PAGE analysis. The two messages were then co-translated. Under these conditions virtually no oligomers were formed, implying that σ 1-1 was preventing the usual association of σ 1-3 monomers into their oligomeric forms.

8.2.5.2 Construction of an S1-3/S1-1 Hybrid Clone

The N-terminal half of the σ 1 protein has been postulated to assume a coiled-coil configuration and hence be responsible for causing (at least in part) the oligomerization of σ 1 (Bassel-Duby *et al.*, 1985 and Nibert *et al.*, 1990). That it possessed an intrinsic oligomerizing property was later demonstrated experimentally for the type 3 protein (Banerjea and Joklik, 1990 and Leone *et al.*, 1991b;c). It was therefore decided to generate a hybrid protein where the N-terminal 1/3 of σ 1-3 would be fused to the C-terminal 2/3 of σ 1-1 by manipulation of the respective genes. The cell-binding properties of σ 1 apparently reside within the C-terminal half of the protein (Nagata *et al.*, 1987 and Yeung *et al.*, 1989). Thus, it was hoped that the protein resulting from the expression of this hybrid gene would oligomerize but retain the cell-binding properties of the type 1 protein.

All three reovirus S1 genes are highly diverged (Gaillard and Joklik, 1983; Cashdollar *et al.*, 1985; Duncan *et al.*, 1990 and Nibert *et al.*, 1990) which prevented an exact replacement of the two N-terminal regions.

However, it was possible to create an in frame hybrid with just 23 extra amino acids and Figure 8.2.8 represents schematically the strategy adopted to produce this clone. A fragment corresponding to approximately the 5' 1/3 of the S1-3 gene was removed from plasmid pG3T3 with the appropriate restriction enzyme digests and DNA modification reactions. The remaining vector/gene fragment was then purified. A fragment of plasmid pBSS1-1ΔL corresponding to the 3' 2/3 of the S1-1 gene isolated and ligated to the pG3T3 vector/gene fragment to create the hybrid clone pGT3S1-1. Plasmid DNA was prepared from the putative recombinant clones and screened by the appropriate restriction enzyme digest analysis to identify this hybrid.

8.2.5.2.1 Oligomerization and Cell Binding Ability of pGT3S1-1




In vitro transcribed mRNA from clone pGT3S1-1 was translated in reticulocyte lysate and analysed by SDS-PAGE under dissociating (boiled in sample buffer) and non-dissociating (incubated at 37°C in sample buffer) conditions to ascertain that a full-length $\sigma 1$ hybrid polypeptide could be produced and that it was capable of oligomerizing. The resulting autoradiograph is shown in Figure 8.2.9 and demonstrates the apparent full length nature of the protein which, as expected, is larger than $\sigma 1-3$ (see lanes A and C for a comparison). It also formed two major oligomeric species (lane D) which were slightly larger than their $\sigma 1-3$ counterparts. The N-terminal region of $\sigma 1-3$ therefore appeared to have conferred upon the hybrid molecule the capacity to oligomerize.

The ability of this protein to attach to L-cells was next examined and it was found to be unable to bind (Figure 8.2.10). Thus, the ability of the hybrid molecule to oligomerize appeared to be insufficient for the proposed cell binding region of $\sigma 1-1$ to manifest cell binding activity.

Figure 8.2.8 Construction of pGT3S1-1

Plasmid pG3T3 was digested with restriction enzyme *NcoI* and the ends of the DNA were filled by Klenow polymerase 1 before digestion with *XbaI* (which cuts in the 3' polylinker). The large, vector fragment containing the 5' S1-3-terminus was then purified from an agarose gel as described in section 2.9.1. Plasmid pBSS1-1ΔL was digested with the enzyme *EcoRI* and the ends made flush as with pG3T3 before *XbaI* digestion to release the 3' - 2/3 of the S1-1 gene. This fragment was isolated from an agarose gel and ligated to the pG3T3 vector fragment to give the recombinant pGT3S1-1. Shown below the map of the hybrid gene is a cartoon depicting the proposed morphological domains contributed by each portion of the hybrid gene based on the assignments for authentic $\sigma 1$ by Nibert *et al.* (1990); see also Figure 1.4.1. The extensions to the stalk are the main features to note and are marked with an asterisk (details given in the text).

Key to $\sigma 1$ cartoon

-  = C-terminal globular head
-  = Cross- β sheet
-  = α -helix

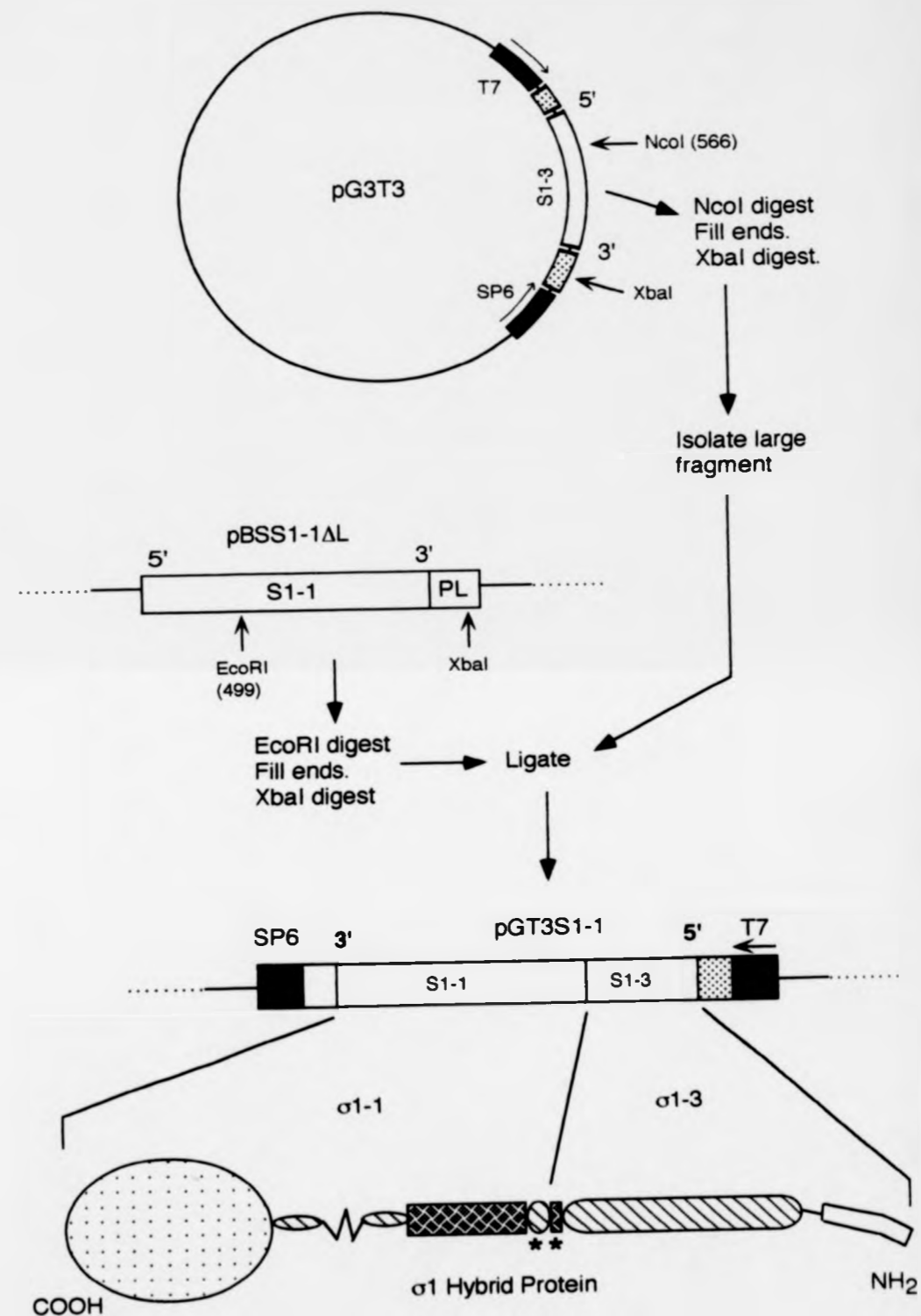


Figure 8.2.9 SDS-PAGE Analysis of the Hybrid σ 1 Protein

In vitro transcriptions were carried out utilizing plasmids pG3T3 and pGT3S1-1 as templates to produce the appropriate σ 1 mRNA. The corresponding σ 1 proteins were synthesized in a commercial reticulocyte lysate as described in section 2.11.1 with the exception that the reaction was performed at 37°C for 30 minutes. Duplicate samples of each protein were either heated to 100°C in sample buffer or incubated at 37°C for 30 minutes in sample buffer before SDS-PAGE (as described in section 2.11.4). The resulting gel was processed by fluorography (section 2.11.5). A photograph of the processed gel is shown opposite. (Reovirus infected cells were prepared as in section 2.2.5)

- Lane R = Reovirus (T3) infected cells
- Lane U = Uninfected cells
- Lane A = σ 1-3 incubated at 100°C
- Lane B = σ 1-3 incubated at 37°C
- Lane C = Hybrid σ 1 incubated at 100°C
- Lane D = Hybrid σ 1 incubated at 37°C
- Lane R' = Reovirus (T1) infected cells

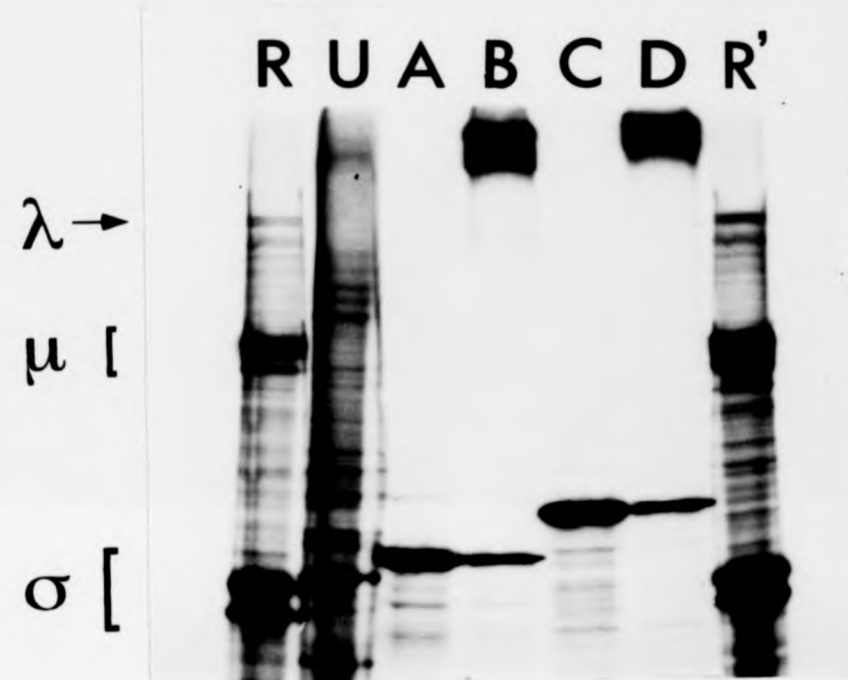
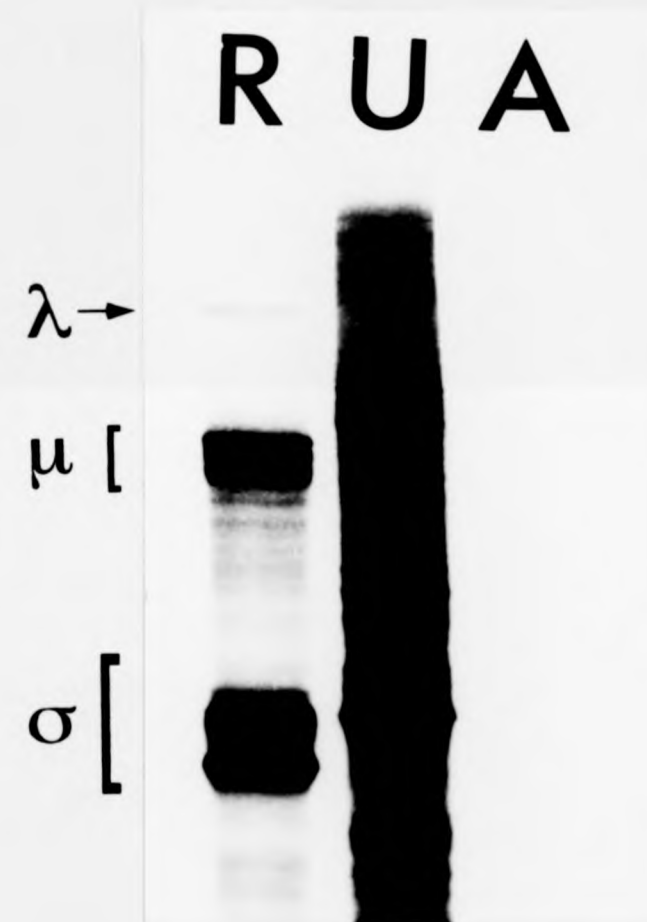


Figure 8.2.10 Assay of the Cell-Binding Ability of the Hybrid $\sigma 1$ Protein

The hybrid-type $\sigma 1$ protein was synthesized *in vitro* as described in the legend to Figure 8.2.9. This was subsequently assayed for L-cell-binding activity as described in section 2.12.2; the resulting "cell-bound" sample was incubated under non-dissociating conditions before SDS-PAGE. A photograph of the processed gel is shown opposite.

- Lane R = Reovirus infected cells
- Lane U = Uninfected cells
- Lane A = Cell bound sample



8.3 DISCUSSION

The expression of full length $\sigma 1$ proteins from cloned copies of the S1-1 and S1-3 genes had been achieved (Chapters 5-7). However, no firm evidence of the cell-binding functions of these proteins was at first obtained, typified in assays where homologous virus did not prevent the apparent attachment of either serotype of $\sigma 1$ protein to L-cells. Thus, the apparent binding was probably non-specific. Two reports by Lee and co-workers, Leone *et al.* (1991b) and Duncan *et al.* (1991), demonstrated the formation of $\sigma 1$ -1 oligomers when synthesized in reticulocyte lysate at 37°C; both authors also alluded to experiments in which $\sigma 1$ was only able to bind to cells as an oligomer. This suggested that possibly, oligomers were not formed at 30°C which would subsequently account for the apparent non-specific binding of the $\sigma 1$ proteins initially observed in this study.

The formation of $\sigma 1$ oligomers in reticulocyte lysate reaction mixes incubated at 30°C and 37°C was therefore investigated. SDS-PAGE analyses under non-dissociating conditions revealed that very little oligomerization occurred at 30°C without incubation in sample buffer at 37°C, whilst in reactions carried out at 37°C this process was obvious but greatly enhanced by the subsequent action of sample buffer (at the same temperature). This should be qualified by stating that only the type 3 protein oligomerized; type 1 remained as monomers under all the conditions employed. Two major oligomeric species that migrated close to the top of the gel were always observed in these assays, although a faster migrating approximately 150 kDa complex was sometimes visualised. Subsequent binding assays utilizing protein translated at 37°C revealed that only the oligomeric form and more specifically, mainly the slowest migrating species was able to bind to L-cells. A similar observation was

made by Leone *et al.* (1991a). Hence, oligomerization of σ 1-3 appears to be necessary for manifestation of the protein's cell-binding ability. The relevance and possible relationship between the different sized oligomers is discussed more fully in the General Discussion, Chapter 11.

These results therefore suggest that the temperature at which the σ 1-3 protein is synthesized influences the oligomerization process, at least *in vitro*. Possibly, the folding of the monomers and any subsequent intersubunit interactions is a largely inefficient process at 30°C. In an *in vivo* study by Bassel-Duby *et al.* (1987), reovirus infected L-cells that had been maintained at 34°C served as a source of σ 1 protein. Oligomerization of the purified σ 1 under non-dissociating conditions was shown to be extremely efficient, with the monomeric species barely evident. Such complete oligomerization was never observed in this study. Hence, in addition to the possible temperature dependence of the process, oligomer formation *in vivo* appears to be a more efficient process compared to that observed *in vitro*. It is possible that factors (or a factor) not present or present in limiting concentrations in the reticulocyte lysate may result in only a small amount of oligomers being able to form.

Thus, the greatly reduced number of oligomers formed when translations are carried out at 30°C would seem to account for the putative non-specific binding observed in the initial binding assays.

The monomeric type 1 σ 1 protein did not manifest a cell-binding ability. However, Duncan *et al.* (1991) have demonstrated binding of σ 1-1 to L-cells but evidence of its oligomeric status was not given or commented upon. The reasons for this discrepancy are unclear, especially as Duncan *et al.* (1991) expressed σ 1-1 in a similar way to that utilized in this study.

As σ 1-1 was only able to form monomers, an attempt was made to oligomerize the protein with the aim of promoting the cell-binding activity

of the protein. An S1-3/S1-1 hybrid gene was therefore created by fusing the 5' 1/3 of the S1-3 gene to the 3' 2/3 of S1-1; these two gene fragments encode the oligomerizing (heptad repeat) region and cell-binding domain of the type 3 and type 1 proteins respectively. The σ 1 hybrid protein derived by the *in vitro* expression of this gene was able to form oligomers but was unable to bind to L-cells. Together with the data concerning σ 1-3, this suggests that oligomerization is necessary but not sufficient for σ 1 to manifest its cell-binding ability (in agreement with a deletion mutant study by Duncan *et al.*, [1991]). The studies with the hybrid molecule also show that the N-terminal one-third of σ 1-3 possesses intrinsic oligomerizing properties as previously demonstrated by Banerjea and Joklik (1990) and Leone *et al.* (1991b;c) and that it does not possess a cell binding function, which is in agreement with its role in anchoring σ 1 to the virion (Mah *et al.*, 1990 and Leone *et al.*, 1991c).

The inability of the hybrid molecule to manifest a cell-binding function may have been the result of a conformational effect. A perfect, non-overlapping hybrid could not easily be created, resulting in a molecule with an N-terminal region elongated by 23 amino acids (Figure 8.2.8) compared to the type 1 protein. This overlap causes an interruption in the coiled-coil and cross- β sheet morphological domains that constitute the N-terminal fibrous rod or stalk (Fraser *et al.*, 1990 and Nibert *et al.*, 1990). It is feasible that this could affect the folding of the C-terminal head region by preventing required folding interactions or possibly promoting incorrect interactions between these two regions. Alternatively, the correct folding of an intact σ 1 molecule may be dependent upon interactions with specific residues of the N-terminal region. In the hybrid molecule, this region is a type 3 structure unlike the remainder of the protein. Thus, although the σ 1-3 N-terminal domain enabled the hybrid protein to oligomerize, other important folding processes may not have been induced.

8.4 SUMMARY

In vitro translation of $\sigma 1$ in a reticulocyte lysate at 37°C was necessary to promote oligomerization of the protein; further incubation in sample buffer increased the amount of oligomerization; only the oligomeric form of $\sigma 1-3$ exhibited cell-binding properties and $\sigma 1-1$ was unable to bind to L-cells as either a monomeric or oligomeric species.

CHAPTER 9

Characterising the σ 1 - L-Cell Interaction

9.1 INTRODUCTION

One of the first steps in the replicative cycle of a virus is attachment to the host cell, a process which is generally accepted as occurring via specific receptor sites or moieties on the cell surface. The presence of a given receptor molecule is often important in determining whether a cell is permissive for infection and hence may be a major factor in determining the tissue tropism and virulence properties of the virus.

Evidence for a defined, homogeneous population of receptor molecules is usually obtained by demonstrating specific, saturable binding of a virus to the host cell. This has been reported for reovirus attachment to a variety of cell types, including L-cells (Armstrong *et al.*, 1984 and Epstein *et al.*, 1984), rat pituitary cells (Maratos-Flier *et al.*, 1983) and rat endothelial cells (Verdin *et al.*, 1989), see also Table 1.6.1. All these studies utilized radiolabelled virions in the binding assays. However, in this study, *in vitro* synthesized $\sigma 1$ was used to analyse the cell-binding process more closely. For this approach to be of relevance to the situation involving virus particles, the cell-binding properties of $\sigma 1$ had to reflect that of native virions and bind in a specific, saturable manner to the chosen host cells, which in this case was L-cells.

In this chapter, some of the parameters of the *in vitro* translated $\sigma 1$ -3 - L-cell binding interaction are described.

9.2 RESULTS

9.2.1 Time Course of the Binding Process

A simple kinetic analysis of the σ 1-3 - cell-attachment process was undertaken as the 1 hour binding period of the previously performed binding assays had been based on the methods of Lee *et al.* (1981b) and Duncan *et al.* (1991). All binding assays were carried out at 4°C (as before) to prevent internalization of the ligand. Accordingly, aliquots of a translation reaction mix containing σ 1-3 were overlaid on monolayers of L-cells for various lengths of time and the proportion of bound material examined by SDS-PAGE, as shown in Figure 9.2.1. After 30-45 minutes approximately half of the protein that was able to bind had done so and by 90 minutes essentially all of this material had bound as further incubation had not significantly increased the intensity of the bound σ 1-3.

It was possible that the relative concentration of σ 1-3 in the translation mix was limiting and that an increase in binding after 90 minutes would have been noticed if more protein had been present in the assay mixes. Alternatively, saturation of the receptor sites could have been achieved (although this did seem unlikely given the relatively small amount of protein synthesized in the lysate). Another assay was therefore performed to examine these alternatives. A similar volume of translation mix to that described in Figure 9.2.1 was bound to L-cells for 1 hour, after which it was transferred to a second monolayer for 1 hour and so on for a further two occasions. Thus, in total, four consecutive bindings with the same mix were performed and the result is shown in Figure 9.2.2. As observed previously, most of the σ 1-3 had bound after 1 hour although a little more did bind to the second monolayer; virtually no bound material

Figure 9.2.1 Time Course of $\sigma 1$ Attachment to L-cells

$\sigma 1$ -3 mRNA was transcribed *in vitro* from plasmid pG3T3 and the encoded $\sigma 1$ -3 protein translated in a commercial reticulocyte lysate at 37°C for 30 minutes. This reaction mix was diluted eightfold into PBS and 100 μ l bound to monolayers of L-cells for periods of time ranging between 15-240 minutes as described in section 2.12.2. Cell bound protein was analysed by SDS-PAGE under non-dissociating conditions (37°C for 30 minutes in sample buffer) essentially as described in section 2.11.4. A photograph of the fluorographed gel (carried out as in section 2.11.5) is shown opposite. The numbers at the top of each lane represent the length of time the samples were bound to the cell monolayers.

Reovirus infected cells were prepared as in section 2.2.5, the viral polypeptides in which acted as size markers; many host cell proteins are also evident in this lane (R) probably due to an incomplete shut-off of host cell protein synthesis. Lane U represents an uninfected cell lysate for comparison.

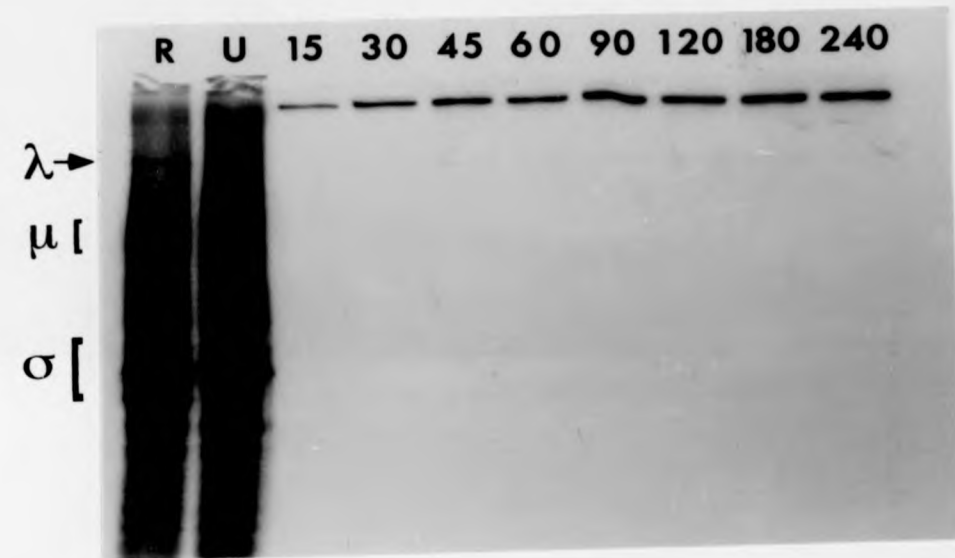
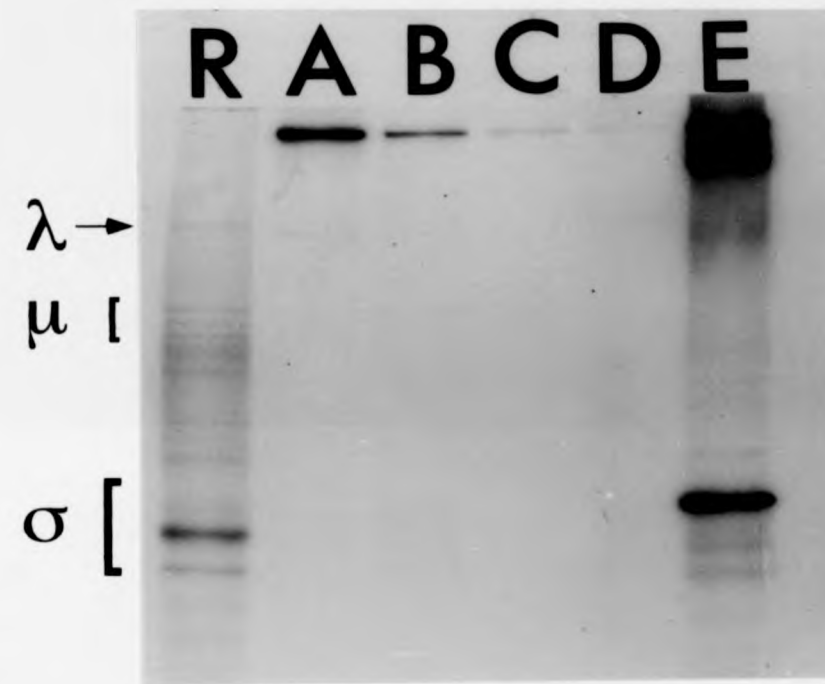


Figure 9.2.2 Consecutive Monolayer Binding Assay

Protein σ 1-3 was *in vitro* translated and diluted into PBS as described in the legend to Figure 9.2.1. 100 μ l of this diluted binding mix was then overlaid on a monolayer of L-cells for 1 hour, after which it was removed and applied to a second monolayer for a further hour. This was repeated for a further two monolayers. During the 1 hour cell incubation periods, the preceding monolayers were washed, solubilized, incubated in sample buffer under non-dissociating conditions and placed on ice until the final monolayer had been processed. An aliquot of the remaining unbound material was similarly incubated in sample buffer. All samples were subjected to SDS-PAGE and the gel processed as in Figure legend 9.2.1. The resulting autoradiograph is shown opposite.

- Lane R Reovirus infected cells
- Lane A First binding
- Lane B Second binding
- Lane C Third binding
- Lane D Fourth binding
- Lane E Unbound sample



was evident on the 3rd and 4th consecutive monolayers. Future binding assays were carried out for 1 hour on the basis of these two experiments.

The presence of a relatively intense oligomer band in the unbound material (Figure 9.2.2, lane E) that comigrated with the cell-bound species, indicates that the monolayers were not saturated but that all of the protein capable of binding had probably done so; the rest of this oligomeric species present in the translation mix was possibly in an incorrect conformation to bind. It was also possible that the amount of oligomer in the unbound sample was over-represented on the gel. It had been incubated in sample buffer under non-dissociating conditions prior to SDS-PAGE and these conditions appear to increase the relative amount of oligomer in σ 1-3 translation reaction mixes, (Figure 8.2.5). This gel also demonstrates that, as observed before, (Figure 8.2.6) it is mainly the slowest migrating oligomeric species that binds to the cells.

9.2.2 Saturation of Receptor Sites

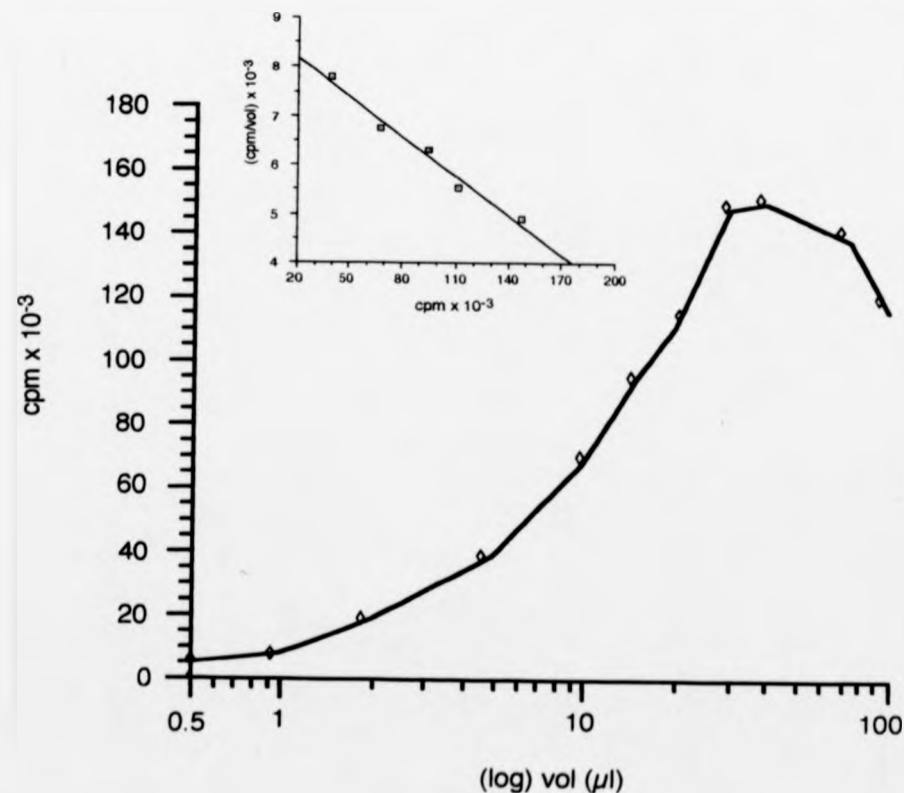
The saturation of the binding sites for protein σ 1-3 was next investigated. Increasing amounts of *in vitro* translated σ 1-3 were bound to cells for 1 hour in an attempt to saturate the appropriate binding sites. Bound material was analysed both by SDS-PAGE (result not shown) and by directly counting aliquots of the resulting solubilized monolayer in a scintillation counter, the results of which are presented graphically in Figure 9.2.3. As can be seen, perhaps surprisingly in view of the amount of protein normally made *in vitro*, saturation was possible.

To calculate the number of "counts bound" that represents saturation of the receptor sites, the data was converted to a Scatchard format, shown as an inset figure in the larger graph. The intercept on the x axis is equivalent to the counts bound at saturation and a value of

Figure 9.2.3 Saturation of L-cell Binding Sites for σ 1-3

a) Protein σ 1-3 was translated *in vitro* as described in Figure legend 9.2.1 but was not diluted immediately into PBS. Increasing volumes of the reaction mix were diluted into 100 μ l of PBS up to a maximum of 100 μ l of lysate (which was not diluted at all). The samples were then bound to monolayers of cells and the cell-membranes solubilized in 100 μ l of 0.5% NP40 as described in section 2.12.2. 10 μ l of each sample was placed in scintillant and counted. These results are shown in the graph below. The linear region was converted to a Scatchard format and this data is shown in the inset graph

The Scatchard data was utilized to calculate the number of L-cell binding sites for σ 1-3. This calculation is shown opposite.



b) Calculation of the Number of σ 1-3 Receptor Sites

Intercept on x axis of Scatchard plot = no. counts bound at saturation
= 176,000 cpm

Specific activity of ^{35}S methionine = 1000 Ci/mmole (or 1 μ Ci/pmole)

Concentration of ^{35}S methionine: 66 μ l = 1mmole or 15pmole/ μ l

Concentration of cold methionine in the lysate = 5 μ M or 5pmoles/ μ l

Ratio of cold : hot methionine in reaction mix = 17 : 1

$$= (17 \times 5) : (1 \times 15)$$

$$= 5.6 : 1$$

1 μ Ci = 2.2×10^6 dpm

8 methionines per molecule of σ 1-3

1/10th of sample counted

Therefore, no. pmoles σ 1-3 = $\frac{176,000 \times 10 \times 5.6}{(2.22 \times 10^6) \times 8}$

$$= 0.55 \text{ pmoles } \sigma 1-3$$

No. molecules σ 1-3 = $0.55 \times 6.023 \times 10^{23}$ (Avogadro's No.)

$$= 3.0 \times 10^{11}$$

There are $\sim 5 \times 10^5$ cells/monolayer

Efficiency of counting of ^{35}S met = 70%

No. receptor sites/cell = $\frac{3.0 \times 10^{11} \times 100}{5 \times 10^5 \times 70}$

$$= \sim 8.57 \times 10^5$$

If σ 1-3 is a trimer, no. receptor sites = 2.86×10^5

If σ 1-3 is a tetramer, no. receptor sites = 2.14×10^5

The efficiency of counting of ^{35}S met was determined in essentially the same manner as for ^3H -UTP in section 2.10.3.

176,000 cpm was obtained. This value was used to calculate the number of σ 1-3 receptor sites/cell and figures of approximately 2.8×10^5 or 2.1×10^5 receptor sites/cell were derived if σ 1-3 binds as a trimer (as proposed by Leone *et al.*, 1991a) or a tetramer (as proposed by Bassel-Duby *et al.*, 1987; Fraser *et al.*, 1990 and Nibert *et al.*, 1990) respectively. The calculation used to obtain these numbers is given in legend b to Figure 9.2.3. The structural order of the oligomer, i.e. a trimer or tetramer is discussed further in section 11.1.1.

9.2.3 Specificity of the Binding Process

The specificity of the cell attachment of σ 1 was assessed by competition assays in which the *in vitro* translated protein was bound to cells in the presence of virus; if protein and virus utilize the same receptor then an excess of virus should inhibit binding of σ 1.

Binding assays were performed in which aliquots of translation mix contained increasing amounts of type 3 reovirus. Bound material was analysed by SDS-PAGE and as can be seen in Figure 9.2.4, increasing the amount of virus in the binding mix resulted in an increasing inhibition of binding until virtually complete inhibition was achieved at the two highest concentrations of virus employed. The small amount of σ 1-3 bound with these two amounts of virus probably represented non-specific binding.

A similar assay was performed with type 1 virus as the competing species, the result of which is shown in Figure 9.2.5. Here, very little competition is evident and only with the highest concentration of virus employed. This could be due to a non-specific blocking effect of the high concentration of virus impairing the interaction of protein with its receptor. Together, these experiments suggest that the T1 and T3 virus bind to different receptors on the same cell type.

Figure 9.2.4 **σ 1-3 : Reovirus Type 3 Competition Assay**

σ 1-3 was translated *in vitro* as described in Figure legend 9.2.1 and diluted sixfold into PBS. A twofold dilution series of reovirus T3 was made (in PBS). 20 μ l of each virus dilution was added to 50 μ l aliquots of the σ 1-3 *in vitro* translation reaction and the volume made up to 100 μ l with PBS. These mixes were then bound to monolayers of L-cells for 1 hour and analysed by SDS-PAGE under non-dissociating conditions as described in section 2.12.2. A photograph of the resulting autoradiograph is shown opposite.

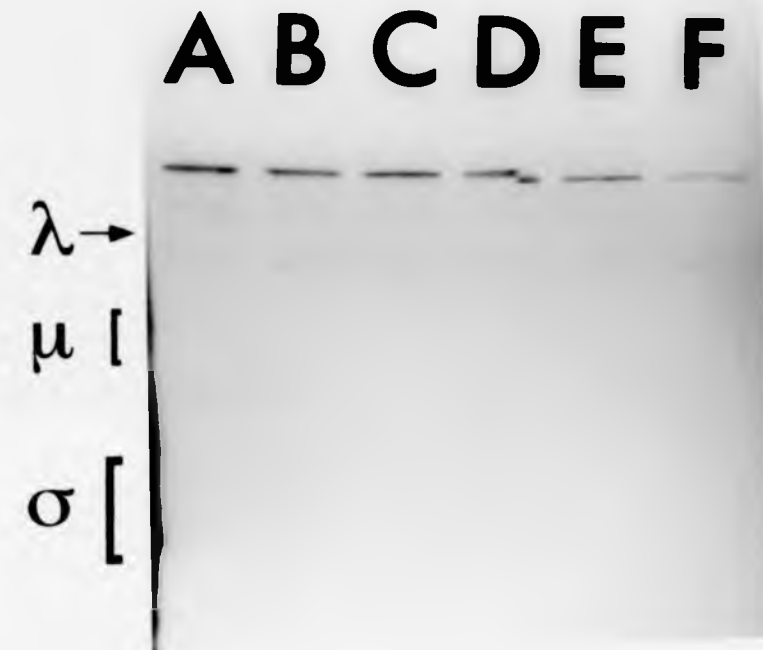
Lane R	Reovirus infected cells
Lane A	σ 1-3 vs 0 μ g T3 virus
Lane B	σ 1-3 vs 10 μ g T3 virus
Lane C	σ 1-3 vs 20 μ g T3 virus
Lane D	σ 1-3 vs 40 μ g T3 virus
Lane E	σ 1-3 vs 80 μ g T3 virus
Lane F	σ 1-3 vs 160 μ g T3 virus



Figure 9.2.5 **σ 1-3 : Reovirus Type 1 Competition Assay**

Protein σ 1-3 was competed against reovirus T1 exactly as described for the T3 virus in Figure legend 9.2.4. The positions of reovirus proteins co-electrophoresed as size markers are indicated.

- Lane A σ 1-3 vs 0 μ g T1 virus
- Lane B σ 1-3 vs 10 μ g T1 virus
- Lane C σ 1-3 vs 20 μ g T1 virus
- Lane D σ 1-3 vs 40 μ g T1 virus
- Lane E σ 1-3 vs 80 μ g T1 virus
- Lane F σ 1-3 vs 160 μ g T1 virus



A small amount of the approximately 150 kDa species of oligomer is noticeable in Figures 9.2.4 and 9.2.5. This species was observed in some of the SDS-PAGE analyses described in Chapter 8 (e.g. Figures 8.2.5 and 8.2.6) and is discussed further in Chapter 11.

9.2.4 SUMMARY

The interaction of σ 1-3 with L-cells was investigated in order to determine whether binding was specific and saturable. This was important for two reasons: to ascertain whether binding reflected the situation observed with native virions and as a prerequisite for the interpretation of intended binding assays with mutant σ 1-3 proteins (Chapter 10).

Maximum binding of σ 1-3 was seen after approximately 90 minutes at 4°C, which compares well with the observations of Armstrong *et al.* (1984) and Epstein *et al.* (1984) who both reported a similar time for the maximum binding of type 3 virus to L-cells. Not all of the slowest migrating oligomeric species present in the σ 1-3 translation mix - (demonstrated in the last chapter to be the major oligomeric σ 1-3 binding species)- was in fact able to bind to cells even after a 4 hour incubation period, as shown by analysis of the unbound proteins (Figure 9.2.2). The construction of a saturation curve also demonstrated that the amount of translation mix utilized in the time course assays was not a saturating quantity. The species unable to bind were possibly composed of incorrectly folded molecules and/or were structures whose subunits had associated improperly; this may reflect a relative inefficiency in these processes in the *in vitro* system. However, it was also possible that the incubation step in sample buffer, prior to analysis by SDS-PAGE increased the relative amount of oligomeric species by causing oligomerization of monomers.

The number of receptor sites/cell reported in the literature for reovirus T3 on L-cells ranges from 1.6×10^4 (Sawutz *et al.*, 1987) to $0.86-1.1 \times 10^5$ (Gentsch and Hatfield, 1984) to a maximum of $3-5 \times 10^5$ (Armstrong *et al.*, 1984 and Ambler and Mackay, 1991). A figure of $\sim 2.8 \times 10^5$ was determined in this study which is in fairly good agreement with those given above considering the range of these values. The tenfold range of values itself may be due to the different methods of assay, such as binding of virus to cells in suspension or monolayer and differences in the specific activities of radiolabelled virions obtained by the various groups. Epstein *et al.* (1984) suggested that the high specific activity of their virus particles allowed for better resolution of their binding curves. Regardless of the experimental method, any such figure is probably best regarded as an approximation, although it was encouraging that the figure obtained in this study using the cell-binding protein did not differ significantly from those determined with virus particles.

Binding of $\sigma 1-3$ to L-cells was found to be specific as competition with homologous type virus (type 3) abolished attachment. However, type 1 virus did not effect specific inhibition of $\sigma 1-3$ binding, which would suggest that these two serotypes utilize different receptors to bind to L-cells. A small decrease in binding was noted at the highest concentration of T1 virus employed in the assay and may represent a non-specific blocking at this concentration or alternatively there could be some overlap in receptor determinants between the two viral serotypes. This is discussed more fully in Chapter 11.

CHAPTER 10

Generation of $\sigma 1$ Mutants and Assessment of their Cell-Binding Ability

10.1 INTRODUCTION

Evidence has accumulated during the last decade to suggest that the cell-binding function of $\sigma 1$ is harboured in its carboxy-terminal region. This was initially proposed by Bassel-Duby *et al.* (1985) who predicted a structural model for $\sigma 1$ in which the C-terminal portion of the protein formed a globular structure, positioned atop an α -helical rod formed by the N-terminal region. It was suggested that the former structure contained receptor recognition domains whilst the latter anchored the molecule to the virion. The structural details were later refined by Nibert *et al.* (1990) who nevertheless suggested no major differences in the essential predicted morphology of the molecule (section 1.4.1 of the Introduction). Furthermore, Furlong *et al.* (1988) visualised reovirus type 2 $\sigma 1$ molecules, both attached to and in isolation from virus particles as having this "lollipop" structure; Fraser *et al.* (1990) made similar observations from computer enhanced micrographs of isolated $\sigma 1$ type 2 protein.

The cell-binding ability of various $\sigma 1$ deletion mutants, expressed as fusion constructs with the β -galactosidase protein in *E. coli* (Nagata *et al.*, 1987) provided more direct proof that this binding function was located in the C-terminal half of the protein. Also, Yeung *et al.* (1989), found that a carboxy-terminal fragment of $\sigma 1$ (generated by trypsin treatment of the intact protein), was capable of binding to L-cells.

A mutational analysis of the $\sigma 1$ protein was therefore undertaken to more precisely map the location of the receptor recognition region(s), the aim of which was to further characterise the virus-host cell attachment process.

10.2 STRATEGY

The initial approach taken in the mutational analysis of the carboxy-terminal region of σ 1-3 was to generate a set of mutants by exonuclease III digestion of the 3' end of the cloned S1-3 gene in plasmid pG3T3. A series of clones that contained an increasing amount of 3' deleted material could then be assayed for their cell-binding function. However, despite the generation of a set of 3' deletion mutants, it was found that all contained deletions of approximately 500 nucleotides or more. Unfortunately, translation of these mutants would have resulted in σ 1 molecules lacking globular head structures (data not shown).

An alternative was to delete specific sequences of nucleotides by oligonucleotide directed mutagenesis. The sequences of the S1 genes of the three viral serotypes and of the predicted σ 1 protein sequences are highly diverged between the three reovirus serotypes (Cashdollar *et al.*, 1985; Duncan *et al.*, 1990 and Nibert *et al.*, 1990). However, Duncan *et al.* (1990) delineated five areas of relatively high sequence homology shared by all three of the proteins and proposed that they could be involved in certain conserved functions of σ 1 (for example oligomerization and cell-attachment). These five regions are distributed throughout the molecule and it was further suggested that one or more of the domains located in the C-terminal region could be involved in the recognition of a putative common L-cell receptor for all three virus serotypes (Duncan *et al.*, 1990). Lee *et al.* (1981b) had originally proposed that the reovirus serotypes utilized a common receptor on L-cells. This issue has already been introduced in section 1.6.1.

It was decided to delete two of the regions proposed by Duncan *et al.* (1990) to be involved in receptor recognition and to ascertain their role in cell-binding. The two areas chosen for deletion both reside within the C-

terminal half of the protein and are labelled D (aa 360-485) and E (aa 418-451) as in the report of Duncan *et al.* (1990). Figure 10.2.1 is a diagram of σ 1-3 showing the relative positions of regions D and E.

The mutagenesis strategy was as follows : subcloning of the cDNA of the S1-3 gene into M13mp19, independent deletion of regions D and E by oligonucleotide directed mutagenesis and replacement of the wild type S1-3 cDNA fragments with those containing the deletions. The method of Vandeyar *et al.* (1988) was utilized to carry out the oligonucleotide directed mutagenesis (previously mentioned in Chapter 7). This protocol is briefly described below and is shown schematically in Figure 10.2.2.

The mutagenic oligonucleotide is annealed to ss DNA prepared from the recombinant M13 clone in which the sequence to be mutated resides. Synthesis of the second (mutant) strand is achieved with a dNTP mix that includes 5-methyl dCTP, instead of dCTP; this ultimately enables selection of the mutant strand. The resulting heteroduplex DNA molecules are digested with the restriction enzymes *MspI* and *HhaI*. The nonmethylated strand of C⁵mCGG/CCGG duplexes can be cleaved by *MspI*; thus the parental strand of the duplex is selectively digested by the action of this enzyme. Single-stranded DNA is cleaved at about half the rate of ds DNA by the enzyme *HhaI* but it is unable to cleave hemi-methylated DNA. This enzyme therefore preferentially digests uncopied and partially copied ss molecules which contribute to the low mutation frequency obtained with the original method of Zoller and Smith (1983). The parental strand of nicked duplex molecules, resulting from the action of *MspI*, is then degraded by the action of exonuclease III. This protocol therefore selects for the mutant strand and results in a mutation frequency of approximately 90% (Vandeyar *et al.*, 1988).

The reaction mixes must be transformed into an Mcr A⁻B⁻ *E. coli* host as the Mcr system is responsible for methylation dependent restriction

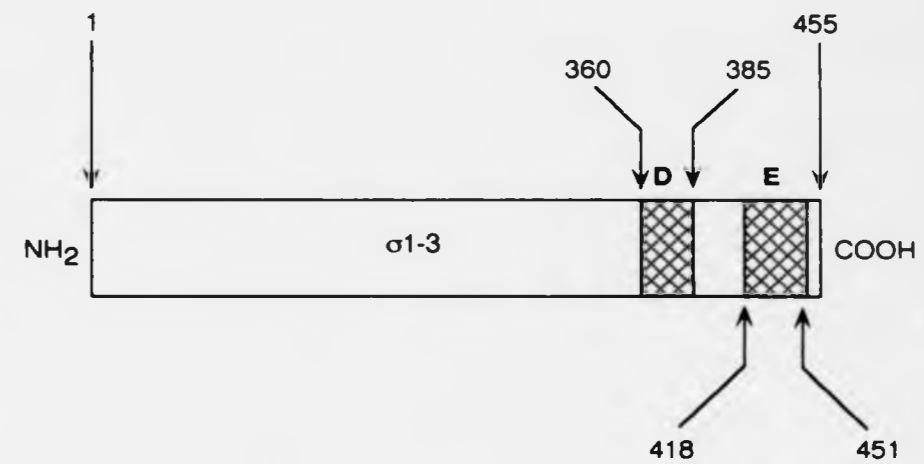
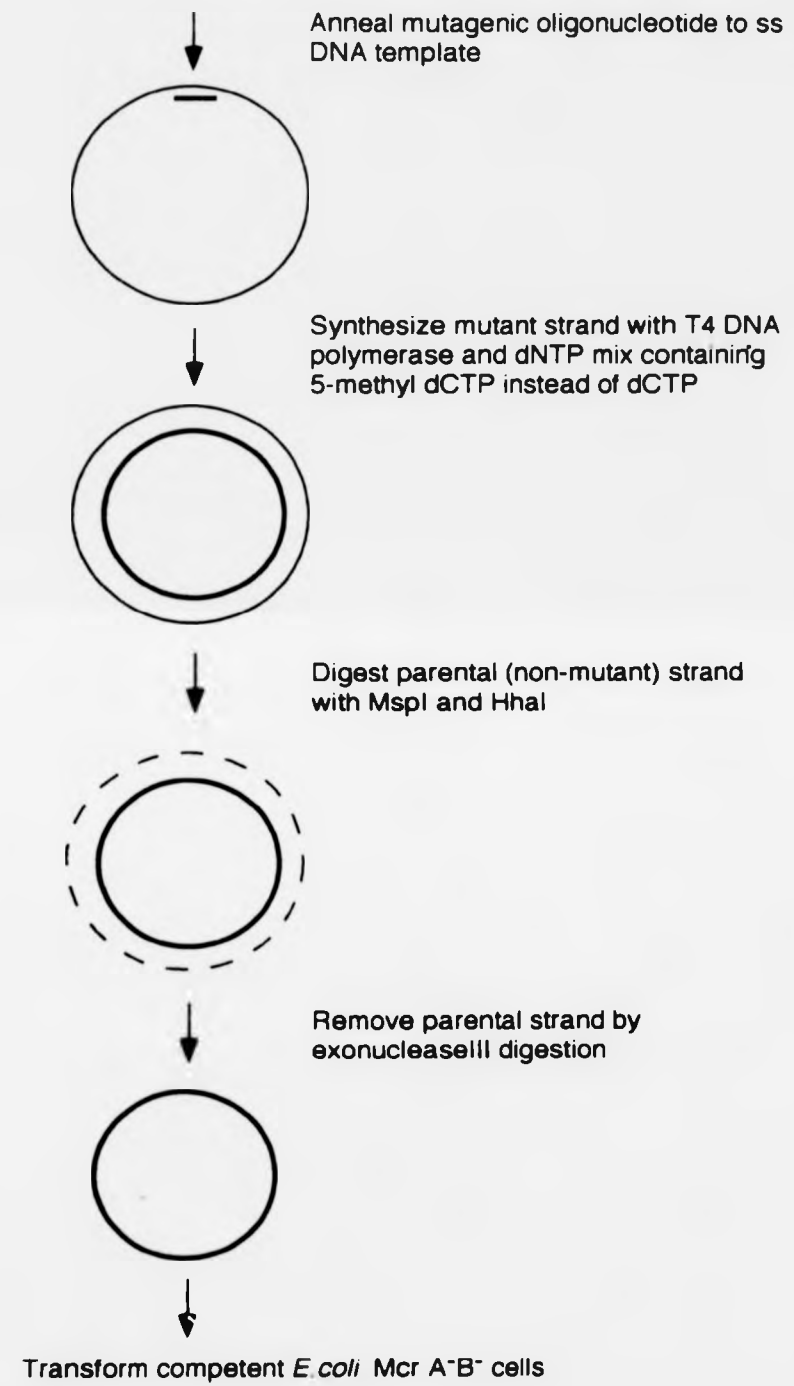


Figure 10.2.1 Schematic Diagram of σ 1-3 Showing the Conserved Regions D and E

The diagram of σ 1-3 shown above has the conserved regions D and E marked upon it. These regions were proposed to be relatively conserved between the three serotypes of σ 1 protein; region D exhibits a 46% homology and region E 32%, whilst the overall homology of the three proteins is 17%. The conserved regions are marked by hatched boxes and their bounding amino acid residues are denoted by the numbers above or below the respective boxes.

Figure 10.2.2 Schematic Representation of the Method of Oligonucleotide Directed Mutagenesis. Adapted from Vandeyar *et al.* (1988). See text for details.



of the methylated target sequences ^5mCG and $\text{G/A}^5\text{mC}$ (Raleigh and Wilson, 1986) and would thus digest the mutant strand (Vandeyar *et al.*, 1988).

10.3 RESULTS

10.3.1 Construction of S1-3 : M13mp19 Recombinant Clones

The entire S1-3 cDNA sequence was subcloned from plasmid pG3T3 into M13mp19 to generate the recombinant clone mp19S1-3, as shown in Figure 10.3.1. Single-stranded DNA prepared from this clone could then be utilized as template in the generation of both of the deletions, which obviated the need to generate two mp19 recombinant clones for the deletion of regions D and E.

10.3.2 Generation and Detection of mp19S1-3 Mutants

Single-stranded DNA from the mp19S1-3 recombinant was prepared and mutagenised using oligonucleotides S1-3 Δ D and S1-3 Δ E in independent reactions (as described in sections 2.8.3-2.8.6) to generate the mutant clones mp19S1-3 Δ D and mp19S1-3 Δ E. The sequences of the mutagenic oligonucleotides are shown in Figure 10.3.2.

A high frequency of mutation is expected with this method of mutagenesis. Accordingly, frequencies of 70% and 80% were observed in the generation of the mp19S1-3 Δ D and Δ E mutants respectively. The corresponding data is shown for the latter mutants only (Figure 10.3.3).

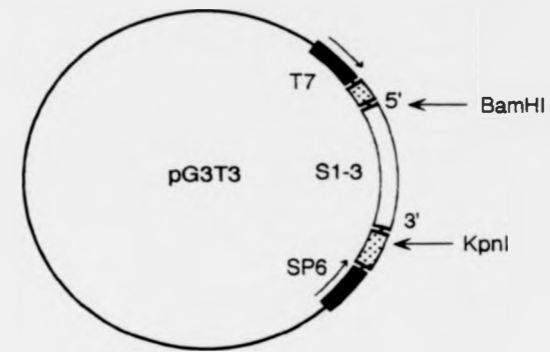
The high frequency of mutation enabled the mutant clones to be directly identified by sequence analysis. Sequence comparison of the appropriate regions of the mutant mp19S1-3 Δ D and Δ E clones with the

Figure 10.3.1 Construction of the Recombinant Clone mp19S1-3

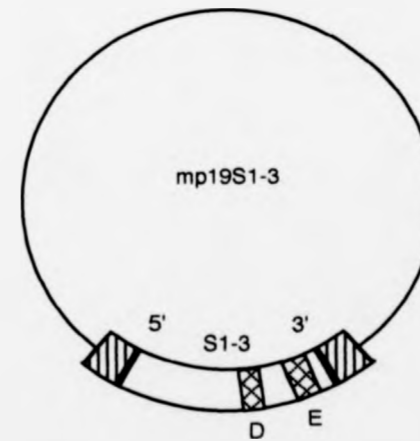
Plasmid pG3T3 was digested with the restriction enzymes *Bam*HI and *Kpn*I which cut in the 5' end of the S1-3 cDNA sequence and in the 3' polylinker respectively. The resulting "gene" fragment was purified from a 1% agarose gel as described in section 2.9.1. M13mp19 RF DNA was digested with these two enzymes which both cleave at sites in the polylinker region; this DNA was similarly purified from an agarose gel. The S1-3 gene fragment was then ligated to the mp19 RF DNA to generate clone mp19S1-3.

Key

- SP6 = SP6 phage promoter sequence
- T7 = T7 phage promoter sequence
- = pGEM-3Z polylinker sequence
- ▨ = M13mp19 polylinker sequence
- ▩ = Region D or E



BamHI/KpnI digest.
Isolate gene fragment.
Ligate into BamHI/KpnI
digested M13mp19



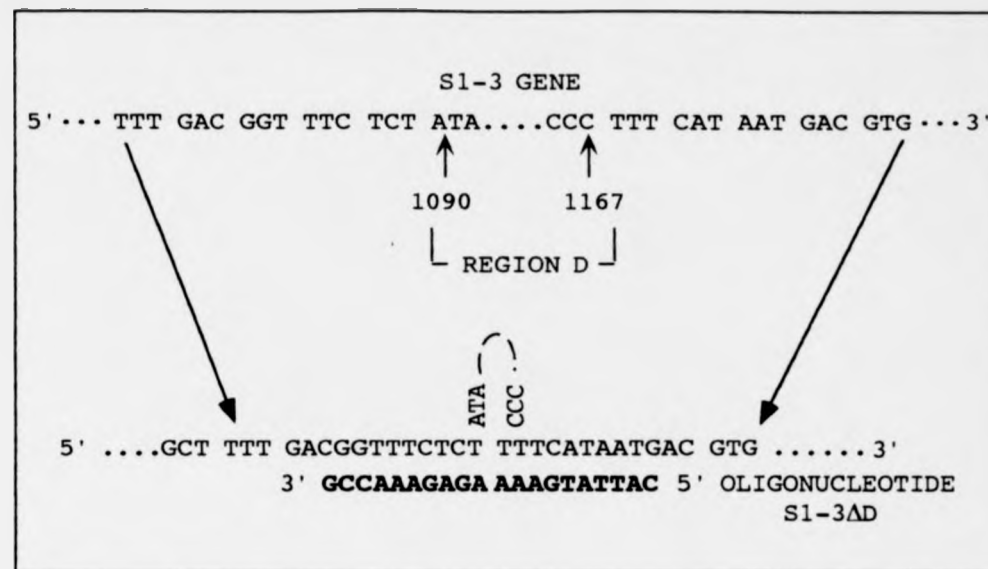
mp19S1-3 single-stranded DNA functions as
template for the independent deletion of
regions D and E.

Figure 10.3.2 Sequences of the Oligonucleotides Utilized in the Deletion of Regions D and E in the Cloned S1-3 Gene

Panel A shows the bounding nucleotides of region D within the S1-3 cDNA. Oligonucleotide S1-3ΔD is shown in bold type and is complementary to two 10 nucleotide sequences located immediately 5' and 3' to these bounding nucleotides. Annealing of the oligonucleotide to the ss DNA template would thus cause the sequence belonging to region D to loop out and ultimately be deleted.

Panel B Similarly, this diagram shows the bounding nucleotides of region E and the sequence of oligonucleotide S1-3ΔE utilized to effect its deletion from the DNA copy of the S1-3 gene.

A



B

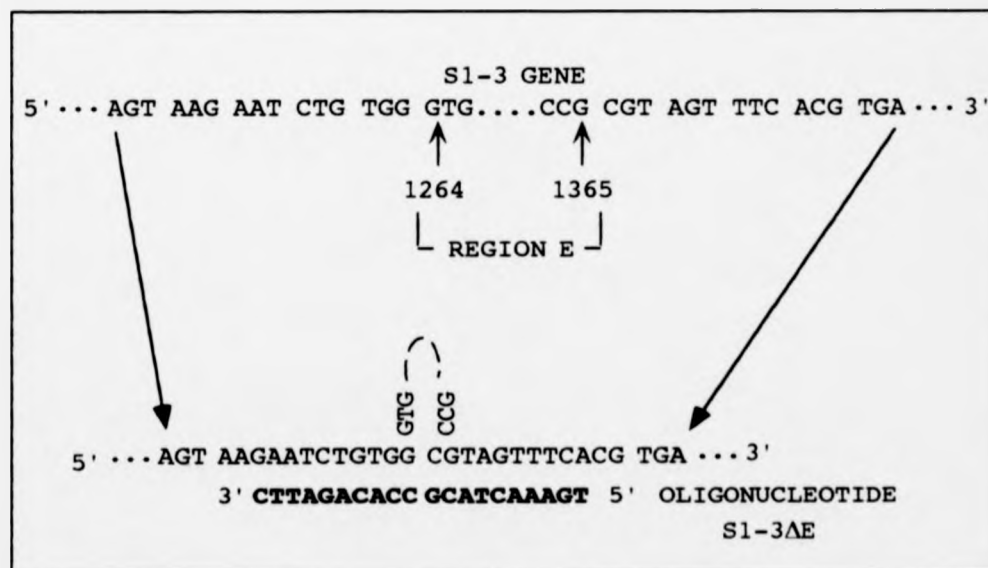



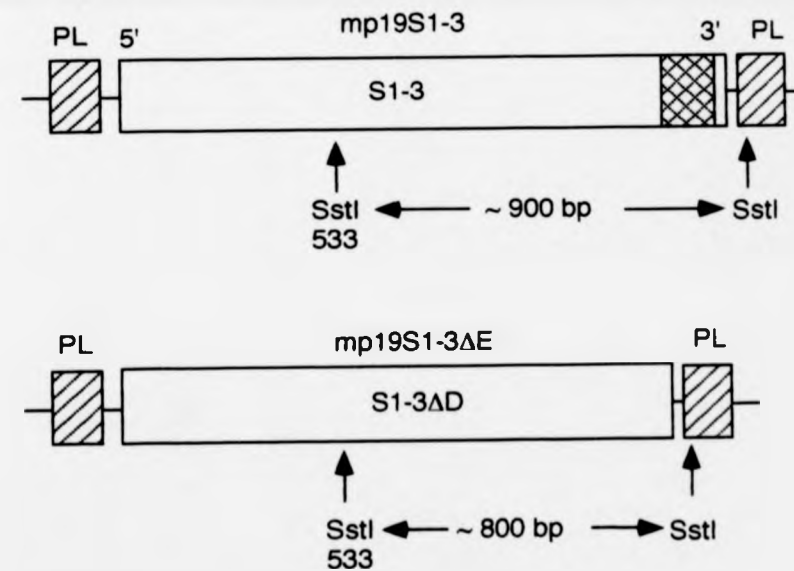
Figure 10.3.3 Restriction Enzyme Digest Analysis of Putative mp19S1-3 Deletion Mutants to Demonstrate the High Level of Mutation Frequency

Ten plaques resulting from the mutagenesis of mp19S1-3 ss DNA with oligonucleotide S1-3ΔE were cultured as described in section 2.8.3. Replicative form DNA was isolated (section 2.7.3), digested with the restriction enzyme *Sst* I and electrophoresed on a 1% agarose gel. A photograph of this gel is shown opposite.

The mutant mp19S1-3ΔE DNA has approximately 100 nucleotides deleted and thus produces an ~ 800 bp band after digestion with *Sst* I; similar digestion of the non-mutated mp19S1-3 RF DNA results in an ~ 900 bp band, shown schematically in the plasmid maps below the photograph. Thus, it can be seen from the photograph that 80% of the putative mutants contained this deletion.

- Lane M Ladder of DNA fragments (BRL)
- Lane 1 mp19S1-3 RF DNA (non-mutated)
- Lanes 2-11 RF DNA from putative mp19S1-3ΔE mutants

 = Region E



corresponding wild type S1-3 cDNA is shown in Figures 10.3.4 and 10.3.5 respectively. Nucleotides 1090-1167 of the S1-3 DNA, corresponding to amino acids 360-385, were removed in mutant mp19S1-3 Δ D, which would result in a σ 1-3 protein truncated by 26 amino acids. The mp19S1-3 Δ E mutant has nucleotides 1264-1365, amino acids 418-451 deleted, resulting in a 34 amino acid truncated σ 1 molecule.

The sequence of the entire region to be utilized in the replacement of the equivalent wild type fragment (demarcated by the enzyme sites shown in Figures 10.3.6 and 10.3.7) was determined to ensure no adventitious mutations had been incorporated during mutagenesis. Figure 10.3.4 does show an apparent insertion of a G residue in the sequence determination of clone mp19S1-3; however several other similar sequence analyses proved this to be a spurious band (data not shown).

10.3.3 Construction of the pG3T3 Mutants

Replicative form DNA from each of the mutant clones was isolated and digested with the restriction enzymes required to release fragments containing the respective deletions. Aliquots of pG3T3 plasmid DNA were digested with the necessary restriction enzymes and recombined with the appropriate mutant fragments to create plasmids pG3T3 Δ D pG3T3 Δ E as shown in Figures 10.3.6 and 10.3.7 respectively. The respective mutant pG3T3 clones were easily identified as restriction enzyme digest analysis of DNA from the putative recombinant clones released a fragment that was truncated in comparison to pG3T3 DNA (results not shown).

Figure 10.3.4 Sequence Analysis of mp19S1-3ΔD

Several mp19S1-3ΔD clones were sequenced, as described in section 2.8.8.1 and the resulting reaction mixes electrophoresed on an 8% polyacrylamide gel as in section 2.8.9. The sequence of one mutant and the non-mutated mp19S1-3 clone (for comparison) is shown opposite. The non-coding strand of S1-3 in both the mutated and non-mutated mp19 clones was analysed. Hence, the sequence obtained was oriented in the 3'-5' direction. Nucleotides 1090-1167 (region D) were deleted in mp19S1-3ΔD resulting in the juxtaposition of nucleotides 1089 and 1168; these latter two nucleotides are marked with arrows. The arrow adjacent to the wild type sequence denotes sequence common to the mutated and non-mutated clones. An interpretation of some of the determined sequence is written below the photograph. Here, S1-3 "wild type" refers to the non-mutated mp19S1-3 DNA.

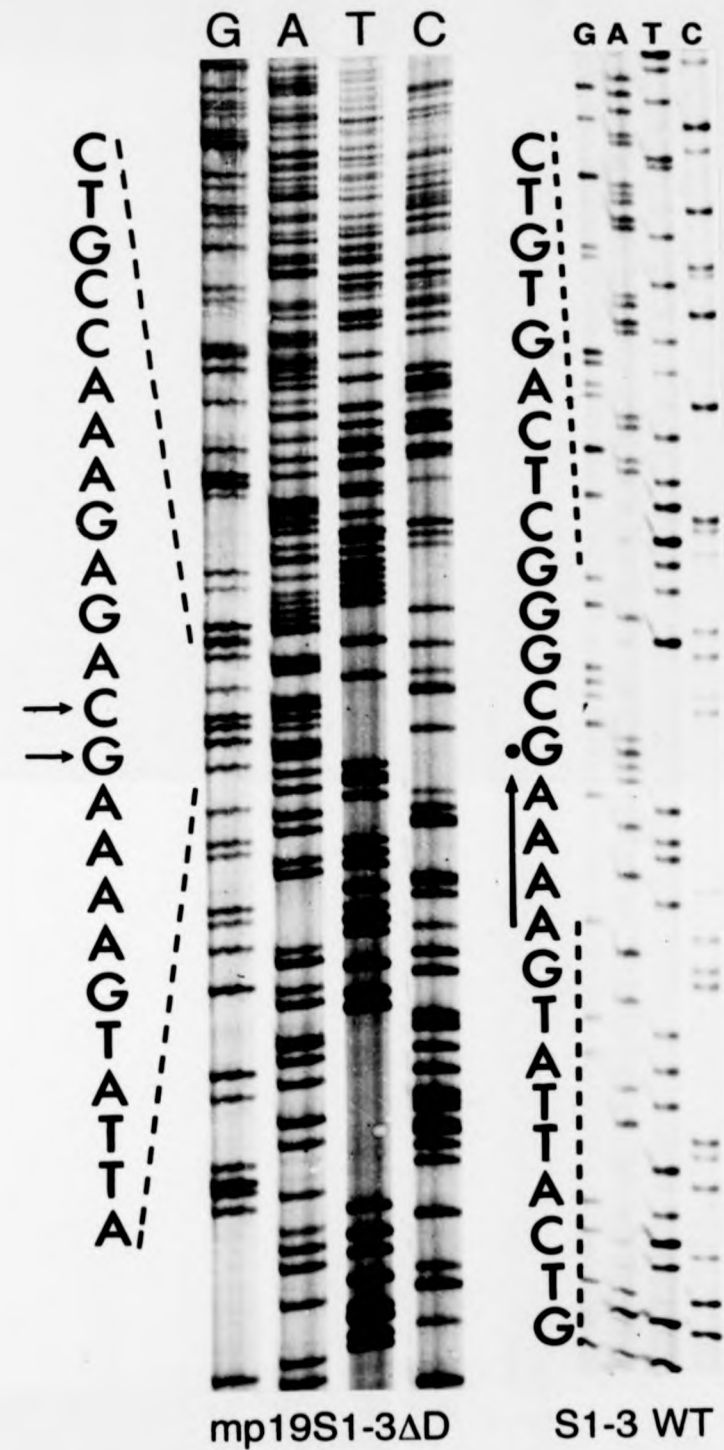
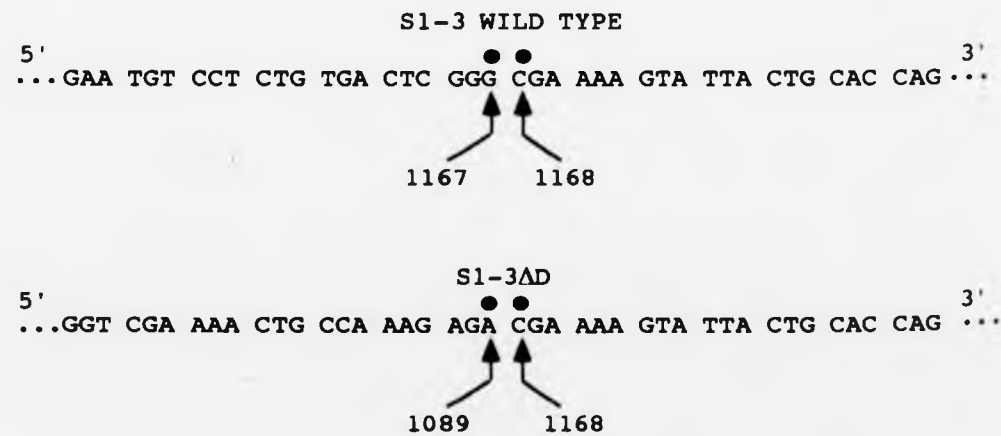


Figure 10.3.5 Sequence Analysis of mp19S1-3ΔE

Several mp19S1-3ΔE clones were sequenced, as described in section 2.8.8.1 and the resulting reaction mixes electrophoresed on an 8% polyacrylamide gel as in section 2.8.9. The sequence of one mutant and the non-mutated mp19S1-3 clone (for comparison) is shown opposite. The non-coding strand of S1-3 in both the mutated and non-mutated mp19 clones was analysed. Hence, the sequence obtained was oriented in the 3'-5' direction. Nucleotides 1264-1365 (region E) were deleted in mp19S1-3ΔD resulting in the juxtaposition of nucleotides 1263 and 1366; these latter two nucleotides are marked with arrows. The arrow adjacent to the wild type S1-3 sequence denotes sequence common to the mutated and non-mutated clones. An interpretation of some of the determined sequence is written below the photograph. Here, S1-3 "wild type" refers to the non-mutated mp19S1-3 DNA.

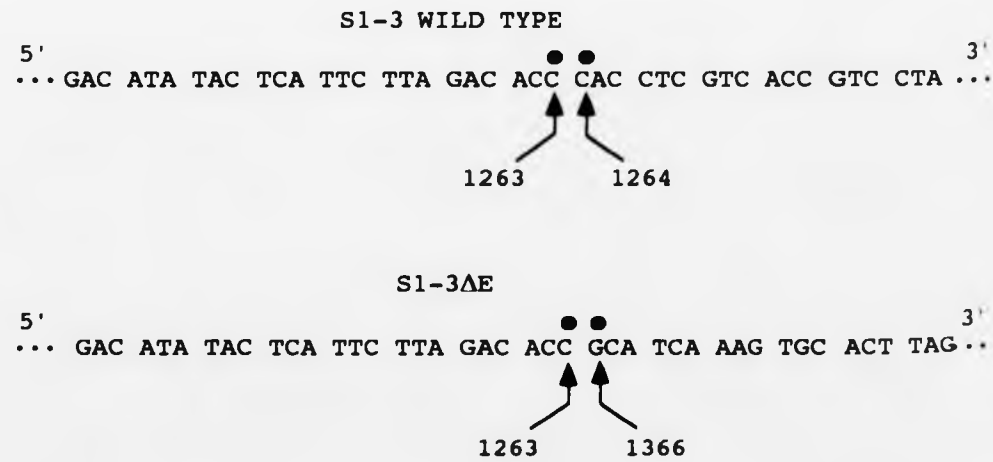


Figure 10.3.6 Construction of Plasmid pG3T3ΔD

The mutant clone mp19S1-3ΔD was cultured as described in section 2.8.3, from which RF DNA was prepared (section 2.7.3). This DNA was digested with the restriction enzymes *Nco*I and *Sca*I which cut at positions 566 and 1284 in the S1-3 DNA respectively. A fragment of DNA from which region D had been deleted was thus released and subsequently purified from an agarose gel (section 2.9.1). Plasmid pG3T3 was partially digested with the enzyme *Sca*I as described in section 2.6.1.1 and then cleaved with *Nco*I. The resulting vector/gene fragment was purified as above and ligated to produce the deletion mutant pG3T3ΔD. This scheme is shown opposite.

The key for the diagram is as for Figure 10.3.1 plus the symbol explained below.



= A fragment, of the S1-3 gene from which region D has been deleted.

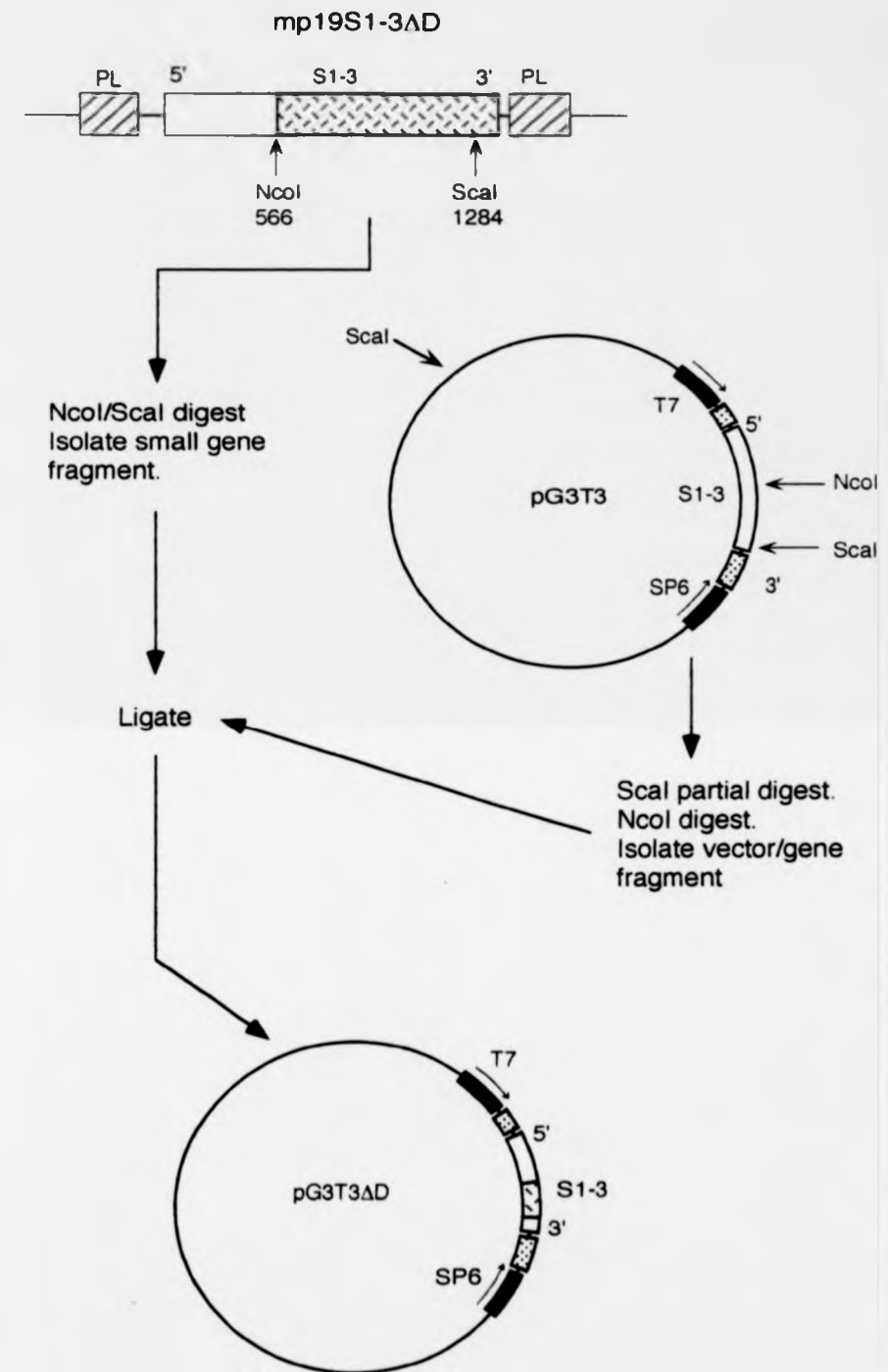

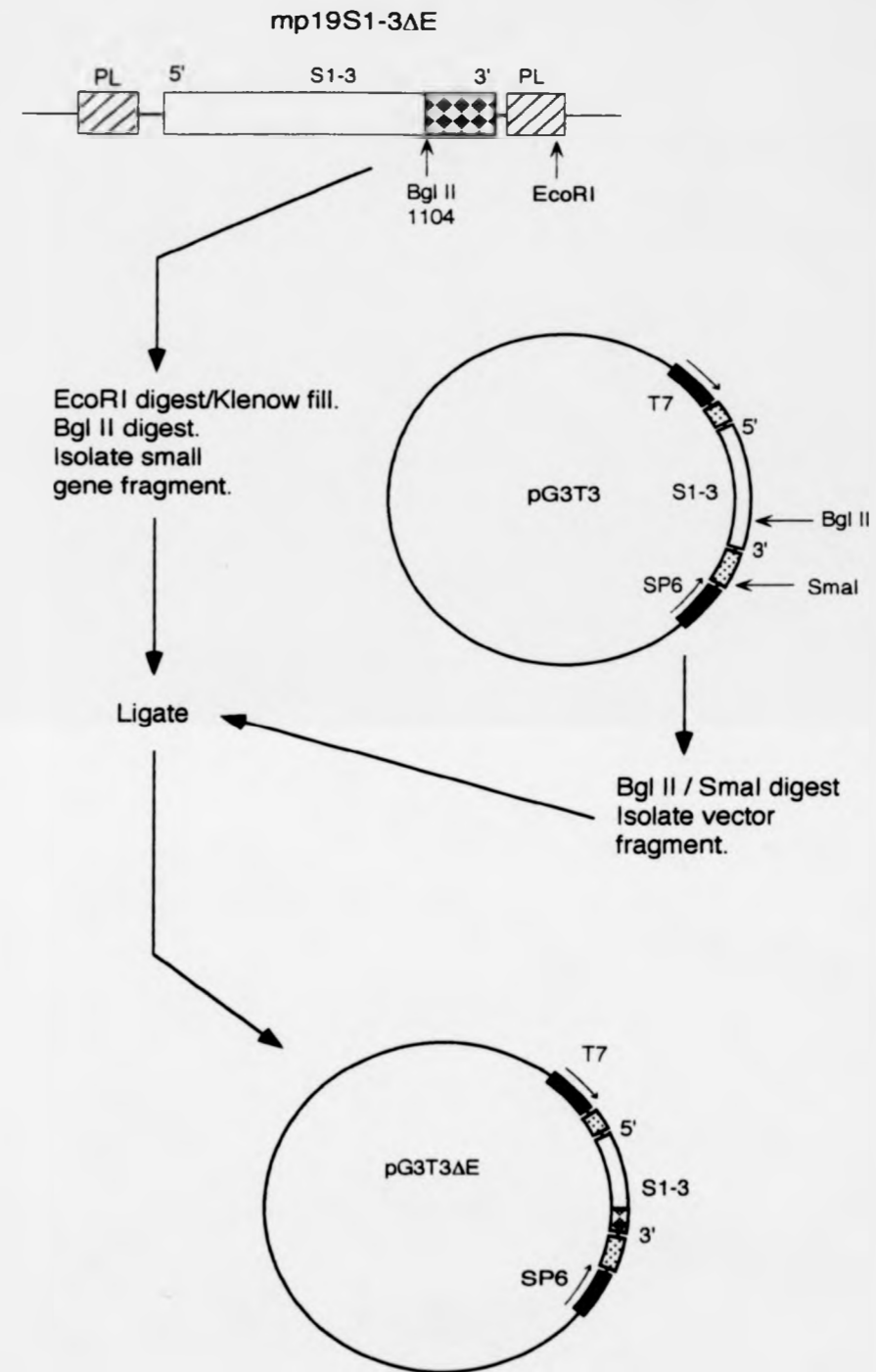


Figure 10.3.7 Construction of Plasmid pG3T3ΔE

Replicative form mp19S1-3ΔE DNA was prepared as described for the mp19S1-3ΔD mutant in Figure legend 10.3.6. This DNA was digested in the 3' polylinker sequence with the restriction enzyme *EcoRI* and the ends of the DNA were filled by the action of Klenow polymerase (section 2.6.2). Subsequent cleavage with the enzyme *Bgl* II (which cuts the S1-3 DNA gene sequence at position 1104) released a fragment of S1-3 DNA from which region E had been deleted; this was purified from an agarose gel (section 2.9.1). Plasmid pG3T3 was digested with the restriction enzymes *Sma*I and *Bgl* II which cut in the 3' polylinker sequence and in the S1-3 gene respectively. The resulting vector/gene fragment was also purified from an agarose gel and ligated to the mutated S1-3 fragment to produce plasmid pG3T3ΔE.

The key is the same as for Figure 10.3.1 plus the symbol explained below.

 = A fragment of the S1-3 gene from which region E has been deleted.



10.3.4 Oligomerization of the Mutant Proteins

It was seen in Chapter 8 that oligomerization of the type 3 $\sigma 1$ protein was required for it to manifest a cell-binding function. It was therefore necessary to determine not only that the mutant proteins were synthesized as truncated products of the expected size but that they could also oligomerize.

Message sense $\sigma 1$ -3 RNA produced by *in vitro* transcription of the mutant plasmids pG3T3 Δ D and pG3T3 Δ E was translated in the reticulocyte lysate system and analysed by SDS-PAGE under denaturing and non-dissociating conditions. These results are shown in Figures 10.3.8 and 10.3.9 respectively and demonstrate that truncated $\sigma 1$ -3 proteins, $\sigma 1$ -3 Δ D and $\sigma 1$ -3 Δ E respectively, were synthesized, with the latter slightly smaller than the former as expected. It can also be seen that these proteins do form oligomers which appear to have lower molecular weights than the non-mutated oligomer.

10.3.5 Cell-Binding Ability of the $\sigma 1$ Type 3 Mutant Proteins

The ability of the mutants to attach to L-cells was examined in comparison to that of the full-length protein. An aliquot of each of the translation mixes utilized in the binding assay was co-electrophoresed with the resulting solubilized monolayers to ensure that the absence of a band representing bound protein was not due to a failure of the protein to be synthesized. As can be seen in Figure 10.3.10, only the full-length protein and neither of the mutants was able to bind to the L-cell monolayers (and each had been successfully translated).

Figure 10.3.8 *In Vitro* Expression of the Mutant σ 1-3 Proteins

Plasmids pGT3, pG3T3 Δ D and pG3T3 Δ E were transcribed *in vitro* to produce full-length and mutant s1-3 mRNAs respectively. These were translated in a commercial reticulocyte lysate as described in section 2.11.1. Aliquots of each translation mix were boiled in sample buffer and analysed by SDS-PAGE (section 2.11.4). The gel was then processed as described in section 2.11.5 and a photograph of the resulting autoradiograph is shown opposite. Reovirus infected cells were prepared as in section 2.2.5.

- Lane R Reovirus infected cells
- Lane U Uninfected cells
- Lane A *In vitro* translated s1-3 mRNA derived from pG3T3
- Lane B *In vitro* translated s1-3 Δ D mRNA derived from pG3T3 Δ D
- Lane C *In vitro* translated s1-3 Δ E mRNA derived from pG3T3 Δ E

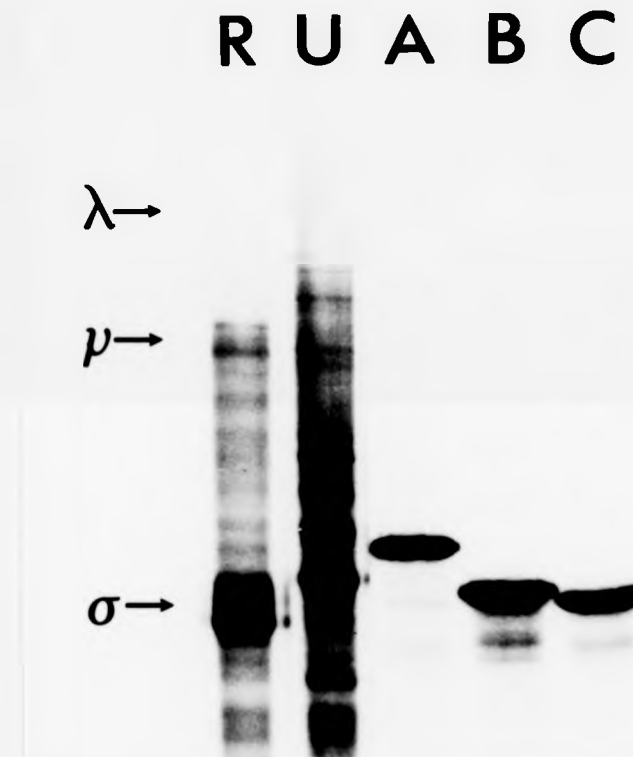


Figure 10.3.9 Oligomerization of the Mutant σ 1-3 Proteins

Plasmids pGT3, pG3T3 Δ D and pG3T3 Δ E were transcribed *in vitro* to produce full-length and mutant σ 1-3 mRNAs respectively. These were translated in a commercial reticulocyte lysate at 37°C for 30 minutes and the resulting proteins analysed by SDS-PAGE under non-dissociating conditions (incubation in sample buffer at 37°C for 30 minutes prior to loading on the gel). A photograph of the gel (which had been processed as in section 2.11.5) is shown opposite.

- Lane R Reovirus infected cells
- Lane U Uninfected cells
- Lane A *In vitro* translated, full-length σ 1-3
- Lane B *In vitro* translated σ 1-3 Δ D
- Lane C *In vitro* translated σ 1-3 Δ E

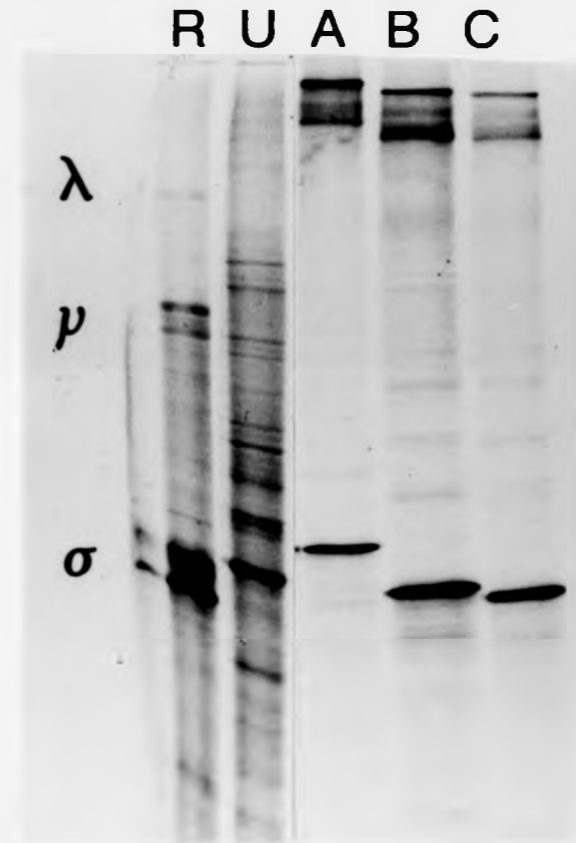
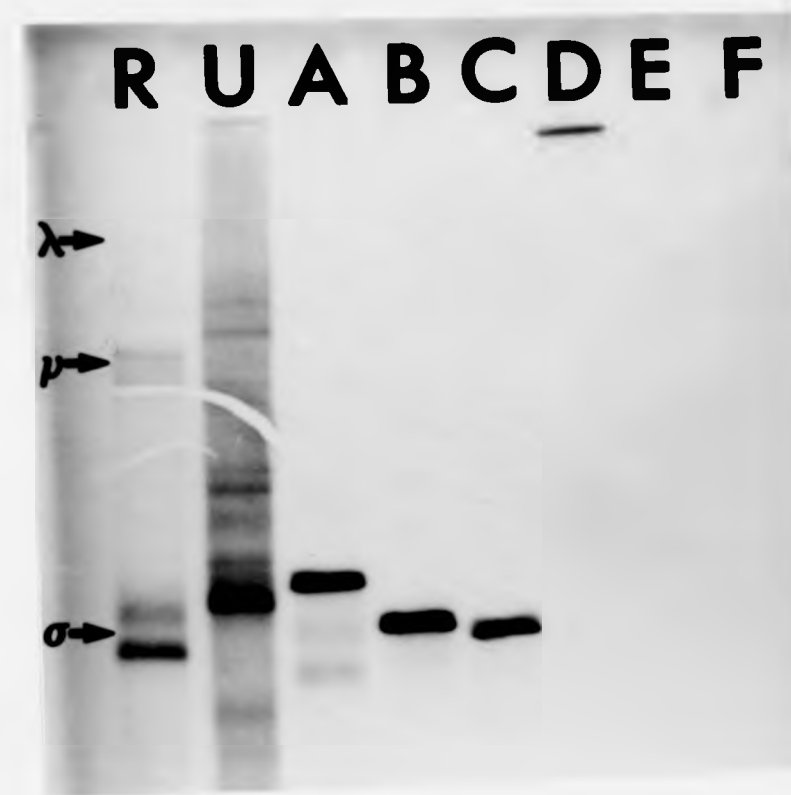


Figure 10.3.10 Assessment of the Cell-Binding Ability of the Mutant σ 1-3 Proteins

Proteins σ 1-3, σ 1-3 Δ D and σ 1-3 Δ E were synthesized as described in the legend to Figure 10.3.9. These were assayed for their L-cell binding ability as described in section 2.12.2; the solubilized, cell-bound material was analysed under non-dissociating conditions of SDS-PAGE. Aliquots of the three *in vitro* translation reaction mixes were analysed on the same gel, but were heated to 100°C (in sample buffer) prior to loading on the gel. A photograph of the resulting gel is shown opposite.

Lane R	Reovirus infected cells
Lane U	Uninfected cells
Lane A	<i>In vitro</i> translated, full-length σ 1-3
Lane B	<i>In vitro</i> translated σ 1-3 Δ D
Lane C	<i>In vitro</i> translated σ 1-3 Δ E
Lane D	Cell-bound σ 1-3
Lane E	Cell-bound σ 1-3 Δ D
Lane F	Cell-bound σ 1-3 Δ E



10.4 SUMMARY

A mutational analysis of the σ 1-3 protein was carried out in order to locate domains of the protein involved in cell-attachment. Two C-terminal σ 1-3 mutants were generated by oligonucleotide directed mutagenesis that were both able to oligomerize but were unable to bind to L-cells. However, a conformational effect caused by the deletion cannot be discounted as an explanation for the inability of the mutant proteins to manifest a cell-binding ability. This matter is more fully discussed in the next chapter.

CHAPTER 11

General Discussion

11.1 General Strategy

In this study, the reovirus $\sigma 1$ protein was expressed *in vitro* to enable an investigation of its cell-binding properties; an attempt was also made to localise the cell-binding domain(s) within the protein. The approach employed was based on exploiting *in vitro* systems. Thus, $\sigma 1$ was synthesized in rabbit reticulocyte lysate from cloned copies of the type 1 and type 3 S1 genes and a binding assay developed utilizing L-cells grown and maintained in tissue culture. This method of $\sigma 1$ expression in this project provided a convenient source of the protein that was virtually free from other, contaminating protein species. Prior to the beginning of this project, the cell-binding activity of $\sigma 1$ had been investigated by a number of other groups. In their work $\sigma 1$ was synthesized in a variety of expression systems, both bacterial and viral (Masri *et al.*, 1986 and Banerjea *et al.*, 1988); isolated from intact virions Yeung *et al.* (1987) or analysed as a crude virus infected cell lysate Lee *et al.* (1981b). These methods are both more time consuming than the method employed in this study and carry the potential risk of problems caused by the presence of contaminating protein species. A further crucial advantage conferred by expressing $\sigma 1$ from a cDNA copy of its encoding gene is that it also allows the opportunity for a mutational analysis of the protein (Chapter 10 and discussed in section 11.1.3).

Whilst the "*in vitro*" approach adopted in this study has advantages, as with any such approach, there are limitations, principally in extrapolating the results to the situation observed *in vivo*. The ideal method of investigating the cell-binding properties of $\sigma 1$ and relating this to viral pathogenesis would be to mutate a cloned copy of the S1 gene and rescue this back into virus in the form of RNA. Binding assays could then be carried out with this "mutant" virus *in vitro* and the effect of the

mutation on the tissue tropism and virulence properties of the virus *in vivo* could also be investigated. A mouse system, described in section 1.3, is available for studies of the latter nature. Although one recent report has described conditions under which reovirus RNA is infectious (Roner *et al.*, 1990) the molecular mechanism(s) involved is poorly understood and the rescue of a single reovirus gene, from a cloned DNA copy into virus particles has yet to be achieved. Thus, the application of *in vitro* methods represented a means by which the characterization of $\sigma 1$ and its cell-binding activities could be further pursued. During the course of this study it became apparent that this approach had also been adopted by the research group of Lee *et al.* (some of whose work is considered in the following sections).

The remainder of this discussion is mainly concerned with the oligomeric nature of $\sigma 1$, its interaction with the L-cell receptor and the current evidence for the nature of the reovirus receptor as issues pertaining to the expression of the type 1 and 3 $\sigma 1$ proteins have already been discussed at the end of the relevant chapters (i.e. Chapters 5, 6 and 7).

11.1.1 Oligomerization of Protein $\sigma 1$

The N-terminal heptad repeat region of $\sigma 1-3$ has been shown to possess an intrinsic oligomerization function (Banerjee and Joklik, 1990 and Leone *et al.*, 1991b). This was also demonstrated in this study by the *in vitro* expression of a plasmid containing a chimeric S1 T1/T3 gene. The resulting hybrid protein, in which the N-terminal region of $\sigma 1-3$ was fused to the C-terminal portion of $\sigma 1-1$ was able to oligomerize, a property not exhibited by the full-length $\sigma 1-1$ protein.

Several oligomeric species of $\sigma 1-3$ were evident when analysed under non-dissociating conditions of SDS-PAGE (pre-incubation in sample

buffer at 37°C). The amount of oligomer formed was greatly increased when the *in vitro* translations were carried out at 37°C as opposed to 30°C. The temperature at which $\sigma 1$ is synthesized *in vitro* therefore appears to be quantitatively important in the oligomerization process. In fact in our work, synthesis of this protein at 37°C was required to generate sufficient quantities of oligomeric $\sigma 1$ for its cell-binding function to be observed when assayed on monolayers of L-cells.

Complete oligomerization was never observed in this study and typically occurred with an approximate 50% efficiency. This is in contrast to the almost complete oligomerization of $\sigma 1$ observed by Bassel-Duby *et al.* (1987) who utilized $\sigma 1$ isolated from intact virions and reovirus infected cell lysates. A factor or factors, may therefore be present *in vivo* that promote efficient oligomerization. Leone *et al.* (1991b) also noted that extrapolation of data concerning the oligomerization property of the heptad repeat region to the folding process should be treated with care as the process involving $\sigma 1$ *in vivo* could well be more complex. Furthermore, these authors have even suggested that the association of intact $\sigma 1$ molecules into oligomers involves chaperones and is ATP dependent (unlike the spontaneous nature of the heptad repeat region event), although the evidence is as yet unpublished. It is feasible that chaperones could be one of the putative factors suggested above as being present in an *in vivo* situation but not in the reticulocyte lysate. Their participation in the oligomerization process could explain the differences in the efficiency of the process observed in this study with that of Bassel-Duby *et al.* (1987). In this respect, it is interesting that another cell-protein, BiP (Binding Protein) thought to be involved in protein folding, (Munro and Pelham, 1986) has been associated with influenza HA molecules. Misfolded HA monomers and oligomers are complexed with BiP and appear to be locked into the endoplasmic reticulum whilst correctly folded

HA molecules are only transiently associated with this protein (Gething *et al.*, 1986).

Several species of oligomer were observed for the *in vitro* synthesized σ 1-3 in this study, of which it was predominantly the oligomeric species migrating slowest on SDS-PAGE analysis that displayed a cell-binding ability. The data of Leone *et al.* (1991a) is in agreement with this observation and these authors also reported that the oligomeric species unable to bind to cells were in fact misfolded molecules and that extensive changes in the conformation of the C-terminal region of σ 1 occur upon oligomerization.

An approximately 150 kDa form of the oligomer was sometimes apparent in this study, most often after analyses of the protein species that had bound to cell monolayers (Figures 8.2.6 and 9.2.4). On most occasions, this lower molecular weight species was not evident in the reticulocyte translation mix that was the source of the σ 1 protein to be assayed on the cell monolayers but was present after the protein had bound to L-cells (e.g. Figure 8.2.6). It therefore seemed likely that it was an alternative form of the slower migrating oligomers, for example either a degraded product, or a lower order species resulting from the dissociation of a σ 1 subunit during the experimental procedure. Another possibility is that the conformation or oligomeric status of the σ 1 oligomer changes following binding to the receptor as a means of locking the protein onto the receptor. The initial binding would therefore be relatively non-specific which would change upon attaching to the actual receptor to a high-affinity interaction. A study by Strong *et al.* (1991) concerning the oligomeric status of σ 1-3 provided an alternative and what appears to be more likely interpretation to this observation. These authors proposed that σ 1 associates into a homotrimer based on gel filtration data and its sedimentation value in sucrose gradients; SDS-PAGE of full-length σ 1 and

a truncated mutant co-synthesized in a reticulocyte lysate revealed multiple hetero-oligomeric species, the composition of which also indicated a trimeric structure. However, a molecular weight of approximately 200 kDa for the $\sigma 1$ oligomer has been assigned by Bassel-Duby *et al.* (1987) and Banerjea and Joklik (1990) for the $\sigma 1$ oligomer based on migration in SDS-polyacrylamide gels. This anomalous molecular weight for a $\sigma 1$ trimer (which should be ~ 150 kDa) was suggested by Strong *et al.* (1991) to be the result of the C-terminal subunits of the head structure opening out from a compact globular shape into a "hydra" like structure under the non-dissociating conditions of SDS-PAGE. Each "tentacle" of the hydra would be equivalent to an unfolded C-terminal subunit (Figure 11.1.1). This shape could easily be responsible for retarding the migration of the proposed trimer through polyacrylamide gels. Strong *et al.* (1991) demonstrated that by reducing the pre-electrophoresis incubation temperature in sample buffer from 37°C to 22°C or lower, the putative trimer migrated to the expected position of approximately 150 kDa. Thus, the species that migrated to a similar position in this study (i.e. just below the reovirus λ proteins in the marker tracks) could represent a more compactly folded form of the slower migrating oligomers. Consistent with this interpretation, an experiment was carried out in this study in which it was found that if the temperature of the $\sigma 1$ lysate/sample buffer mix was reduced to 4°C prior to analysis by SDS-PAGE, a similar downshift in gel migration of the oligomer resulted (data not shown).

Further characterisation of the factors involved in stabilising the $\sigma 1$ oligomers under non-dissociating conditions was beyond the scope of this study. However, Bassel-Duby *et al.* (1987) and Leone *et al.* (1991b) have investigated the oligomeric status of $\sigma 1$ under similar conditions. The former suggested that the unique solvent properties of β -Me (present in the sample buffer) enabled $\sigma 1$ to form multimers, whereas the latter

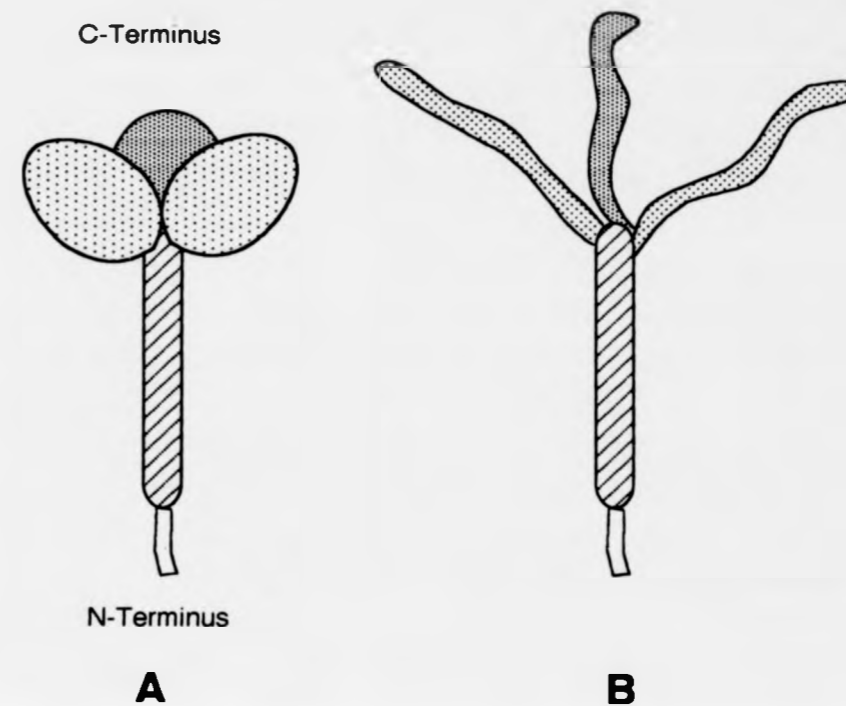


Figure 11.1.1 Putative Oligomeric Form of Protein σ 1-3

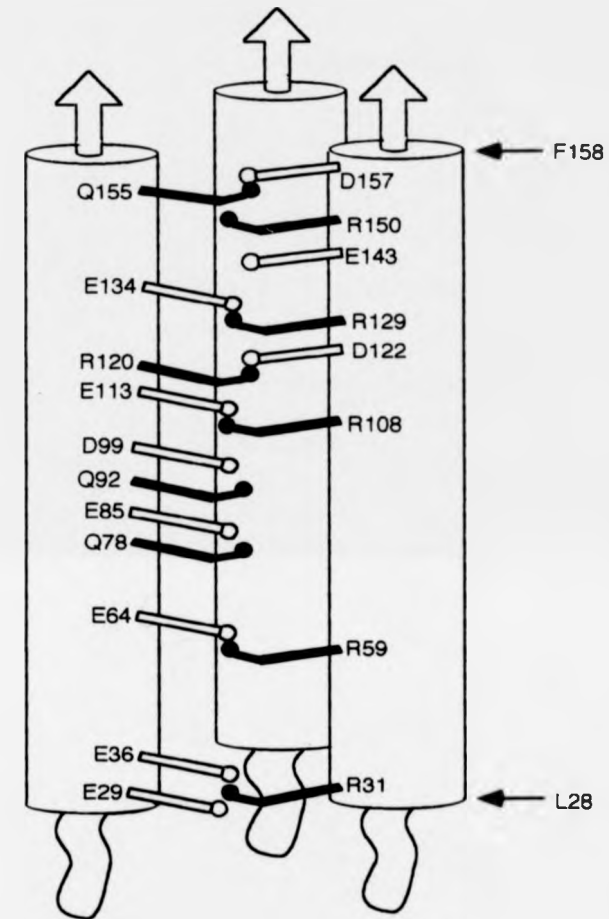
The two forms of σ 1-3 shown above are those proposed by Strong *et al.* (1991). The oligomer is thought to be a homotrimer with the C-terminal heads of the three subunits folded into compact globular structures, stabilised by inter and intrasubunit interactions (diagram A). Under non-dissociating conditions of SDS-PAGE, the trimer remains associated through the N-terminal rods, but the globular heads dissociate; this is shown in diagram B. The dissociated form would probably exhibit a retarded migration upon SDS-PAGE analysis, thus causing an overestimation of the molecular weight of the oligomer (Strong *et al.* 1991).

suggested it was due to the little known property of β -Me as a weak chelating agent; thus divalent cations and β -Me were shown to have antagonistic effects on the stability of oligomeric $\sigma 1$ N-terminal tryptic fragments. The latter study also reported that EDTA was able to replace β -Me in sample buffer to similar effect. The heptad repeats of the N-terminal region of $\sigma 1$ are taken to be indicative of a coiled-coil structure (Bassel-Duby *et al.*, 1985) where residues *a* and *d* of the heptad are characteristically apolar and thought to play a role in stabilising oligomeric structures via hydrophobic interactions between the strands of the oligomer (McLachlan and Stewart 1975). Leone *et al.* (1991b) suggested that the effect of the divalent ions would be to disrupt ionic interactions in the N-terminal region that are involved in contributing to oligomer stability. Parallel alignment of the strands of the oligomer revealed that the oppositely charged residues in the *e* and *g* positions (or sometimes the *b* and *d* positions) of the heptad are appropriately positioned to form acid-base pairs (Nibert *et al.*, 1990 and Leone *et al.*, 1991b); this is shown in Figure 11.1.2. However, these ionic interactions are thought to be secondary to the influence of the hydrophobic interactions.

The aforementioned suggestion that $\sigma 1$ exists as a trimer is in direct contradiction to all previous proposals, which favoured a dimeric or a tetrameric structure. It was originally believed that $\sigma 1$ formed a dimer based on stoichiometric data and its distribution at the vertices of the viral icosahedron (Smith *et al.*, 1969 and Lee *et al.*, 1981b); sequence analysis of the S1 gene (Bassel-Duby *et al.*, 1985) and chemical cross-linking experiments of purified $\sigma 1$ (Yeung *et al.*, 1987) further supported this idea. Analysis of the protein under non-dissociating conditions of SDS-PAGE (Bassel-Duby *et al.*, 1987; Banerjea *et al.*, 1988 and Banerjea and Joklik, 1990) as well as under the electron microscope (Fraser *et al.*, 1990) have resulted in the updated proposal of $\sigma 1$ as a tetramer. The variance of the

Figure 11.1.2 Schematic Representation of the Putative Ionic Interactions Involved in Stabilizing the σ 1 Oligomer

Shown opposite is a diagram of the amino acid residues in the σ 1 heptad repeat region assigned as forming acid base pairs on two adjacent helices of the σ 1 oligomer. These interactions are thought to occur between amino acids in positions *e* and *g* of the heptad repeat. The positively charged residues are shown in black and the negatively charged in white; amino acids in position *e* are marked on the left hand helix and those in position *g*, on the right. The diagram is reproduced from Leone *et al.* (1991b). Three helices are shown as these authors believe the oligomer to be a trimeric structure.



suggested trimeric nature of $\sigma 1$ (Strong *et al.*, 1991) with the SDS-PAGE based tetramer proposals of the above studies can be explained by the hydra model of Strong *et al.* (1991). The reason for the discrepancy with the electron microscopy study of Fraser *et al.* (1990) is less clear. Strong *et al.* (1991) also found that the N and C-terminal fragments produced after trypsin cleavage of oligomeric $\sigma 1$ also migrated as trimeric particles in polyacrylamide gels.

The cell-attachment proteins of several other viruses, both enveloped and non-enveloped, have been reported to be trimers. Examples are : the influenza HA (Wilson *et al.*, 1981), the coronavirus spike protein (Cavanagh, 1983 and Delmas and Laude, 1990), the vesicular stomatitis virus (VSV) G protein (Doms *et al.*, 1987 and 1988), the rabies virus G protein (Gaudin *et al.*, 1992), the adenovirus fibre protein (van Oostrum *et al.*, 1987 and Devaux *et al.*, 1990) and the envelope (*env*) glycoprotein of HIV-1 (Gelderblom *et al.*, 1989 and Weiss *et al.*, 1990). However, the oligomeric status of *env* is controversial and has been proposed to be a dimer, or multiple of a dimer, (Thomas *et al.*, 1991) or a tetramer (Schawaller *et al.*, 1989). The shape of these attachment proteins also appears to represent a common theme of a globular, cell-binding region atop a rod-like structure. Two segments in each of the rod sections of the spike proteins of several different coronaviruses have also been suggested to form coiled-coil ropes based on the identification of heptad periodicities in their predicted protein sequence (de Groot *et al.*, 1987 and Rasschaert and Laude, 1987). Analysis of x-ray crystallographic data of the influenza HA demonstrated that it possesses complex coiled-coil structures (Wilson *et al.*, 1981) whilst Devaux *et al.* (1990) have inferred that the stalk of the adenovirus fibre may be α -helical in nature rather than the previously proposed β -sheet (Green *et al.*, 1983). These authors have suggested that an α -helical coiled-coil structure could confer flexibility to the particular

protein and thus aid in the "detection" of and binding to host cell receptors. In contrast to this strategy, many of the picornaviruses appear to have buried their host-cell receptor recognition structures in the virus architecture (Hogle *et al.*, 1985, and Rossmann *et al.*, 1985), possibly to avoid detection by the host's immune system (Rossmann, 1989).

Whilst this study and that of Leone *et al.* (1991a) has shown that oligomerization of $\sigma 1$ is undoubtedly crucial for manifestation of its cell-binding function, a quaternary structure for the other virus cell-attachment proteins mentioned above, may not represent a similar functional prerequisite but may be a consequence of other processes affecting these proteins. Such an example is provided by the enveloped viruses where oligomerization appears to be important in the intracellular transport of their cell-binding proteins. Thus, trimerization of the influenza HA and of the VSV G protein has been shown to be necessary for transport from the endoplasmic reticulum to the golgi complex (Gething *et al.*, 1986; Copeland *et al.*, 1986; 1988; Kreis and Lodish, 1986 and Doms *et al.*, 1987 and 1988).

11.1.2 The Reovirus L-Cell Receptor

The density of putative reovirus receptors ($\sim 2.8 \times 10^5$ /cell) and the kinetics of the $\sigma 1$ -3-cell binding interaction observed in this study were similar to the data of other groups in which whole virions were used to investigate reovirus L-cell interactions (Epstein *et al.*, 1984; Gentsch and Hatfield, 1984 and Gentsch and Pacitti, 1985). This suggested that the isolated $\sigma 1$ -3 protein was effectively mimicking virus in the cell-binding process. Cell receptor densities have been calculated for several other viruses, including rhinovirus (major receptor group): 1-2,000 sites/HeLa cell (Colonno *et al.*, 1988), adenovirus type 41: 4.3×10^4 sites/HEp-2 cell (Yeh

and Luftig, 1991), adenovirus type 2: 3-6,000 sites/HeLa cell (Persson *et al.*, 1983), Theiler's murine encephalomyelitis virus: 1.5×10^3 sites/BHK-21 cell and 2.5×10^3 sites/oligodendrocyte (Rubio *et al.*, 1990) and herpes simplex virus (HSV): 5×10^5 sites/human R970-5 cell (Johnson *et al.*, 1990). A broad range of densities is evident. This is probably a reflection of the fact that viruses utilize cell surface molecules that are normally involved in a particular cellular function; the specific densities may therefore be a reflection of the level of expression required for a particular cell type.

The nature of the receptors for T1 and T3 reovirus on L-cells is controversial in that there is evidence favouring a common receptor for the two viruses and there are results pointing clearly to each virus type using a distinct receptor. The results obtained in this study suggest that these two virus serotypes in fact bind to different receptor moieties, as the attachment of σ 1-3 to L-cells could be competed with type 3 virus but not type 1 (section 9.2.3). A small decrease in the amount of σ 1-3 bound to the cells was caused by the highest concentration of T1 virus and may therefore indicate some overlap in the receptor molecules or merely a non-specific inhibition due to the high concentration of competing virus. Epstein *et al.* (1984) and Ambler and Mackay (1991), produced similar data in competition experiments involving whole virions of these two serotypes. Epstein *et al.* (1984) observed competition only with homologous type virus although some inhibition of the T1 binding was noted with the very highest amounts of competing T3 virus. In addition, a different number of receptor sites for reovirus T1 and T3 was determined by both Epstein *et al.* (1984) and Ambler and Mackay (1991). This would also appear to support the idea of two distinct sets of receptors, although an alternative is that a single receptor exists for which T1 and T3 have different affinities. Ambler and Mackay (1991) have even suggested that there is one receptor for

reovirus T3 and another to which T1 and T3 can both bind, but for which T3 has the higher affinity.

A situation involving two distinct receptor moieties for type 1 and type 3 reovirus would be compatible with the $\sigma 1$ mediated serotype specific neurotropism and virulence patterns displayed by these viruses in the mouse model (Weiner *et al.*, 1977 and 1980b). In this system, T1 virus infects ependymal cells (Kilham and Margolis, 1969; Margolis and Kilham, 1969 and Weiner *et al.*, 1977) and T3 neuronal cells (Walters *et al.*, 1963; Margolis *et al.*, 1971; Gonatas *et al.*, 1971; Raine and Fields, 1973 and Weiner *et al.*, 1977). These serotype specific differences in target host cell was shown to be a property of the respective $\sigma 1$ proteins. A similar binding pattern was observed *in vitro* utilizing cultured neurones and ependymal cells (Tardieu and Weiner, 1982). Thus, distinct sets of receptor molecules, specific to each of these cell types likely determines the different cell-binding properties of the T1 and T3 viruses.

The conclusion that the T1 and T3 viruses bind to different receptors contradicts the conclusions reached by Lee *et al.* (1981b) and Choi *et al.* (1990) who have proposed that the T1 and T3 viruses utilize the same receptor. Lee *et al.* (1981b) demonstrated that $\sigma 1$ protein from each serotype could be competed by homologous and heterologous virus (i.e. T1, T2 and T3) equally effectively in binding assays. Choi *et al.* (1990) found that type 1 and type 3 reovirus bound to similar sets of L-cell membrane proteins in Western blot analyses although there were some minor differences between the sets of proteins identified by each virus. Specificity of binding was demonstrated in assays where the virus used to probe the blots was prevented from identifying the appropriate membrane proteins by the presence of homologous type virus. Also, wheat germ agglutinin, a lectin with specificity for sialic acid, was found to inhibit binding of the T3 virus to L-cells and in Western blot assays, recognized

many of the proteins similarly identified by reovirus T3. Thus, it was reasoned that sialic acid is a major receptor determinant for reovirus and as it is a common cell surface component, accounted for the fact that multiple membrane proteins were identified by the virus in the Western blots. A pertinent point here is that protein samples to be analysed in Western blots are often boiled as part of the blotting procedure (as in the study of Choi *et al.* [1990]) and so the proteins are unlikely to remain in their native conformations. Therefore, when this approach is utilized to identify virus receptor proteins, it is probable that the virus may not be binding to the same structure it recognizes on the cell surface.

Prior to the study of Choi *et al.* (1990), others had accumulated evidence to suggest a role for sialic acid in reovirus receptor recognition. Gentsch and Pacitti (1985) reported that neuraminidase treatment of L-cells reduced the binding of reovirus by 60-70% and sialyl-oligosaccharides also mediated inhibition of binding. Subsequently, the α -anomeric form of sialic acid was defined as the minimal reovirus receptor determinant on the basis of experiments in which sialyloligosaccharides specifically inhibited the attachment of reovirus to L-cells and BSA-sialic acid conjugates were able to bind virus particles whereas control conjugates were not (Paul *et al.*, 1989).

However, the precise role of sialic acid is unclear. Armstrong *et al.* (1984) and Epstein *et al.* (1984) showed that binding of reovirus types 1 and 3 was not affected by pre-treatment of L-cells with neuraminidase, whilst Gentsch and Pacitti (1985) were still able to estimate a receptor density for and demonstrate specific binding of the virus (in competitive binding assays) after neuraminidase treatment of L-cells. The anti-idiotypic antibody -87.92.6- which has been reported to recognize the same receptor determinant as σ 1-3 (Kauffman *et al.*, 1983a; Noseworthy *et al.*, 1983 and Co *et al.*, 1985a) was unaffected in its binding to L-cells by their

prior incubation in tunicamycin (Noseworthy *et al.*, 1983). Also, Verdin *et al.* (1989) found that only a single membrane protein from rat endothelial cells was able to bind reovirus in Western blot assays and that both type 1 and type 3 virus could detect this protein. This contrasts with the study of Choi *et al.* (1990) where multiple membrane proteins were identified by reovirus in similar Western blot analyses (owing to a proposed recognition of sialic acid). Thus, whilst the study of Verdin *et al.* (1989) appears to support the claim of Choi *et al.* (1990) of a common receptor for the T1 and T3 viruses, it does not support their proposed role for sialic acid as *the* common receptor.

If sialic acid is a receptor determinant for both T1 and T3 virus, then other factors must be involved in manifesting the specific binding and infection patterns observed *in vitro* and *in vivo*, as it seems unlikely that either neuronal or ependymal cells (the serotype specific targets *in vivo*) do not possess sialylated cell surface structures. Possibly, sialic acid could mediate an initial attachment to the cell surface whereupon specific protein interactions could occur. Alternatively, sialic acid may form part of the receptor entity with greater specificity determined by proteinaceous components. It is, of course, also feasible that the receptor(s) present on L-cells are different to those with which reovirus interacts *in vivo*.

11.1.3 Mapping the Cell-Binding Domain of $\sigma 1$

Two regions of the C-terminal half of the $\sigma 1$ -3 protein were mutated in this study in an attempt to locate a cell-binding domain or domains involved in reovirus receptor recognition. Duncan *et al.* (1990) identified five regions of the $\sigma 1$ polypeptide that showed relatively high levels of conservation between the three serotypes and suggested a possible role for those within the C-terminal portion of the protein in receptor binding.

Two of these regions, designated D and E, were those deleted in this study to evaluate their putative function in binding to the reovirus receptor. Thus, the nucleotides of the cloned S1-3 gene corresponding to these areas of the protein were removed by site directed mutagenesis and the proteins expressed from the mutant genes named $\sigma 1-3 \Delta D$ and $\sigma 1-3 \Delta E$. A similar study was not possible $\sigma 1-1$ as the full-length protein was unable to either oligomerize or bind to L-cells (Chapter 8).

Neither $\sigma 1-3\Delta D$ or $\sigma 1-3\Delta E$ were able to bind to L-cells suggesting that either regions D and E are involved in the binding process or that the deletion of the residues in these sites caused the mutant proteins to fold incorrectly. If both regions are involved in receptor recognition, it is possible that amino acids from each contribute to a conformational binding site on $\sigma 1$ responsible for binding to the receptor. Alternatively, if sialic acid forms part of the receptor moiety, then one region could bind to the carbohydrate and the other to a protein component by which specificity between serotypes could be attained. If the data reported by Duncan *et al.* (1991) is taken into account (see below), it would appear that the most likely explanation is that the ΔD and ΔE mutant proteins were not correctly folded.

Duncan *et al.* (1991) generated a set of C-terminal truncated $\sigma 1-3$ mutants by deleting various lengths from the 3' end of a cloned copy of the S1-3 gene. None of these mutant proteins retained a cell-binding activity. Evidence derived from protease and immunoprecipitation analyses of these proteins suggested that the deletions had caused gross conformational changes in the $\sigma 1$ molecules which were postulated as leading to non-functional proteins. Thus, full-length $\sigma 1$ was cleaved by trypsin to generate a 26 kDa N-terminal fragment and a 23 kDa C-terminal fragment, whilst chymotrypsin treatment resulted in the production of a 46 kDa polypeptide. In contrast, all the mutant proteins were degraded by

chymotrypsin digestion and trypsin treatment resulted in the generation of the 26 kDa N-terminal fragments but the complete degradation of the C-termini. The mutant proteins were thus proposed to be misfolded molecules, a consequence of which was the exposure of cryptic protease cleavage sites. Further evidence was provided by immunoprecipitation data in which only full-length $\sigma 1$ was recognized by a neutralizing monoclonal antibody. Similarly, the deletions introduced in $\sigma 1-3$ in this study, may have effected major conformational changes in the mutant proteins $\sigma 1-3\Delta D$ and $\sigma 1-3\Delta E$ leading to their loss of cell-binding activity. This would require further work to be confirmed.

There have been several other reports concerning attempts to identify the cell-binding domain(s) of $\sigma 1$, one of which also concentrated on analysing amino acid residues in the conserved regions of this protein delineated by Duncan *et al.* (1990).

Investigations utilizing a monoclonal anti-idiotypic antibody - 87.92.6- (Bruck *et al.*, 1986 and Williams *et al.*, 1988 - explained in greater detail in section 1.6) resulted in the proposal that amino acids 317- 332 of $\sigma 1-3$ are involved in receptor recognition. A synthetic peptide, designated V_L as it corresponded to the CDRII sequence of the light chain variable region of 87.92.6, was found to inhibit the binding of reovirus T3 to L-cells (Williams *et al.*, 1988). In a subsequent study, Williams *et al.* (1991) utilized variants of peptide V_L in similar binding assays and from these suggested that the amino acids in positions 325, 326, 327 and 328 (serine, tyrosine, serine and serine respectively) of the $\sigma 1-3$ protein were likely to interact through their hydroxyl oxygens with the reovirus receptor (which was regarded as being sialic acid). These amino acids lie within one of the five conserved regions (region C) identified in the sequence analysis of Duncan *et al.* (1990) of which tyrosine 326 is conserved in all three virus serotypes.

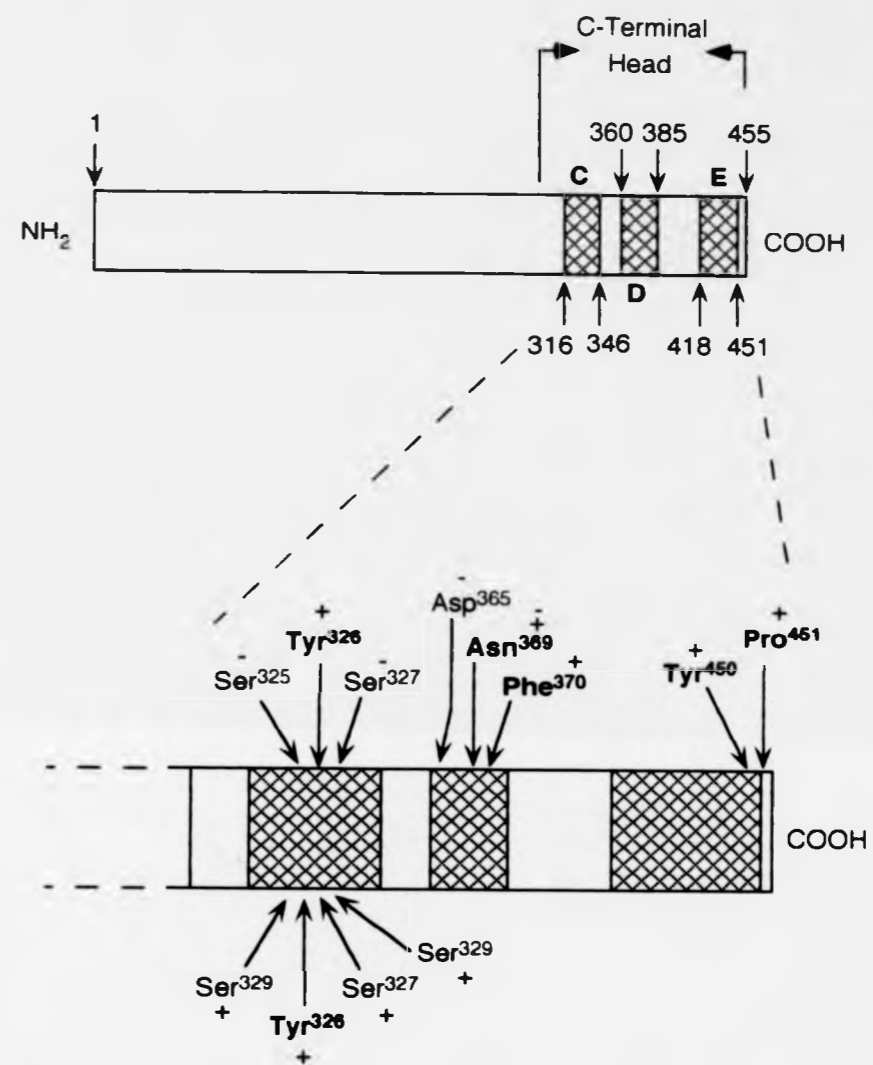
In a more direct assessment of these residues, Turner *et al.* (1992) mutated the appropriate amino acids of σ 1-3 and tested the resulting protein variants for their ability to bind to L-cells; amino acids within regions D and E were similarly targeted with both conserved and non-conserved amino acids being mutated. The mutation of conserved residues in regions C, D or E resulted in proteins that had lost their cell-binding activities and was associated with conformational changes in the C-terminal globular heads of these proteins. However, mutagenesis of the non-conserved residues, including those in positions 325 and 327 suggested by Williams *et al.* (1991) to be involved in receptor recognition, had no effect on the ability of the resulting mutant proteins to bind to L-cells. The results from these two studies are summarised in Figure 11.1.3.

Turner *et al.* (1992) concluded that the conserved amino acids were probably not all involved in receptor binding but played important roles in maintaining the C-terminal globular head in a functionally correct conformation and that the receptor binding site was therefore likely to be conformationally defined. These authors also accepted that sialic acid is a receptor moiety for reovirus and that as a conformational sialic acid binding pocket has been defined for two well characterized sialic acid binding proteins, i.e. the influenza HA (Weis *et al.*, 1988) and wheat germ agglutinin (Wright *et al.*, 1987), it would not be unexpected for the reovirus σ 1 receptor binding site to be conformational. Furthermore, since it was the amino acids conserved between all three serotypes that were proposed to be important in receptor recognition, Turner *et al.*, (1992) suggested sialic acid to be a receptor determinant not only for T3 reovirus but for T1 and T2 as well. Thus, despite the contradictory conclusions of Williams *et al.* (1991) and Turner *et al.* (1992) that the same amino acid residues either do or do not contribute to the σ 1-host cell receptor binding site, each regarded their studies as evidence for sialic acid as a component

Figure 11.1.3 σ 1-3 Amino Acids Putatively Involved in Receptor Recognition

The above diagram shows the amino acid residues of σ 1-3 investigated in the studies of Williams *et al.* (1991) and Turner *et al.* (1992) as being potentially involved in reovirus receptor recognition. The conserved regions C, D and E of the σ 1-3 protein (Duncan *et al.* 1990) are marked as hatched boxes. The residues above the enlarged representation of σ 1-3 are those mutated in the protein by Turner *et al.* (1992) whilst those below, are the amino acids in σ 1-3 corresponding to those mutated in the synthetic peptides utilized by Williams *et al.* (1991).

The amino acids marked in bold type are conserved residues in all three virus serotypes. "+" indicates that mutation of the amino acid diminished either the cell-binding ability of σ 1-3 or that the synthetic peptide with the equivalent residue mutated, was less able to inhibit binding of reovirus to L-cells. "-" indicates that mutation of these residues had no effect on these functions of σ 1-3 or the synthetic peptides.



recognized by reovirus in the cell-binding process. However, for reasons discussed previously in section 11.1.2, the evidence that this carbohydrate molecule does function in a capacity of a reovirus receptor moiety would appear to be inconclusive.

In summary, the *in vitro* approach taken in this study to the expression and characterisation of the cell-binding properties of the $\sigma 1$ protein of reovirus has proved to be a useful model of the virus-cell interaction and mimicked many of the binding characteristics displayed by virus particles. The main advantages with this system are :

i) Synthesis of $\sigma 1$ in rabbit reticulocyte lysate is a convenient and reliable means of producing the "substrate" for the cell-binding assays. Virus preparations are altogether more inconvenient, time consuming and expensive, especially if radiolabelled virus is required.

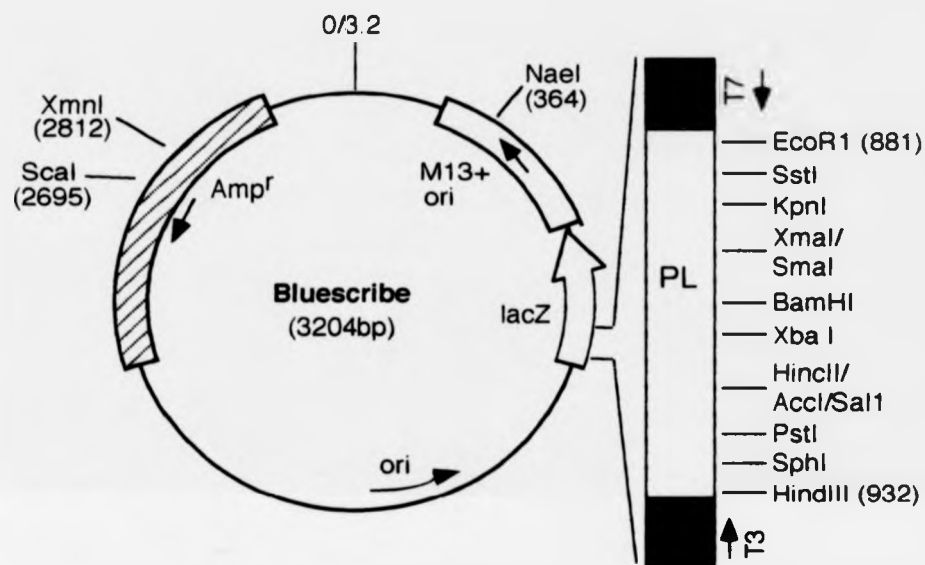
ii) Expression of $\sigma 1$ from cloned copies of the appropriate S1 genes enabled the relatively easy generation of $\sigma 1$ mutants in the attempt to identify amino acids critical for receptor recognition. At present, the generation of such specific mutants is not technically possible with virus particles.

However, despite the fact that the mutant proteins were unable to bind to L-cells, it was not possible to define a cell-binding domain owing to the high probability that they were misfolded molecules. Other researchers have taken a similar mutational approach to this problem and were also unable to discount a conformational effect of the mutation as the reason for the protein being non-functional. Possibly, the most convincing data concerning the identification of $\sigma 1$ residues involved in binding to the reovirus receptor would be obtained if a $\sigma 1$ /receptor or virus/receptor complex could be analysed by x-ray crystallography.

APPENDICES

Appendix A: Map of Bluescribe Vector (Stratagene)

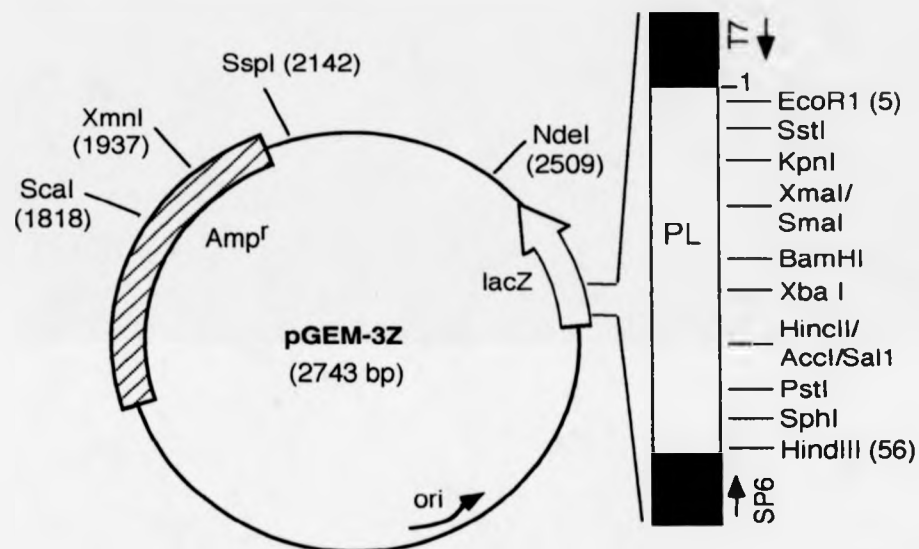
The map below shows the relevant features of the vector Bluescribe.



- T7 = T7 RNA polymerase promoter
T3 = T3 RNA polymerase promoter
lacZ = Portion of *lacZ* gene (encoding the α -peptide of β -galactosidase)
amp^r = β -lactamase gene conferring ampicillin resistance
PL = Polylinker region
M13+ = Origin of replication of the bacteriophage M13.
The + indicates that the plasmid strand rescued by a helper phage will contain the coding region of the *lacZ* gene.

Appendix B: Map of pGEM-3Z Vector (Promega)

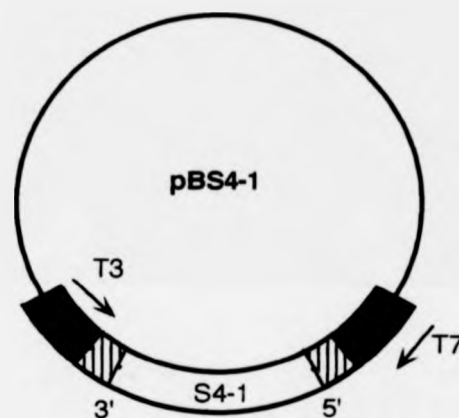
The map below shows the relevant features of pGem-3Z. The number 1 adjacent to the T7 bacteriophage promoter (black box) indicates the T7 RNA polymerase transcription initiation site and also represents the first base in the numbering scheme of the plasmid.



- T7 = T7 RNA polymerase promoter
 SP6 = SP6 RNA polymerase promoter
 lacZ = Portion of *lacZ* gene (encoding the α -peptide of β -galactosidase)
 amp^r = β -lactamase gene conferring ampicillin resistance
 PL = Polylinker region

Appendix C: Map of Clone pBS4-1

Clone pBS4-1 consists of a cDNA sequence of the reovirus S4-1 gene engineered into the vector Bluescribe and was constructed by P. Martin (University of Warwick). The 5' end of the gene is adjacent to the T7 bacteriophage promoter. Plus sense s4-1 RNA can therefore be synthesized utilizing T7 RNA polymerase.



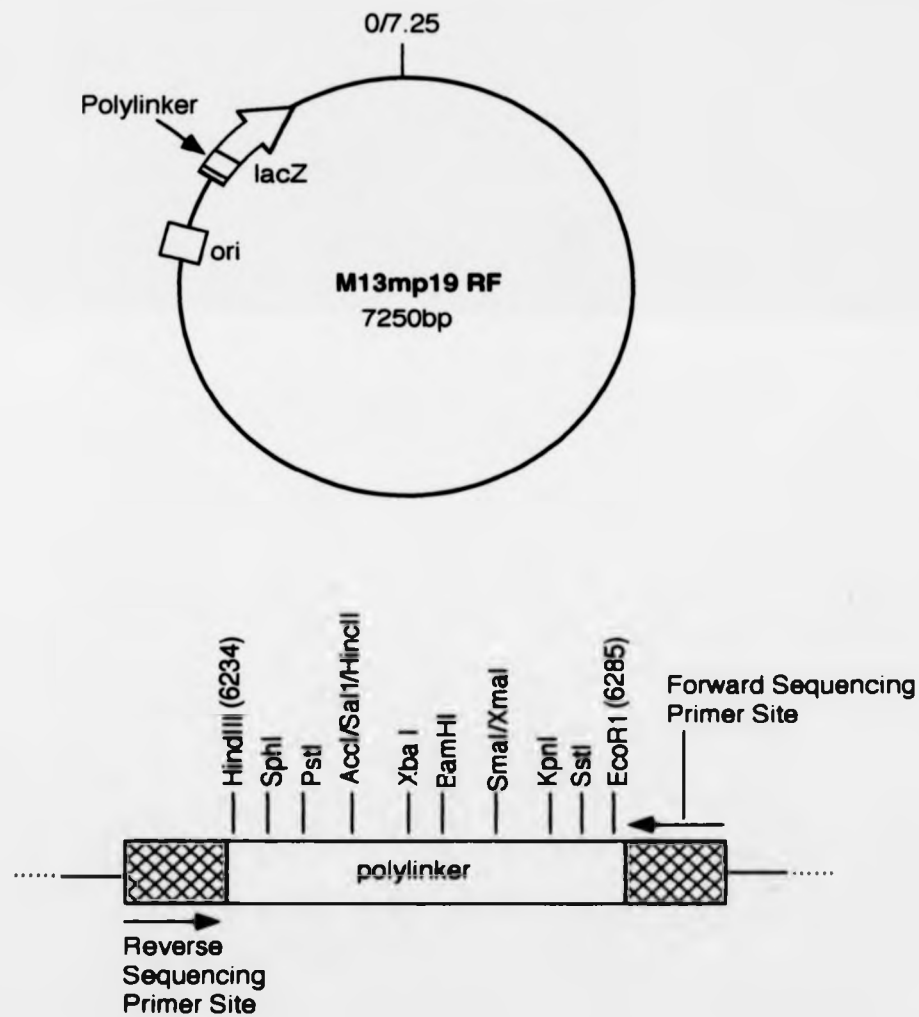
T7 = T7 RNA polymerase promoter

T3 = T3 RNA polymerase promoter

 = Polylinker sequence

Appendix D: Map of Vector M13mp19

M13mp19 (Yanisch-Perron *et al.*, 1985) was utilized as the cloning vector to effect the oligonucleotide directed mutageneses of the cloned reovirus S1 genes described in this study (Chapters 7 and 10). The linear map below the RF map shows the recognition sites of the polylinker and the positions of the forward and reverse primer sequencing sites.



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