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ANALYSIS OF THE RECEPTOR-BINDING DOMAIN OF THE REOVIRUS σ1 PROTEIN

By

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Abstract

Oligonucleotide site-directed mutagenesis has been used to modify the reovirus cell attachment protein σ1 of serotype 3 at residues located in regions C, D, and E that are highly conserved between the three reovirus serotypes. Mutant σ1 forms were synthesized in *in vitro* transcription and cell-free translation systems and assayed for (i) host cell (mouse L fibroblast) binding activity, (ii) stability against limited proteolysis, and (iii) the ability to be recognized by a neutralizing monoclonal antibody.

An inability or reduced ability to bind L-cells is observed to occur for mutant σ1 forms that have single amino acid changes in any of the highly conserved regions C, D, or E, throughout the C-terminus of σ1. While other single amino acid changes in regions C and D produce mutant σ1 forms that are capable of binding L-cells at WT levels. Limited proteolysis using trypsin and chymotrypsin reveals that those mutant σ1 forms incapable of binding L-cells also show proteolytic fragment patterns that differ from WT T3 σ1 in that the C-terminal fragments are completely degraded; WT T3 σ1 being cleaved to produce stable C-terminal and N-terminal fragment populations. Those mutant σ1 forms capable of binding exhibit the WT T3 σ1 proteolytic fragment patterns. Those mutant σ1 forms that are incapable of binding are also incapable of interacting with the neutralizing mAb G5 that specifically interacts with an epitope in the C-terminal half of the functional multimer form of T3 σ1 that subsequently prevents binding of reovirus T3 to host cells. These data indicate that the loss of σ1 binding is accompanied by a conformational change of the σ1 protein and suggests that conserved residues throughout the C-terminus of T3 σ1 are involved in the structural and/or functional integrity of the receptor-binding domain. Another mutant σ1 form capable of binding host cells at WT levels

demonstrated a conformational change that prevented the protein from being recognized by the neutralizing mAb G5. This data indicates that the neutralization and cell-attachment epitopes of T3 σ1 are not identical.

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Abbreviations and Symbols

A_{260}	absorbance measured at 260 nm
aa	Amino acid
Abs	Antibodies
BSA	bovine serum albumin
bp	base pair
CHY	Chymotrypsin
Δ	refers to the non-WT S1 gene or non-WT σ1 protein
DEPC	diethylpyrocarbamate
dH_2O	glass distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid (as the disodium dihydrate)
EtBr	ethidium bromide
FCS	fetal calf serum
HA	hemagglutinin
HI	Hemagglutination inhibition
IPTG	isopropyl-thio-β-D-galactopyranoside
JMEM	Joklik's minimal essential media
mAb G5	Monoclonal antibody G5
MOI	multiplicity of infection
NSI	Mouse myeloma cell line BALB/c P-3-NSI/1-Ag4-1
Nt	Nucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PEG	polyethylene glycol 8000
pfu	plaque forming units

PMSF	pheylmethylsulfonylfluoride
REN	restriction endonuclease
RF	replicative form
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
ssDNA	single-stranded DNA
T1	Serotype 1
T2	Serotype 2
T3	Serotype 3
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
TRP	Trypsin
WT	Wild Type
x g	relative centrifugal field
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Amino Acid One Letter Codes

A	alanine
D	aspartic acid
F	phenylalanine
G	glycine
K	lysine
N	asparagine
P	proline
S	serine
T	threonine
V	valine
Y	tyrosine

CHAPTER I: Introduction

A function essential to all viruses is the recognition of a susceptible host cell and the specific binding of the virus to this cell, thereby initiating the infection process. The molecular basis for this recognition event involves the selective interaction of virus attachment proteins (VAP's) with specific surface components of the host cell.

The specificity of the virus-cell interaction is a major determinant of virus tropism in viral pathogenecity. Indeed, the tissue specificity and host range of many viruses are dependent on this specific viral recognition and attachment to host cell surface receptors, or viral receptors. The elucidation of this viral attachment process is then essential for understanding viral pathogenecity and for the prevention and treatment of viral disease. This improved understanding will have an equally powerful impact on the study of eukaryotic cellular mechanisms, due to the close association of viral replication with host cellular functions, and on understanding the broader class of macromolecular recognition reactions represented by the specificity of the virus-host interaction.

I.1 The Reoviridae

Reoviruses are members of the genus *Orthoreovirus* of the family Reoviridae. There are five other genera comprising this virus family and include, (i) Orbivirus, which cause arthropod-borne diseases of humans and animals (Colorado tick fever, bluetongue disease and African horse sickness), (ii) Rotavirus, which also infects a wide variety of mammals and vertebrates, including man, and which cause acute infantile gastroenteritis in humans, (iii) Cypovirus, or cytoplasmic polyhedrosis virus, which are important insect

pathogens, (iv) and two genera of plant viruses, Phytoreovirus and Fijivirus, which are arthropod-transmitted.

The name "reovirus" was introduced by Sabin in 1959 to describe this group of viruses which were typically isolated from the respiratory and gastrointestinal tracts and were not associated with any known disease state; thus the acronym 'reo', derived from respiratory enteric orphan virus. They were differentiated from other enteroviruses at the time due to several distinguishing features: size about 75 nm, exhibited unique cytopathic characteristics in monkey cells, pathogenecity for newborn mice, and hemagglutination of type O human red blood cells. Today viruses of each genus are classified together because they have similar structural features, nucleic acid type and composition and replicative strategies. In general, virions are non-enveloped, have a double-shelled capsid - each with icosahedral symmetry - that contains 10 to 12 linear double stranded (ds) RNA genome segments.

The best studied and most well understood members of the orthoreovirus genera are the mammalian reoviruses. They are divided into three distinct serotypes (type 1 [Lang strain], type 2 [Jones strain], and type 3 [Dearing strain]) based upon antibody-neutralization and hemagglutination-inhibition tests (Rosen, 1960). Reovirions consist of an inner protein shell, enclosing ten double-stranded (ds) RNA genomic segments (core), surrounded by an outer protein shell (outer capsid) (Caspar & Klug, 1962; Jordan & Mayor, 1962). The ten dsRNA segments encode the ten primary viral proteins (McCrae & Joklik, 1978; Mustoe et al., 1978): two of the proteins are nonstructural (NS), found only in the infected cell cytoplasm (σNS & μNS), while the other eight proteins comprise the core [λ1, λ3, μ1, μ2 & σ2, and λ2 which forms the 12 spikes (projections) which extend from the core to the outer surface of the virion (Hayes et al., 1981)] and the outer capsid (μ1c, σ3 & σ1). The σ1 protein is a minor

outer capsid protein that is found as a multimer (Bassel-Duby et al., 1987; Banerjea et al., 1988) at the 12 vertices of the virion in association with the λ2, "spike", protein (Lee et al., 1981a; Joklik, 1983). The outer capsid proteins, core proteins and nonstructural proteins have been reviewed in detail by Tyler and Fields (1985a; 1985b) and Mah (1989). These viruses are ubiquitous in their geographic distribution and naturally infect a wide range of mammalian species. Mammals possessing antibodies to reovirus constitute an even larger group - includes most mammals studied to date. In the human population the ubiquitous nature of reovirus is shown by the fact that by the age of 16, 50 to 80 % of the general American population have antibodies to reovirus (Jackson & Muldoon, 1973; Stanley, 1974). These viruses have been isolated from persons with mild respiratory diseases, diarrhea, rashes or fever, although the majority of isolates are from completely healthy individuals. Two categories of symptomatic disease then may be related to reovirus infection: upper respiratory infections and enteritis. Reovirus has also been implicated as a possible cause of biliary atresia in human newborns (Bangaru et al., 1980; Morecki et al., 1982). Experimental infection of human volunteers with reovirus is usually asymptomatic, in individuals with symptoms (only 25 to 30 % of volunteers) the illness is manifested as a mild cold with sneezing, malaise, rhinitis and headaches (Rosen et al., 1963). The direct importance then of mammalian reoviruses in human disease is difficult to ascertain because of their ubiquity and the common absence of symptoms associated with infection.

On the other hand, in mice specific disease processes have been directly linked to mammalian reoviruses (reviewed by Sharpe & Fields, 1985 and Tyler & Fields, 1985b). All three serotypes can cause acute infections in newborn mice - in the central nervous system (CNS), heart, lungs, liver and pancreas tissue - with clinical syndromes varying with the amount of virus inoculated, the

viral serotype and the route of infection, as well as with the age and strain of the mouse. Young mice inoculated intracerebrally, subcutaneously or by inhalation of T1 reovirus results in the infection of ependymal cells of the CNS to produce a non-lethal ependymitis followed by hydrocephlus. T3 reovirus given by these same routes results in the infection of neuronal cells producing a lethal encephalitis. Given orally, however, T3 produces no neurotropic effects, whereas T1 still produces the same neurotropic effects observed after injection or inhalation of virus (Rubin & Fields, 1980). Reovirus of all three serotypes can produce myocardial injury in suckling mice, cause alveolar hemorrhage in the lungs, with associated pulmonary edema, and have been observed to produce hepatic injury via hepatic necrosis. Liver damage is thought to be related to the oily hair-runting syndrome in reovirus infected mice, which results in diarrhea, oily hair, growth retardation, jaundice and ataxia. Serotypic differences in immunopathogenicity are observed upon the infection and damage of the pancreatic islet cells, with T1 infection alone resulting in the production of insulin autoantibodies.

The mammalian orthoreoviruses provide a valuable and workable model system in which to study the cell-virus interaction at the plasma membrane level, specifically the mechanism of virus attachment to host cells, for a number of reasons. Primarily, because the viral protein that serves as the cell attachment protein, σ1, has been identified. Reovirus is also easily cultured and large yields of virus are obtainable for study. Finally, the relatively innocuous nature of the mammalian reoviruses provide a good laboratory model that is easily managed and manipulated, therefore, also providing an excellent prototype system in which to lay down the initial ground work needed to study the more highly infectious and economically and socially important members of the reoviridae family. Mammalian reoviruses have also been important in

illuminating some of the basic concepts and molecular mechanisms of microbial pathogenecity. Furthermore, their ubiquitous nature and ability to produce serotype specific disease processes allows for studies into the nature and specificity of virally induced disease states in a variety of animal systems.

I.2 Reovirus Pathogenesis

Using mice as an animal model a great deal of information has been gathered concerning reovirus pathogenesis. A brief description of the stages in reovirus pathogenesis will be given (reviewed by Sharpe & Fields, 1985 and Tyler & Fields, 1985b). Under natural conditions reoviruses behave primarily as enteric viruses, entering the host via the upper alimentary tract. After oral inoculation both T1 and T3 can be sequentially visualized adherent to the surface of the intestinal microfold cells ("M cells"), internalized within vesicles of M cells, in the intercellular space between M cells and mucosal mononuclear cells, and on the surface of mucosal mononuclear cells. The M cells, therefore, appear to be a translocation mechanism carrying reovirus across the intestinal mucosa into Peyer's patches. Viral entry is followed by two other stages, primary replication and viral spread. Mammalian reovirus causes few local symptoms, however, limited viral growth within the intestine is observed: following oral innoculation into suckling mice T1grows well in intestinal tissue, whereas, T3 reovirus progressively drops in titre. Reovirus then enters the circulation and produces disease at a distant site. This stage of reovirus pathogenesis has not yet been studied in depth, however, preliminary evidence suggests a variety of pathways may be used by reovirus. Hematogenous, lymphoid and neural routes have all been suggested.

Viral tropism, the specificity of a virus for a particular host tissue, is the final stage of pathogenesis. Cell and tissue tropism is determined in part by the

interaction of viral surface structures with cell-surface receptors on host cells. These virus-receptor interactions enable virus to penetrate and damage the host cell and are a major determinant of virulence. The cell and tissue tropism of reovirus is determined by a single viral protein, the σ1 polypeptide. Much of the data gathered on the role of σ1 as the viral protein responsible for tissue targeting has come from the study of genetic reassortant reoviruses - generated by the coinfection of host cells with two different reovirus serotypes, resulting in the reassortment of the dsRNA segments. For example, it has been shown that the serotype specific tropism of the CNS, T1 infecting ependymal cells and T3 infecting neuronal cells, is determined by the S1 gene segment (which encodes the σ1 protein), as deduced by intracerebral injection of reassortant viruses (Weiner et al., 1980a). Intracerebral innoculation of reassortants also reveals that viruses containing the T3 S1 dsRNA segment are able to grow in the eye and infect retinal ganglion cells (Tyler et al., 1983). Genetic analysis of reassortant viruses has further revealed that σ1 is responsible for determining the following cell and tissue tropisms: (i) the ability to infect pituitary cells (Onodera et al., 1981); T1 infects the growth hormone-producing cells in the anterior lobe of the pituitary gland and in some cases infection of the pituitary results in "runting syndrome"; T3 does not infect cells in the anterior pituitary but can result in the runting syndrome (Stanley & Leak, 1963; Walters et al., 1973), and (ii) the pattern of tissue injury in skeletal muscle; after intramuscular injection in suckling mice both T1 and T3 grow to high titre in muscles, however, T3 produces necrotizing myositis whereas T1 produces only mild interstitial inflammation.

The role of σ1 in determining cell and tissue tropism is only one of many important functions exhibited by this protein. Since it is the σ1 protein of

reovirus that is the topic of study in this thesis project it will now be discussed at length.

I.3 Functions of the σ1 Protein

The use of reovirus as a model system for the study of virus-cell interactions took an important step forward upon the finding that the σ1 protein serves as the reoviral attachment protein (Lee et al., 1981b). The σ1 protein possesses several other properties that make it one of the most functionally important viral proteins. It is the viral hemagglutinin (Weiner et al., 1978), agglutinating erythrocytes in a type specific manner: T1 agglutinates human erythrocytes and T3 agglutinates bovine erythrocytes (Eggers et al., 1962). It is responsible for tropism and specific cell injury in the CNS, pituitary and retina, and skeletal muscle, as discussed previously, and specifies virulence (Weiner et al., 1977; Spriggs & Fields, 1982; Spriggs et al., 1983b). Sigma 1 is responsible for evoking a number of host immune responses, including: the eliciting of type specific neutralizing and hemagglutination inhibition (HI) antibodies (Abs) in the host (Weiner & Fields, 1977; Gaillard & Joklik, 1980; Hayes et al., 1981; Lee et al., 1981a; Burstin et al., 1982), the development of T-cell-dependent delayed-type hypersensitivity (Weiner et al., 1980b; Rubin et al., 1981; Greene & Weiner, 1980), the generation of cytotoxic T lymphocytes (Finberg et al., 1979; Finberg et al., 1981; Finberg et al., 1982; London et al., 1989), and suppressor T cells (Fontana & Weiner, 1980; Greene & Weiner, 1980), and in the case of reovirus T1 the induction of autoimmunity with Abs being directed against insulin and growth hormone (Onodera et al., 1981; Onodera et al., 1982; Haspel et al., 1983) and T1 induction of immuno suppression (Garzelli et al., 1985).

The σ1 protein has also been demonstrated to facilitate virus binding to microtubules *in vitro* although the *in vivo* significance of this phenomenon is not clear (Babiss et al., 1979; Sharpe et al., 1982). It is a key determinant in the ability of reovirus to survive in the gut (Bodkin & Fields, 1989) - an important feature for an enteric virus - and for clearance and mode of spread of reovirus in the mouse (Verdin et al., 1988; Tyler et al., 1986). Finally the σ1 protein has been indirectly linked by genetic studies (Sharpe & Fields, 1981) to the process by which reovirus inhibits host DNA synthesis, although evidence that directly links σ1 (and/or σ1s, see below) to this inhibition effect has not yet been obtained (Mah, 1989).

To further probe the cell attachment property of σ1 a number of studies have been specifically directed at the characterization and isolation of cellular receptors recognized by σ1, and therefore by reovirus. The ability of σ1 to bind and thus agglutinate erythrocytes indicates the presence of receptors on these cells that are recognized by σ1. Early studies suggested that the erythrocyte receptor for reovirus was a surface glycoprotein and protein in nature (Lerner et al., 1963; Gelb and Lerner, 1965; Lerner et al., 1966; Tillotson and Lerner, 1966; Lerner & Miranda, 1968). Later studies showed that reovirus receptors on erythrocytes are susceptible to neuraminidase as well as to proteases (Armstrong et al., 1984; Gentsch and Pacitti, 1985), suggesting that the receptor for hemagglutination was a sialic acid-bearing glycoprotein. More recently, Paul and Lee have shown that the viral receptor on erythrocytes is the M, N blood group antigen, glycophorin, a sialoglycoprotein (Paul & Lee, 1987).

Through the use of an anti-idiotypic Ab (anti-anti-σ1) the T3 receptor on both mouse thymoma cell line R1.1 and the rat neuroblastoma cell line B104 was identified as a 67K protein (Co et al., 1985a). Evidence was also presented which showed this 67K protein to be structurally similar to the

mammalian β-adrenergic receptor (Co et al., 1985b). However, upon further examination a number of findings show that it is unlikely that β-adrenergic receptors serve as universal cell determinants for specific reovirus binding: (i) studies using the human epidermoid carcinoma cell line A431, known to possess large numbers of β-adrenergic receptors and which is susceptible to reovirus infection, showed that cellular responses to β-adrenergic agonists and antagonists was not affected by the presence of reovirus, nor was reovirus binding and internalization able to trigger cellular responses normally triggered by β-adrenergic agonist binding (Choi & Lee, 1988), (ii) sequestration of β-adrenergic receptors from the cell surface of A431 cells has no effect on reovirus binding (Choi & Lee, 1988), and (iii) reovirus can bind and infect cells that possess few, if any, β-adrenergic receptors (Choi & Lee, 1988; Sawutz et al., 1987). Binding of both T1 and T3 reovirus to a 54K protein on the membrane of rat endothelial cells has also recently been shown (Verdin et al., 1989). This protein is believed to be distinct from the 67K protein described by Co et al (1985a).

Evidence that carbohydrate-bearing structures are important for reovirus host recognition, as well as for hemagglutination, has come from a number of biochemical studies. First, reovirus receptor activity can be destroyed by pretreatment of intact host cells with neuraminidase or by oxidation with low concentrations of periodate (Armstrong et al., 1984; Gentsch & Pacitti, 1985). Second, reovirus binding to host cells is inhibited in the presence of a high concentration of N-acetylneuraminic acid and N-acetylneuraminyl lactose, and that reovirus interact with a number of sialic acid-bearing glycoproteins (Gentsch & Pacitti, 1985; Paul & Lee, 1987). Finally, using a solid phase binding assay it has been demonstrated that α-sialic acid alone is recognized by reovirus at a level comparable to that of the other sialoside conjugates (Paul

et al., 1989). Given the fact that many glycoproteins present on the cell surface are sialylated this last result raises the question of whether reovirus can recognize not one but many cell surface receptor molecules. Indeed results from this lab have shown that reovirus binds to multiple proteins on the L cell plasma membrane (Choi et al., 1990); this binding is specific, showing inhibition in the presence of excess unlabelled virus, shows the involvement of a single determinant (ie sialic acid), and both T1 and T3 reovirus recognize the same set of proteins.

Recognition of a sialic acid component on a cell surface molecule would theoretically be a very non-specific binding phenomenon, which could explain the ubiquitous nature of reovirus and the ability of reovirus to bind to and infect a variety of cell types *in vitro*. However, it cannot be ruled out that additional elements other than the simple recognition of sialic acid residues must also be involved in the specific interaction of reovirus with a receptor, otherwise the serotype specific tropism exhibited by reovirus in *in vivo* model systems and mediated by σ1 could not be rationalized.

I.4 Characteristics of the S1 Gene and the σ1 Protein

As previously mentioned, the σ1 protein is encoded by the viral gene segment S1 and in view of the various functions displayed by the protein σ1, and its significance in viral pathogenesis, the S1 gene of reovirus T3 was cloned and sequenced (Nagata et al., 1984; Bassel-Duby et al., 1985; Cashdollar et al., 1985), followed by its functional expression in *Escherichia coli* (Masri et al., 1986; Pelletier et al., 1987); the expressed protein is capable of attaching to mammalian cells (mouse L fibroblasts) in a specific manner, of competing with reovirus particles for cell surface receptors and of aggregating human erythrocytes. The results indicate that the T3 S1 gene is 1416

nucleotides (nts) long and contains a long open reading frame (LORF) of 1365 nts which encodes the 455 amino acid (aa) long σ1 protein. A second short overlapping ORF of 360 nts encodes the 120 aa long σ1s protein (also termed σ1ns or p14). The σ1s protein has been detected among the translation products of melted S1 genome segments in *in vitro* protein synthesizing systems, as well as in reovirus-infected cells (Ernst & Shatkin, 1985; Jacobs et al., 1985; Sarkar et al., 1985). The T1 and T2 σ1 proteins have also been cloned and sequenced (Cashdollar et al., 1985; Munemitsu et al., 1986). In view of several incongruities observed in these published sequences the cDNA clones of the T1 and T2 genome segments were resequenced (Duncan et al., 1990). The anomalies of the originally published sequences were corrected upon resequencing, with the following features of the S1 genes obtained. The T1 S1 gene is 1463 nts long and contains a LORF of 1410 nts which encodes the 470 aa long T1 σ1 protein. The T2 S1 gene is 1440 nts long and contains a LORF of 1386 nts which encodes the 462 aa long T2 σ1 protein. Similar to the T3 S1 gene, both the T1 and T2 genes contain a second short overlapping ORF of 357 nts and 375 nts encoding σ1s proteins of 119 aa and 125 aa long, respectively. Analysis of the predicted amino acid sequences of the three S1 genes reveals proteins of comparable size with molecular weights of 51.5 KDa, 50.4 KDa and 49.1 KDa, respectively.

The resequencing of the S1 genes confirmed the previous reports that the T1 and T2 S1 genome segments are much more closely related to each other (57 % nt matches) than to the T3 S1 genome segment (37 and 39 % nt matches, respectively). Corrected for random coincidence (25 % background random matching), the "true" percentage of similarity between the serotype pairs 1:2, 1:3 and 2:3 are 32, 12 and 14 %, respectively. Further analysis reveals the evolutionary divergence pattern of the three S1 genome segments

(Duncan et al., 1990). Based on the percentage mismatches in each codon position and taking into account that 25 % matches would be found when comparing completely unrelated sequences, the value of mismatches expressed as a percentage of (codons X 0.75) provides an estimate of the extent of divergence towards complete randomness. Results indicate that the third base codon positions have diverged to an extreme extent for the S1 genome segments (for the serotype pairs 1:2, 1:3 and 2:3, the percentage divergence towards complete randomness in the third base codon position is 79, 96 and 91 %, respectively), more than for any other reovirus genome segment (Weiner & Joklik, 1989). For example, comparing the third base codon position mismatch of serotypes 1 and 2, the most closely related serotype pair, the S1 genome segments have diverged 79 %, whereas the S3, M2 and L1 genome segments have diverged only 48, 53 and 13 %, respectively. The difference in evolutionary divergence patterns is even greater for the first and second base codon positions, where the highest divergence figures for any first or second base codon position for the S3, M2 and L1 genome segments is 25 and 8 %, respectively; by contrast, the corresponding figures for the S1 genome segments vary from 40 to 85 %. From this analysis it was concluded that evolutionary divergence has been greatest for the S1 gene segment than for any other genome segment; most likely because the σ1 structure is more tolerant of sequence change. Furthermore, it was concluded that the S1 genome segment has diverged for the longest period of time; since the more closely related the genome segments, the higher the proportion of transitions among codon mismatches and the S1 genome segments show % transition mismatches lower than for other reovirus genome segments.

Analysis of the amino acid sequence of σ1 (Bassel-Duby et al., 1985), as determined from the cloned T3 S1 gene, predicts an α-helical structure at the N-

terminal end spanning about one third of the molecule and a globular head structure at the C-terminal end. Furlong and coworkers have reported similar α-helical structures for the T1 and T2 σ1 proteins (Furlong et al., 1988), thus suggesting an amino-terminal fibrous domain is common to all three reovirus σ1 proteins. The σ1 protein is, therefore, predicted to have a morphology of a long fibrous tail topped with a globular head. The α-helical coiled-coil structure is typical of other α-helices (Crick, 1953; Creighton, 1984) in showing the following features: a heptapeptide repeat with the correct position of hydrophobic amino acids and the absence of both proline and aromatic amino acids from the heptapeptide repeat. These features have also been observed in a number of fibrous proteins in which the coiled coils of 2 of 3 α-helices are wound around each other and appears to contribute to the multimer formation of these proteins: examples include myosin (McLachlan and Karn, 1983), α-keratins (Steinert, 1978; McLachlan, 1978), tropomyosin (Talbot and Hodges, 1982), and fibrinogen (Doolittle, 1978). It has been hypothesized that the α-helical coiled-coil structure of σ1 is also involved in the formation of multimers and for subsequent stabilization.

On a gross scale σ1 has been shown to exist as a multimer (Bassel-Duby et al., 1987; Banerjea et al., 1988) and is associated with the λ2 protein of reovirus at the twelve vertices of the virion (Lee et al., 1981b). Although σ1 is known to exist as a multimer, the exact numerical state of the multimer form is being debated. Early evidence suggested σ1 to exist as a dimer (Lee et al., 1981b; Yeung et al., 1987; Bassel-Duby et al., 1985) or tetramer (Bassel-Duby et al., 1987; Banerjea et al., 1988). More recent evidence from this lab, based on SDS-PAGE analysis of σ1 produced in a rabbit reticulolysate *in vitro* translation system, shows σ1 as a trimer (unpublished observations). Further analysis is needed to confirm that σ1 also exists as a trimer in the virion particle.

The proposed morphology of σ1 has been confirmed with EM studies of reovirus particles and isolated T2 σ1 protein (Furlong et al., 1988) in which σ1 is seen as a rod (fiberous) structure topped with knobs extending from the surface of the viral particles. Purified σ1 isolated from virions (Furlong et al., 1988) or from a mammalian expression system (Banerjea et al., 1988) also assumes this morphology. From these EM studies it has been proposed that much of the fibrous domain of σ1 and the globular head can exist in two states in the virion: 1) unextended; buried in the λ2 spikes, or 2) extended outward from the surface of the virion. Furthermore, whereas isolated σ1 measures 41.4 nm in length, the maximum extended length from the surface of viral particles is 36 to 40 nm, indicating that a small portion of σ1 is buried within the structure of the virion, anchoring to the λ2 protrusions. Recent analysis of σ1 deletion mutants (Mah, 1989) confirms that σ1 is anchored to the reovirion via its amino-terminus and further shows that the N-terminal 121 amino acids alone dictates the incorporation of σ1 into the virion; a hydrophobic region within this 121 amino acid stretch is postulated to have a function in the anchoring process.

Overall, the morphology of σ1 resembles the hemagglutinin protein (HA) of influenza virus (Wilson et al., 1981; Wiley and Skehel, 1987; Weis et al., 1988) and even more closely the adenovirus fiber protein (Greene et al., 1983; Devaux et al., 1987), all of which are viral attachment proteins responsible for virus-host interactions. On more general terms, the morphology of σ1 appears to follow and overall evolutionary scheme common to the development of viral attachment proteins.

I.5 The Functionally Distinct Regions of σ1

Major research efforts have been directed at defining the structure-function relationship of the σ1 protein; initial studies revealed that there are functionally distinct domains on the σ1 protein. This finding is supported by a number of lines of evidence. The use of monoclonal antibodies directed against σ1 has allowed the identification of at least three distinct functional domains (or epitopes) on the σ1 protein (Burstin et al., 1982; Spriggs et al., 1983a). In these studies, one class of monoclonal antibodies exclusively neutralized reovirus infectivity (neutralization), a second class only inhibited virus-mediated hemagglutination (hemagglutination inhibition or HI), while a third class exhibited both neutralization and HI activity. These epitope mapping studies were the first indication that the various functions of σ1 could be assigned to specific regions of the protein.

A second approach used to study the functional domains of the σ1 protein was to select antigenic variants that were resistant to neutralization by anti-σ1 reovirus type 3 monoclonal antibodies (Spriggs and Fields, 1982; Spriggs et al., 1983b; Bassel-Duby et al., 1986; Kaye et al., 1986). Several variants have been isolated that are attenuated for neurovirulence and restricted cell tropism. Sequencing of these variants revealed a single point mutation in each variant with the altered amino acid residue of four attenuated variants, F, K, A14 and A17, being at residue 419, and the fifth variant, A, altered at amino acid 340, all in the carboxy-terminus. These results established that amino acids 419 and 340 of the σ1 protein carboxy-terminus are important sites affecting reovirus virulence, and further implicated the function of the carboxy-terminus as being distinct from the amino-terminus.

To further probe the functional domains on the σ1 protein a series of in-frame deletion mutants were generated using a set of restriction enzymes which

roughly divide the S1 gene into four segments (5'-I-II-III-IV-3') of similar size (Nagata et al., 1987). These in-frame deletion mutants were expressed in *Escherichia coli* and assayed for, (i) host cell (mouse L fibroblasts) binding activity, and (ii) glycophorin (reovirus erythrocyte receptor) binding activity (as a measure of hemagglutination activity). It was found that mutant σ1 forms with segment III or IV deleted did not exhibit any detectable L-cell binding activity, whereas mutants with these two segments intact (but lacking segments II or segments I and II) were capable of attaching to L-cell receptors, albeit with reduced efficiencies. On the other hand, only full length σ1, but none of the mutants, could bind immobilized glycophorin. Results thus indicated that the host cell attachment domain is localized in the C-terminal-half of the molecule (segments III and IV), and is distinct from the hemagglutination domain which involves residues in the middle of the protein (segments II and III).

The final line of evidence showing the existence of functionally distinct domains on σ1 comes from receptor binding studies using proteolytic fragments of σ1 (Yeung et al., 1989). In these studies purified native σ1 protein from [^{35}S]methionine-labeled T3 reovirions were subjected to limited trypsin and chymotrypsin digestion. It was found that T3 σ1 (49K molecular weight) was cleaved by trypsin to yield an ~24K and an ~25K fragment, and by chymotrypsin to yield a 42K fragment. The 24K tryptic fragment, but not the 25K tryptic fragment, was shown to possess L-cell binding capacity, and represents the carboxy-terminal half of T3 σ1 since it contains the single cysteine residue (amino acid 351) as revealed by tryptic analysis of [^{35}S]cysteine-labeled σ1. Neither tryptic fragment was able to bind to glycophorin, the reovirus receptor on human erythrocytes. Therefore, the function of reovirus host cell attachment is distinct from that of reovirus hemagglutination. Furthermore, the two tryptic fragments were recognized by different neutralizing monoclonal anti-σ1

antibodies, again indicating that neutralizing and cell attachment sites are not necessarily equivalent.

These observations are all in absolute agreement, and clearly indicate that different recognition mechanisms are involved in reovirus host cell attachment and hemagglutination. Furthermore, it can be concluded that the receptor-binding domain maps to the carboxy-terminus of the σ1 protein.

I.6 Structure/Function Relatedness of the T1,T2 & T3 σ1 Proteins

The structure/function relatedness among the σ1 proteins of the three serotypes was not immediately apparent from early studies of reovirus and the σ1 protein. In fact early studies revealed striking differences between these proteins: (i) genetic reassortment analysis revealed that type-specificity is determined by the S1 genome segment which encodes σ1, with σ1 being the most type-specific of all reovirus-coded proteins (Weiner and Fields, 1977; Gaillard and Joklik, 1980), (ii) genetic reassortment studies also revealed that the S1 gene defines tissue tropism (Weiner et al., 1977), and (iii) characterization of anti-σ1 immunoglobulins secreted by cloned hybridoma cell lines showed that the σ1 proteins of the three serotypes have quite different antigenic determinants (Lee et al., 1981a; Hayes et al., 1981).

With the continued analysis of the three serotypes, and more specifically of the three σ1 proteins, it was soon apparent that along with the differences exhibited, the three σ1 proteins also show marked functional and structural similarities. Evidence for their relatedness was first demonstrated upon the isolation and characterization of recombinant clones derived from mixed infection of L cells with reovirus types 1, 2 and 3 (Sharpe et al., 1978; Ramig et al., 1978; Weiner et al., 1978). These studies showed the ability to form viable

(infectious) recombinants between the serotypes, and specifically showed that σ1 could be assembled into virions of different serotypes while retaining the viability of the recombinant.

Competition experiments between polypeptide σ1 from infected L cell lysates and reovirus particles demonstrated that each of the three serotypes interfered to the same extent with the attachment not only of the homologous σ1 protein, but also with that of heterologous σ1 (Lee et al., 1981b). These studies indicate that reovirus particles and free protein σ1 of all three serotypes attach to the same cell surface receptor on mouse L fibroblasts.

Final evidence for the relatedness of the σ1 proteins has come from the direct analysis of the amino acid sequences of the σ1 proteins of reovirus serotypes 1, 2, and 3 (Duncan et al., 1990). Analysis of the sequences reveals that although the three σ1 proteins have diverged very extensively, they still share 79 of their approximately 470 amino acid residues (the length of the T1, T2 and T3 σ1 proteins are 470, 462 and 455 amino acids, respectively). Significant clustering of 49 of the 79 triply conserved residues into five regions, designated as regions A-E, is observed (Fig. 12). In regions A, B, C and E, 32% of the residues are common to all three σ1 proteins, while region D exhibits an even greater homology of 46%. This homology is approximately two to three times higher than the overall homology of 17% exhibited by the three proteins. When conserved amino acid replacements are taken into account, the five regions exhibit residue similarities that range from 39 (region C) to 55% (region B).

The tertiary structures of the three σ1 proteins are also predicted to resemble each other (Duncan et al., 1990). All three proteins have an α-helix content of ~17%, A β-sheet content of ~37%, a random coil content of ~30%, and a turn content of ~16%. The α-helix plots for the proteins resemble each

other, with serotypes 1 and 2 showing a greater resemblance to each other than to T3 protein. For the T1 and T2 proteins about two-thirds of the α-helices are in the amino-terminal one-third of the protein; for the T3 protein this fraction is about 90%. Hydropathy plots of the σ1 proteins show the carboxy-terminal regions, spanning ~130 residues and covering region C, D and E, have very similar profiles; again the profiles of T1 and T2 appear to be more similar to each other than to that of T3 σ1. Region A is predominantly of α-helical structure and represents the carboxy-terminal limit of the predicted α-helical coiled-coil structure (Bassel-Duby et al., 1985; Furlong et al., 1988) that is the major structural feature of approximately one-third of the amino-terminal end of all three σ1 proteins. Region B exists predominantly in a random coil/β-sheet with numerous turn configurations. Regions C, D and E all reside in the carboxy terminal domain of σ1 that harbours the host cell attachment function. Secondary-structure predictions reveal no dominant structural pattern in this domain, but instead shows this area to be a mixture of short α-helices, β-sheets and random coils and contains many turns; 12 proline residues and bulky aromatic amino acids are also present (Bassel-Duby et al., 1985). All these features suggest a globular-like structure for the carboxy-terminal domain of σ1.

With the demonstration of a structure-function relatedness among the three σ1 proteins it was concluded that certain functional sites, such as cell attachment, hemagglutination, oligomerization and anchorage, are presumably conserved among the σ1 proteins of the three serotypes. The five highly conserved regions, A-E, are also suggestive of regions that have been conserved through extensive evolution of the S1 gene due to some functional significance. The direct importance of the cell attachment domain of σ1 to the overall fidelity of reovirus as an infectious agent necessitates further studies to elucidate more specifically the structure-function relationship of this protein.

The identification of conserved regions among the three σ1 proteins provides a good basis for these studies.

I.7 The Research Project

The major direction of research in this laboratory is aimed at the elucidation of virus-host interactions at the plasma membrane level via studies of the well characterized reovirus model system. Study of the σ1 protein is essential to this goal as it is the viral attachment protein of reovirus. In this project the reovirus viral attachment protein σ1 has been studied in order to more fully define the receptor-binding domain of this protein. *In-vitro* site-directed mutagenesis has been utilized as a tool for this undertaking. This method is an extremely powerful research tool and has been used to reveal structure-function relationships of a number of proteins (reviewed by Dalbadie-McFarland and Richards, 1983; Smith, 1985; Botstein and Shortle, 1985). The regions of the σ1 protein being studied are located in the carboxy-terminal half of the protein, previously shown to be involved in the cell binding function of σ1, and include the C, D, and E conserved regions. Amino acids in region C have been targeted since it has been shown that a synthetic peptide corresponding to this region of the σ1 protein is capable of cell binding (Williams *et al.*, 1988). Residues in region D have been targeted because of the high homology between serotypes exhibited in this region, 46% (Duncan et al., 1990); with the possibility that this region is involved in receptor recognition. Deletion mapping studies have also shown that removal of regions D and E destroys L cell binding (Nagata et al., 1987). Recent fine structure mapping studies in our laboratory have shown that removal of as few as four amino acids from the carboxy-terminus of serotype 3 σ1 completely abrogates L cell binding, further indicating the significance of region E for cell attachment. Two of three serotype

conserved amino acids spanning the terminal twelve amino acids of the protein were targeted for mutagenesis. Mutagenesis of the cloned S1 gene was followed by expression of the mutated genes using an *in vitro* transcription vector system and cell-free translation. The mutant σ1 protein forms were then analyzed for altered cell-binding capability and altered structure. The successful completion of this project has provided further information concerning the structure-function organization of σ1 and has allowed for a more critical analysis of potential regions involved in the σ1 receptor-binding function. Together with the rapidly growing body of knowledge concerning σ1 protein functions, the information ascertained in this study, therefore, provides a good basis for future studies aimed at fully understanding the structure-function relationships of this protein at the molecular level.

CHAPTER II: Materials and Methods

II.1 Propagation of Bacteriophage M13 and Host Cells

The basic strategies for the construction of M13 bacteriophage plasmids, their growth and maintenance in host cells containing an F' episome were those described by Messing (1983). Since the protocols were in some cases significantly altered the procedures used in this project will be described in detail.

Bacterial Host for Bacteriophage M13 vectors

All M13 vectors were propagated in the *E. coli* host strains JM109 or DH5αF', which carry F' episomes and a number of genetic markers that make them useful to work with M13 vectors [e.g., lacZΔM15, Δ(lac-proAB), and proAB] (Yanisch-Perron et al., 1985). Master stocks of JM109 were streaked onto minimal (M9) agar plates and incubated for 24 to 36 hours at 37°C. Master stocks of DH5αF' were streaked onto 2xYT agar plates and incubated for 16 to 24 hours at 37°C. Master stocks of JM109 and DH5αF' were also stored at -70°C in M9 medium or 2xYT medium containing 15 % glycerol (see Appendix: Frozen Storage of Bacteria).

Phage Plaquing

An isolated colony of *E. coli* DH5αF' (or JM109) growing on 2xYT medium were inoculated into 5 mL of 2xYT broth and grown overnight with shaking at 37°C. This culture was diluted 1 in 100 into 5 mL of 2xYT broth and grown with shaking at 37°C for 4 hours. Serial 10-fold dilutions (10^{-1} to 10^{-9}) of the phage stock were prepared in 2xYT and 100 µL samples of the 10^{-7} to 10^{-9} dilutions were added to test tubes containing 200 µL of log phase (or an overnight culture) *E. coli* DH5αF', 4 mL of B-broth top agar at 42°C, 40 µL of a 2 % Xgal

solution and 20 µL of 100 mM IPTG. The solution was mixed by swirling and poured onto prewarmed (37°C) 2xYT agar plates. The agar plates were left at room temperature for a minimum of 15 minutes to set, followed by incubation in an inverted position at 37°C overnight. Plaques were generally visible within 4 hours but an overnight incubation was needed to allow for colour development. M13 phage produced blue plaques while M13 recombinant phage produced colourless plaques.

Preparation and Storage of M13 Phage Stocks

M13 phage and all recombinant M13 phage stocks were prepared from single well isolated plaques as follows. To 5 mL of 2xYT medium in a sterile 15-mL culture tube was added 50 µL of log phase DH5aF' (or JM109) and an agar plug (picked with a sterile pasteur pipette) containing a single phage plaque, formed on a lawn of *E. coli* DH5aF' (or JM109). The infected culture was incubated for 4 to 5 hours at 37°C with vigorous shaking (200 rpm). The entire culture was centrifuged at 10K x g for 5 minutes at 4°C, and the supernatant aliquoted into sterile 1.5-mL microfuge tubes. These phage stocks were then stored at 4°C or -20°C. M13 phage particles can be stored frozen at -20°C for many years without the loss of infectivity (Messing, 1983).

II.2 Preparation of Bacteriophage M13 DNA

Preparation of M13 Replicative Form DNA

Large amounts of the replicative form DNA (RF DNA) of M13 were isolated from 250 mL cultures of phage-infected *E. coli* DH5αF' (or JM109) as follows. An isolated plaque was picked with a sterile pasteur pipette, added to 3 mL of 2xYT in a 15-mL test tube and grown for 6 hours at 37°C with vigorous shaking. After 3 hours, 2.5 mL of an overnight culture of *E. coli* DH5αF' was added to 250

mL of 2xYT in a 1-L flask and grown for 3 hours at 37°C with shaking. After this final 3 hours, the infected cell culture was added to the log phase *E. coli* DH5αF' culture and incubated overnight at 37°C with shaking. The infected cells were harvested by centrifugation at 10K x g for 15 min at 4°C; the phage-containing supernatant from these cultures were also used for large scale preparations of phage ssDNA (see below). The RF DNA was isolated using a modified large scale alkaline-SDS lysis procedure (see Appendix: Preparation of Plasmid and Phage RF DNA).

For small scale isolations of phage RF DNA, an overnight culture of *E. coli* DH5αF' (or JM109) grown in 2xYT medium was diluted 1 in 100 in 5 mL of 2xYT broth in 15-mL test tubes. A 10 μL aliquot of a phage stock or a freshly picked plaque was added to each tube and the infected cells were grown for 5 to 6 hr at 37°C with vigorous shaking. A 1.5 mL volume of the cell culture was transferred to a 1.7-mL microfuge tube and the cells were pelleted in a microfuge at 16K x g for 30 sec at room temperature. The supernatant was decanted, an additional 1.5 mL of culture was added, and the tubes were again spun for 30 sec. The supernatant was decanted and the RF DNA was isolated using a modified small scale alkaline-SDS lysis procedure (see Appendix: Preparation of Plasmid and Phage RF DNA).

Large Scale Preparation of Phage DNA

Large amounts of ssDNA were isolated from phage-containing supernatant prepared from 50 mL cultures. The 50 mL cultures of *E. coli* DH5αF' (or JM109) were phage-infected by one of two methods:

Method I: An isolated plaque was picked with a sterile pasteur pipette, added to 3 mL of 2xYT in a 15-mL test tube and grown for 3 hours at 37°C with vigorous shaking. At the same time, 500 μL of an overnight culture of *E. coli* DH5αF' was

added to 50 mL of 2xYT in a 1-L flask and grown for 3 hours at 37°C with shaking. After 3 hours, the infected cell culture was added to the log phase *E. coli* DH5αF' culture and incubated a further 5 to 6 hours at 37°C with shaking. Method II: An overnight culture of *E. coli* DH5αF' (or JM109) was added at a dilution of 1 in 100 to 50 mL of 2xYT and grown to an A_{550} of 0.3. An appropriate volume of phage stock was then added for a multiplicity of infection (MOI) of 4 x 10^4 pfu/mL (ie. 2 x 10^6 pfu in 50 mL of broth culture or ~50 to 100 µL of phage stock in 50 mL of broth culture). The infected cell culture is grown for a further 5 to 6 hours at 37°C with shaking.

The phage-containing supernatant was harvested by centrifugation at 10K x g for 15 min at 4°C to pellet the cells. The supernatant was transferred to sterile 50-mL conical centrifuge tubes and stored at 4°C until needed. If the phage-supernatant became turbid during storage it was cleared by centrifugation at 10K x g for 15 min at 4°C before use. The phage were then precipitated by mixing 32 mL of phage stock with 8 mL of 20 % PEG 8000 - 3.5 M ammonium acetate in a sterile 50-mL polyethylene centrifuge tube and incubating at room temperature for 30 min. The precipitate was collected by centrifugation at 13K x g for 15 min at 4°C and the supernatant was decanted. The inside of the tube was wiped out with a Kimwipe to remove the remaining PEG solution and the pellet resuspended in 0.5 mL of phage lysis buffer (PLB: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.25 M NaCl, 0.2% SDS). The solution was incubated at 68°C for 30 min and the proteins removed by phenol (once) and phenol:chloroform (three times) and chloroform (twice) extractions. The final aqueous phase was transferred to a 1.5-mL microfuge tube and the phage DNA precipitated by the addition of a 1/10 volume of 3.5 M sodium acetate and two volumes of ethanol and incubation at room temperature for 30 min or an overnight incubation at 4°C. The DNA was recovered by centrifugation in a microfuge at 16K x g for 15

min at 4°C, washed with 70 % ethanol and the tube inverted to allow excess supernatant to drain away from the pellet. The pellet was dried in a vacuum desiccator, resuspended in 20 µL of TE (pH 8.0) and stored at 4°C or -20°C until needed. The yield of DNA was determined by standard A_{260} measurements (Maniatis et al., 1982).

Small Scale Preparation of Phage DNA

For small scale isolations of phage ssDNA, an overnight culture of *E. coli* DH5αF' (or JM109) grown in 2xYT medium was diluted 1 in 100 in 5 mL of 2xYT broth in 15-mL test tubes. A 10 µL aliquot of a phage stock or a freshly picked plaque was added to each tube and the infected cells were grown for 5 to 6 hr at 37°C with vigorous shaking. A 1.5 mL volume of the cell culture was transferred to a 1.7-mL microfuge tube and the cells were pelleted in a microfuge at 16K x g for 30 sec at room temperature. A 1.2 mL volume of the supernatant was carefully removed to a 1.7-mL microfuge tube and the phage were precipitated by adding 400 µL of 20% PEG 8000 - 3.5 M ammonium acetate. The tube was vortexed to mix the solution and incubated for 30 min at room temperature. The precipitate was collected by centrifugation at 16K x g for 15 min at room temperature and the supernatant was decanted. The tube was recentrifuged for 1 min and the residual supernatant removed. The pellet was resuspended in 300 µL of phage lysis buffer (PLB), vortexed well and incubated at 68°C for 30 min. The proteins were removed by extraction with phenol and chloroform as follows: a half volume of phenol was added, vortexed well and the tube allowed to stand at room temperature for 5 min; a half volume of chloroform was then added, vortexed well and the tube centrifuged at 16K x g for 5 min at room temperature. The aqueous phase was transferred to a 1.7-mL microfuge tube and extracted twice with an equal volume of chloroform. The

final aqueous phase was transferred to a fresh tube and the phage ssDNA was precipitated by the addition of a 1/10 volume of 3.5 M sodium acetate and two volumes of ethanol followed by an overnight incubation at 4°C. The DNA was recovered by centrifugation in a microfuge at 16K x g for 15 min at 4°C, washed with 70 % ethanol and the tube inverted to allow excess supernatant to drain away from the pellet. The pellet was dried in a vacuum desiccator, resuspended in 14 µL of TE (pH 8.0) and stored at 4°C or -20°C until needed. The yield of ssDNA was ~ 1 - 3 µg, enough for 2 sequencing reactions.

II.3 Transformation of Phage DNA

M13 RF DNA, 1 to 10 ng in a volume of approximately 10 µL of water or TE buffer, was added to 300 µL of prechilled competent cells (see Appendix), and incubated on ice for 40 minutes. The cells were heat shocked by incubation in a 42°C water bath for 90 seconds, then immediately placed on ice for 2 to 3 minutes; the 300 µL of cells and DNA was then added to B-broth top agar. During the 40 minute incubation, B-broth top agar is melted and 4 mL is added to sterile disposable centrifuge tubes (15 mL capacity) prewarmed in a 42°C water bath. To the 4 mL of B-broth top agar was added 40 µL of 2 % X-gal solution, 20 µL of 100 mM IPTG, 200 µL of log phase DH5αF' (or JM109) cells and the 300 µL of cells and DNA. The contents were mixed immediately by gently swirling 2 or 3 times, and then poured onto prewarmed (37°C) 2xYT agar plates. The agar plates were left at room temperature for a minimum of 15 minutes to set, followed by incubation in an inverted position at 37°C overnight. Plaques of 1 to 2 millimeters in diameter were visible within 16 to 24 hours. M13 phage produced blue plaques while recombinant M13 phage produced colourless plaques. Plaques were picked and added to 2xYT broth for DNA isolation as described above.

II.4 Subcloning of S1 Gene Fragment into M13

The carboxy-terminal portion of the T3 S1 gene was subcloned from the plasmid pES1T3, containing the entire S1 coding sequence, into M13 mp19 to be used as the ssDNA template for mutagenesis; this plasmid was referred to as pSE0.9-19. Plasmid pES1T3 DNA was isolated as outlined in the Appendix (Preparation of Plasmid and Phage RF DNA) and M13 mp19 RF DNA was isolated as described above. Plasmid pES1T3 DNA (6 μg) was REN digested with 5 units of Sst I and 5 units of EcoRI, simultaneously, in a 50 μL reaction (see Appendix for standard REN digestion procedures), and a sample was checked by agarose gel electrophoresis for complete digestion. The M13 mp19 RF DNA (1.5 μg) was first REN digested with 5 units of Sst I in a 20 μL reaction volume for 1 hr at 37°C. To this reaction was added 1 μL of BRL REact Buffer 2 (500 mM Tris-Cl, pH 8.0, 100 mM $MgCl_2$, 500 mM NaCl), 3 μL 500 mM NaCl, 5.5 μL H_2O and 5 units of EcoRI in 0.5 μL for a final reaction of 30 μL and 100 mM NaCl; the reaction was incubated at 37°C for 1 hr and a sample was checked by agarose gel electrophoresis for complete digestion. The fragments of interest from these REN digestion were isolated by agarose gel electrophoresis using low melting point agarose (Appendix). The DNA pellet was resuspended in a small volume (10 to 20 μL) of sterile TE (pH 8.0) and the DNA concentration was estimated by the degree of EtBr fluorescence relative to a series of known standards (Appendix). The S1 gene fragment was ligated into the Sst I/EcoRI digested M13 mp19 vector using T4 DNA ligase and insert:vector molar ratios of 2:1 and 3:1, as outlined in the Appendix. The ligated phage DNA was transformed into competent *E. coli* DHFαF' cells as described above (section II.3). Colourless plaques were picked from the B broth agar plates, the cultures grown in 5 mL of 2xYT broth and RF DNA

isolated as described previously. The DNA was ethanol-precipitated and resuspended in 50 µL of TE (pH 7.5) containing DNase-free RNase A at a concentration of 20 µg/mL. Detection of the M13 clone containing the insert was done by REN mapping as follows. A sample of this DNA, typically 2 to 5 µL, was digested with 5 units of an appropriate REN (a double digest using Sst I and EcoRI; a single digest using Nci I) and the DNA fragments were separated by gel electrophoresis through 0.7 % agarose slab gels in TBE buffer at 120 volts for 20 to 30 min (Appendix). DNA molecular weight standards (λ HindIII DNA fragments or a 1 Kbp DNA ladder) and REN digests of the plasmid pES1T3 and M13 mp19 (as described above) were also included on the gel.

II.5 Phosphorothioate-Based Oligonucleotide Mutagenesis

Mutagenesis of the cloned S1 gene was accomplished using the technique described by Nakamaye & Eckstein (1986; Taylor et al., 1985a, b; Sayers et al., 1988), which utilizes phosphorothioate-modified DNA. The details for the preparation of the buffers and reagents used in the mutagenesis protocol are outlined in the Appendix.

Synthesis of Mutagenic Oligonucleotides

The eight mutant oligonucleotides used in the mutagenesis procedure were custom made by Dr. R. T. Pon of the Regional DNA Synthesis Laboratory, Department of Medical Biochemistry, The University of Calgary, Calgary, Alberta. The mutant oligonucleotides were made using solid phase chemical synthesis on an Applied Biosystem 380 or 381 DNA Synthesizer, gel purified and lyophylized to a powder form. Before use all mutant oligonucleotides were resuspended in dH_2O to a concentration of 20 pmol/µL for a working stock solution, and stored at -20°C.

The four mutant oligonucleotides, S325Δ, S327Δ, Y450Δ and P451Δ, were chemically 5'-phosphorylated during synthesis. The four mutant oligonucleotides, Y326NΔ, D365YΔ, N369YΔ and F370VΔ, were phosphorylated enzymatically according to the procedure of Zoller and Smith (1983), as follows. The mutant oligonucleotide, 10 μL of the 20 pmol/μL oligonucleotide stock solution, was added to 20 μL of 100 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 330 μM ATP, followed by the addition of 5 units of T4 polynucleotide kinase (PNK). The reaction mixture was incubated for 45 min at 37°C, followed by heat inactivation of the enzyme at 65°C for 10 min; 3 μL of this reaction mixture (oligonucleotide concentration at 6.7 pmol/μL) was removed and diluted in 17 μL of H_2O for a final oligonucleotide concentration of 1 pmol/μL. This diluted solution was stored at 4°C and used in the mutagenesis procedure; the undiluted reaction mixture was stored at -20°C until needed.

Mutagenesis

The single-stranded M13 DNA pSE0.9-19, isolated as described above (Large Scale Preparation of Phage DNA), was used as the template DNA in all mutagenesis experiments. Into a 0.5-mL microfuge tube was placed 10 μL of buffer 1 (600 mM NaCl, 600 mM Tris-Cl, pH 8.0), 10 μL of ssDNA template at a concentration of 1.0 μg/μL (10 μg of ssDNA pSE0.9-19), 8.0 μL of 5'-phosphorylated mutant oligonucleotide at a concentration of 1 pmol/μL (8.0 pmol) and 22 μL of H_2O for a final volume of 50 μL. Annealing of the mutant oligonucleotide to the ssDNA template was done by heating this reaction mixture to 70°C in a heating block for 10 min, immediately transferring to a 37°C heating block for 30 min and then placing the mixture on ice. To this solution was added 10 μL of 100 mM $MgCl_2$, 10 μL of nucleotide mix 1 (10 mM ATP, 2.5 mM dCTPαS, 2.5 mM each of dATP, dGTP and dTTP), 18 units of T4 DNA

ligase, 12 units of DNA polymerase I large fragment (Klenow enzyme) and dH_2O to a final volume of 100 µL. The solution was mixed by pipetting up and down and then incubated in a 16°C waterbath overnight (~ 16 hr), for the polymerization and ligation of the mutant DNA strand (a 1 µL fraction of this reaction mixture was removed for gel analysis). Non-mutant ssDNA was removed by nitrocellulose filtration as follows: the polymerization reaction mixture was made 300 mM in sodium acetate and filtered through 2 nitrocellulose filters using a Sartorius syringe filter holder; the filters were washed two times with 80 µL of 500 mM NaCl and the combined filtrates ethanol precipitated. The DNA was recovered by centrifugation in a microfuge at 16K x g for 15 min at 4°C, washed with 70 % ethanol and the tube inverted to allow excess supernatant to drain away from the pellet. The pellet was vacuum dried and resuspended in 50 µL of buffer 2 (5 mM Tris-Cl, pH 8.0, 0.5 mM EDTA). A 15 µL aliquot of this RF DNA solution (~ 1.0 µg of RF DNA) was removed to a 0.5-mL microfuge tube containing 7.5 µL of buffer 3 (300 mM NaCl, 120 mM Tris-Cl, pH 8.0, 60 mM $MgCl_2$, 100 mM DTT), 9.0 units of the restriction endonuclease Nci I and dH_2O to a final volume of 75 µL. This reaction mixture was incubated at 37°C in a heating block for 90 min for the nicking of the non-mutant strand of the DNA molecule, after which a 10 µL fraction was removed for gel analysis. To the remaining 65 µL of the nicking reaction was added 14.1 µL 500 mM NaCl, 10 µL of buffer 4 (522 mM Tris-Cl, pH 8.0, 21 mM $MgCl_2$, 35 mM DTT), 2.0 µL of a working stock of exonuclease III at a concentration of 25 units/µL (50 units; exonuclease III was diluted immediately before use in a 1 X solution of buffer 4 to a working stock of 25 units/µL, excess working stock was discarded), and 8.9 µL of dH_2O for a final volume of 100 µL. This reaction mix was incubated at 37°C in a heating block for 15 min to remove ~1500 bases from the non-mutant strand of the DNA

molecule, which included the non-mutant S1 gene sequence. After the incubation was complete the reaction mix was heated to 70°C in a waterbath for 15 min to inactivate the exonuclease III enzyme and a 17 μL fraction was removed for gel analysis. To the remaining 83 μL of the gapped solution was added 10 μL of nucleotide mix 2 (3.5 mM ATP, 2.5 mM each of dATP, dCTP, dGTP and dTTP), 5.0 μL 100 mM $MgCl_2$, 3.0 units T4 DNA ligase and 2.5 units DNA polymerase I. This reaction mixture was incubated at 16°C in a waterbath for 3 hr for repolymerization of the gapped DNA to form homopolymeric mutant duplex DNA; a 15 μL fraction was then removed for gel analysis.

All samples removed for gel analysis during the mutagenesis procedure were analyzed on a 0.7 % agarose gel as described in the Appendix (Agarose Gel Electrophoresis). After gel analysis, 2 μL and 10 μL fractions of the repolymerized reaction mixture were used directly to transform DH5αF' competent cells according to the procedure described above for the transformation of phage DNA. Well isolated colourless plaques were picked from the B-broth agar plates, added to 2 mL of 2XYT medium and incubated at 37°C for 6 hr with shaking. The cells were pelleted at 3.3K x g for 15 min at 4°C and the supernatant was transferred to sterile 5-mL polyethalene tubes and stored at 4°C. This phage-containing supernatant (also referred to as phage stock) was used for (1) the screening of mutants, (2) sequencing, to confirm and identify mutants, and (3) for replaquing and further isolation of mutant DNA for cloning purposes. The cell pellets were used for the isolation of M13 RF DNA by a modified small scale alkaline-SDS lysis procedure (see Appendix).

II.6 Screening for Mutants

Restriction Endonuclease (REN) Analysis

The screening of putative mutants for two of the sites targeted, Y326 and D365, was done by restriction endonuclease analysis of the M13 RF DNA purified from ten well isolated phage plaques. RF DNA isolated by the small scale alkaline-SDS lysis procedure was resuspended in a final volume of 50 μL of TE (pH 7.5). Y326N S1 DNA was screened for the presence of a new EcoRI site by REN digestion of 5 μL of the DNA sample followed by gel electrophoresis, as described in the Appendix. D365Y S1 DNA was screened for the loss of a Sau3AI site by first digesting 20 μL of the RF DNA sample with Sst I and EcoRI and purifying the 880 bp fragment from agarose gels (see Appendix). The purified Sst I/EcoRI fragment was then digested with Sau3AI in a final reaction volume of 10 μL followed by TBE-5% PAGE (5% polyacrylamide, 1 X TBE) analysis; prior to gel analysis a 1/6 volume of 6 X DSB (60 mM Tris-HCl, pH 8.0, 120 mM EDTA, 15 % ficoll-400, 0.25 % bromophenol blue, 0.25 % xylene cyanol) was added to the restriction digest sample. DNA fragments were visualized by soaking the gel for 15 - 30 min in 1 X TBE containing 0.5 μg of EtBr/mL. The gel was briefly destained by soaking in 1 X TBE and photographed as for agarose gels (Appendix).

Dot Blot Hybridization

Putative mutants of all other sites targeted were screened by dot blot hybridization. A Bio-Rad Bio-dot SF Blotting apparatus was assembled according to the recommended procedure using a 9 cm x 12 cm Hybond N nylon filter that was pre-wetted with 2 X SSC buffer (1 X SSC: 0.15 M NaCl, 0.015 M sodium tricitrate, pH adjusted to 7.0 with 10 N NaOH). Phage-

containing supernatants (100 μL) were loaded as individual samples to each of the wells on the dot blot apparatus; duplicate controls of recombinant pSE0.9-19 and M13 mp19 phage supernatants (100 μL) were also added. Samples were absorbed onto the nylon filter by vacuum aspiration, the apparatus disassembled and the nylon filter removed. The nylon filters were rinsed with 2 X SSC and air dried followed by heating in a microwave at maximum power for 1 min. The nylon filters were then placed, inverted (ie. DNA side down), onto a sheet of plastic wrap and UV irradiated on a UV transilluminator for 5 min. The nylon filters were placed inside a plastic storage bag and prehybridized using 10 mL of prehyb solution [6 X SSC, 10 X Denhardts solution (0.2% Ficoll-400K, 0.2% polyvinylpyrrolidone, 0.2% BSA), 0.2% SDS]. The plastic bags were heat sealed and incubated at room temperature, overnight with gentle rocking. The prehyb solution was removed from the plastic bags and the nylon filters rinsed with 50 mL of 6 X SSC each for 1 min. Hybridization of the ssDNA with the [^{32}P]-labelled mutagenic oligonucleotide (see below under 'Preparation of Labelled Probe') was done by adding 4 mL of the appropriate labelled mutagenic probe to each of the plastic bags containing the nylon filters. The bags were heat sealed and incubated at 68°C for 30 min and cooled to room temperature over 30 min. The labelled primer was removed and the filters washed 3 times at room temperature with 100 mL of 6 X SSC for 5 min. The nylon filters were removed from the plastic bags, blotted on one side to remove excess liquid, and covered with plastic wrap. Autoradiography was done using Kodak XAR-5 film and exposed overnight (16 - 20 hr).

The nylon filters were then washed at increasing temperatures of stringency (see Results) in sealable plastic bags using 50 mL of preheated 6 X SSC and incubating at the appropriate temperature for a minimum of 5 min. The nylon filters were autoradiographed as described above.

Preparation of Labelled Probe

Mutagenic oligonucleotides were [^{32}P]-labelled by one of two methods, depending on whether or not the oligonucleotides were chemically labelled during synthesis. The four mutagenic oligonucleotides Y326NΔ, D365YΔ, N369YΔ and F370VΔ were not 5'-phosphorylated during synthesis and were therefore [^{32}P]-labelled using the T4 polynucleotide kinase (T4 PNK) forward reaction (Grunstein and Hogness, 1975; Cobianchi and Samuel, 1987). Reactions were set up in 1.5-mL microfuge tubes containing 30 pmol of the mutagenic oligonucleotide, 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 7 mM DTT, 2 - 5 units of T4 PNK, 100 μCi [γ ^{32}P]-ATP (5000 Ci/mmol), and dH_2O to 30 μL final. The reaction mixture was incubated at 37°C for 30 min, diluted with 3 mL of 6 X SCC and filtered through a 0.45 μm syringe filter unit to remove material that might cause 'hot spots'. The filter unit was washed through with 1 mL 6 X SSC and the filtrates pooled. The four mutant oligonucleotides, S325Δ, S327Δ, Y450Δ and P451Δ, were chemically 5'-phosphorylated during synthesis and were therefore [^{32}P]-labelled using the T4 PNK-exchange reaction as modified from the protocols of van de Sande et al. (1973) and Cobianchi and Samuel (1987). Reactions were set up in 1.5-mL microfuge tubes containing 50 pmol of the mutagenic oligonucleotide, 5 μL 10 X kinase exchange reaction buffer [500 mM imidazole-HCl (pH 6.8), 120 mM $MgCl_2$, 100 mM DTT, 2 mM ADP, 20 μM ATP], 125 μCi [γ ^{32}P]-ATP (5000 Ci/mmol), 20 units T4 PNK, and dH_2O to 50 μL final. The reaction mixture was incubated at 37°C for 30 min, diluted with 3 mL of 6 X SCC and filtered as described above.

Sequencing of Phage DNA

The final identification of all mutants was done by sequencing the phage DNA of the phage stock corresponding to the mutant DNA detected by REN

analysis or dot blot hybridization. The appropriate ssDNA template for sequencing was isolated from phage-containing supernatant as described in section II.3 (Small Scale Preparation of Phage DNA). A Sequenase kit from the United States Biochemical Corporation (USB), Cleveland, OH., was used for the sequencing reactions. The procedures followed were those outlined in the Step-By Step Protocols for DNA Sequencing With Sequenase using the dideoxy chain-termination method of Sanger et al. (1977) and modified T7 DNA polymerase (Sequenase); sequencing reactions were done according to the published procedures of Tabor and Richardson (1987). A typical reaction was done using 1 - 2 μg of ssDNA template, 0.5 pmol of the appropriate primer, 5 μCi [α-^{35}S]-dATP (1000 Ci/mmol), and 3.5 units of the Sequenase enzyme. For sequencing S325, S327, Y326N, D365Y, N369Y and F370V mutant DNA a synthetic oligonucleotide corresponding to nucleotides 1156 - 1170 of the S1 gene sequence was used. For sequencing Y450 and P451 mutant DNA the Universal M13 Sequencing primer was used.

Sequencing Gel Electrophoresis

DNA sequence analysis was routinely done using electrolyte gradient gels (Lurquin, 1988) as follows. Conventional 8% polyacrylamide sequencing gels were prepared in 1 X TBE buffer containing 8 M urea, polymerized by the addition of 0.05% ammonium persulphate and 0.1% TEMED (final concentrations), and cast between 20 cm x 50 cm glass plates separated by 0.4 mm spacers; wells were made during the polymerization step using a slot forming comb inserted at the top of the gel. Gels were allowed to polymerize for at least 30 min, the comb was removed and the wells cleared of any debris. The gels were run on a DNA Sequencing System SVG-20 apparatus (Tyler Research Instruments, Edmonton, Alberta) in which the upper buffer reservoir

was filled with 0.5 X TBE and the lower buffer reservoir was filled with 0.5 X TBE containing 1 M sodium acetate. Urea that had leached into the wells was flushed out with running buffer immediately prior to loading the samples. A plastic coated aluminum plate was clamped onto one side of the glass plates to aid in heat distribution during electrophoresis. Electrophoresis was carried out at a constant current of 35 mA until the bromophenol blue in the sample buffer had reached the bottom, ~ 3 hr. After electrophoresis, the apparatus was disassembled and the gel transferred to a sheet of Whatman 3MM filter paper and fixed by streaming a 500 mL solution of 10% acetic acid and 12% methanol over the entire gel (also leaches out the urea). Excess fixing solution was drained off, the gel covered with plastic wrap, and dried at 80°C for 30 - 60 min under vacuum. The plastic wrap was removed and the DNA fragments detected by autoradiography using Kodak XAR-5 X-ray film exposed overnight (16 - 24 hr) at -70°C.

II.7 Transcription Plasmid Subcloning

Mutant S1 gene fragments were subcloned from the M13 vector into the Riboprobe Gemini transcription system pGEM-4Z vector (Promega, Bio/Can Scientific Inc., Mississauga, Ont.). The phage-containing supernatant of confirmed S1 gene mutants was used to prepare purified recombinant M13 RF DNA according to standard procedures (section II.2). This mutant RF DNA, isolated from small scale preparations and resuspended in 50 µL of TE (pH 7.5), was digested with the restriction enzymes Sst I and Kpn I in 100 µL reaction mixtures according to standard procedures (Appendix). The reaction mixtures contained 45 µL of the DNA sample, 10 µL of 10 X BRL REact Buffer 4 (1 X Buffer 4: 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 50 mM KCl), and 20 units each of Sst I and Kpn I. The Sst I/Kpn I S1 gene inserts were purified from

agarose gels using low melting point (LMP) agarose, as outlined in the Appendix, and the insert DNA resuspended in 20 μL of H_2O. The plasmid pG4T3 (a plasmid containing a full-length DNA copy of the serotype 3 S1 gene cloned into the pGEM-4Z transcription vector), isolated according to standard procedures for large scale preparation of plasmid DNA (Appendix), was also digested with the restriction enzymes Sst I and Kpn I in 100 μL reaction mixtures containing 20 μg of pG4T3 DNA, 10 μL of 10 X BRL REact Buffer 4, and 10 units each of Sst I and Kpn I. This Sst I/Kpn I pG4T3 vector was also agarose gel purified and the vector DNA resuspended in 50 μL of TE (pH 7.5) buffer diluted 1:1 with dH_2O. DNA concentrations of the gel purified mutant S1 gene inserts and the pG4T3 vector were estimated by the degree of EtBr fluorescence relative to a series of known standards (Appendix). The mutant S1 gene inserts were ligated into the Sst I/Kpn I pG4T3 vector using T4 DNA ligase and an insert:vector molar ratio of 3:1, and the ligated plasmid used to transform competent *E. coli* DH5αF' cells according to standard procedures (Appendix). The site-specific mutation within these pG4T3-Δ (Δ refers to non-WT) clones were reconfirmed by double-stranded DNA sequencing using Sequenase (Kraft et al., 1988) as follows. Isolated transformed colonies were added to 5 mL of 2xYT medium and grown at 37°C, with shaking, overnight. The overnight cultures were then added at a dilution of 1 in 50 to 5 mL of 2xYT medium and grown for 6 hr at 37°C with shaking. A volume of 3 mL of this culture was used for sequencing of isolated dsDNA; according to the procedures of Kraft et al. (1988), plasmid DNA was isolated by standard small scale plasmid preparation techniques (Appendix), denatured under alkaline conditions (0.2 N NaOH), collected by standard ethanol precipitation procedures, and sequenced according to the Step-By Step Protocols for DNA Sequencing With Sequenase. The remaining 2 mL of these cultures were stored at 4°C and later used as an

inoculant to prepare 250 mL cultures used for large scale preparations of the pG4T3-Δ DNA.

II.8 In Vitro Transcription and Cell-Free Translation

Transcription

Capped mRNA was prepared *in vitro* using SP6 RNA polymerase (Bethesda Research Laboratories, BRL) according to a procedure modified from the Promega Riboprobe Gemini Technical Manual and the protocol of Dasso and Jackson (1989). Purified pG4T3-Δ DNA and wild type (WT) pG4T3 DNA were linearized with HindIII in 200 µL reaction mixtures according to standard procedures (Appendix). The reaction mixtures contained 20 µg of DNA, 20 µL of 10 X BRL REact Buffer 2 (1 X Buffer 2: 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl), and 3 units Hind III/µg of DNA. Complete linearization was checked for by agarose gel analysis. Linearization was followed by digestion with 1 mg of proteinase K/mL in 0.1% SDS, 5 mM Tris-HCl (pH 7.5), 10 mM EDTA in a final volume of 400 µL. Incubation was at 37°C for 30 min, with subsequent phenol and phenol:chloroform extractions and ethanol precipitation. Linearized plasmid DNA was resuspended in 200 µL of RNase-free TE (pH 7.5), the concentration of DNA checked by agarose gel analysis, followed by ethanol precipitation and resuspension of the DNA samples to a final concentration of 0.5 µg/µL in diethylpyrocarbonate (DEPC, Sigma)-treated dH_2O.

The linear pG4T3-Δ and WT DNA were used as templates for SP6 polymerase in 50 µL reactions containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 1 mM ATP, CTP, and UTP, 0.1 mM GTP, 0.5 mM $m^7G(5')ppp(5')G$, 4 mM dithiothreitol, 10 µg/mL bovine serum albumin (BSA), 1.6 units/µL placental RNase inhibitor (Promega), 5 µg linear plasmid

DNA, and 1.8 units/μL SP6 RNA polymerase. Reactions were incubated at 37°C for 45 min, with the subsequent addition of GTP to a final concentration of 0.75 mM, and incubation for a further 30 min. RNase-free DNase (BRL) was added to 20 μg/mL, followed by incubation at 37°C for 15 min. Full length capped RNAs were purified by phenol, phenol:chloroform and chloroform extractions, followed by three cycles of precipitation with ethanol. The resulting pellets were dried under vacuum, resuspended in DEPC-treated dH_2O at 0.1 mg/mL, and stored at -70°C. The concentration and quality of the RNA was checked by agarose gel electrophoresis (Appendix). Yields were typically between 2 and 10 μg RNA/ μg DNA.

Translation

Capped SP6 transcripts were translated *in vitro* in nuclease-treated rabbit reticulocyte lysates (Promega). Reactions were prepared according to manufacturer's specifications in the presence of 1 μCi/μL [^{35}S]methionine (New England Nuclear; >800 Ci/mmol), 1.6 units/μL placental RNase inhibitor (Promega), and 20 μM amino acid mixture minus methionine. Typically, 30 - 100 ng of RNA were used in a 25 μL translation reaction. Incubations were carried out for 20-30 min at 37°C and translation stopped by the addition of 3 volumes of phosphate-buffered saline (PBS), to a final volume of 100 μL, followed by centrifugation in a microfuge at 16K x g for 3 min at 4°C. The supernatant was transferred to a fresh tube and the diluted translation lysates were used directly in the assays described in section II.9. Translation lysates were stored at -70°C, avoiding repeated freeze/thaw cycles.

II.9 Assays for Analysis of σ1 Forms

Expression Analysis

The [^{35}S]-methionine labelled *in vitro*-expressed wild-type and mutant σ1 proteins were analyzed by SDS-10% PAGE and autoradiography (Appendix). To a 1/50 fraction of the diluted translation lysate was added 6 μL of 5 X Laemlli loading buffer (0.2 M Tris-HCl, pH 6.8, 5% SDS, 50% glycerol, 10% β-mercaptoethanol, 0.5% bromophenol blue) and dH_2O to 30 μL final. The samples were incubated at 37°C for 30 min just prior to gel loading.

Binding Assay

Binding studies were carried out using mouse fibroblasts L-929 (L-cells). L-cells were grown in Joklik-modified minimal essential medium (JMEM, containing penicillin G and streptomycin at 75 units/mL and 50 μg/mL, respectively; Gibco Laboratories) supplemented with either 5% fetal calf serum (FCS) or 5% of 4:1 newborn calf serum (NBCS):FCS (vol:vol). Stock cultures of L-cells were maintained as monolayers in tissue culture plastic flasks incubated at 37°C with >80% relative humidity and 5% CO_2. At confluence, cells were rinsed once with PBS, trypsinized using trypsin and ethylene diaminetetraacetic acid (EDTA) [Gibco; 0.05% trypsin, 1 mM EDTA in PBS], resuspended in 5% FCS in JMEM, and transferred to new flasks; according to established procedures (Jakoby and Pastan, 1979; Bird and Forrester, 1981). Alternately, suspension cultures of L-cells were grown in sterile flat bottom boiling flasks (containing magnetic stir bars) incubated in 37°C waterbaths. Cells in suspension were maintained at a concentration of 5 x 10^5 to 10^6 cells/mL.

L-cell binding assays were performed essentially as described previously (Lee et al., 1981b; Masri et al., 1986). Confluent L-cell monolayers were

prepared one day prior to use by plating 0.36 x 10^6 cells/well in a 24-well tissue culture plate (15 mm diameter). The media was removed, the monolayers washed with 1 mL of PBS, and the L-cell monolayers in each well preincubated with 200 µL of a PBS solution (5 mg/mL BSA, 30 µg/mL cycloheximide in PBS) at 4°C for 10 min. The PBS solution was removed by aspiration and 75 µL of diluted translation lysates containing 5 mg/mL BSA, 30 µg/mL cycloheximide, and 0.5 mM cold L-methionine were added to the monolayers. The plates were incubated at either 4°C or 39°C for 1 hr, with intermittent rocking; for binding studies done at 39°C all samples and solutions were preheated to 39°C prior to use. After incubation, 1 mL of PBS was added to each well and the solution aspirated off. This washing step was repeated 5 more times. After the final wash, 300 µL of PBS was added to each well, the monolayers were scraped off using a rubber policeman, and the cell suspension transferred to a prechilled 0.5-mL microfuge tube. The wells were rinsed with 150 µL of PBS and pooled with the appropriate sample. Cells were pelleted by centrifugation in a microfuge for 10 sec at ~5K x g, the PBS aspirated off, and the cells lysed by the addition of 25 µL of solubilization buffer [50 mM Tris-HCl, pH 7.9, 25% sucrose, 1% nonidet P-40 (NP 40), 0.5% sodium deoxycholate (DOC), 2 mM DTT, 5 mM EDTA, 0.1% Aprotinin (protease inhibitor), 1 mM PMSF]. The solution was mixed (by gently swirling and by pipetting up and down), incubated on ice for 2 min, followed by the addition of 35 µL of PBS and swirling to mix. Nuclei and cell debris were removed by centrifugation in a microfuge at 16K x g for 2 min at 4°C. The supernatant (50 µL) was transferred to a fresh tube containing 12 µL of 5 X Laemlli loading buffer and incubated at 37°C for 30 min just prior to analysis by SDS-10% PAGE and autoradiography. One half of the sample (25 µL) was loaded on a standard polyacrylamide gel.

Proteolysis Assay

Limited proteolysis analysis was carried out using the proteases trypsin and chymotrypsin, essentially as described previously (Yeung et al., 1989). Stock solutions of TPCK-treated trypsin (Sigma Chemical Co.), 0.1 mg/mL, or TLCK-treated chymotrypsin (Sigma Chemical Co.), 0.5 mg/mL, were made up in PBS and stored at -70°C. Proteolytic reactions contained 20 μL of diluted translation lysate, 4 μL of TPCK-treated trypsin (0.01 mg/mL final) or TLCK-treated chymotrypsin (0.05 mg/mL final), and PBS to a final volume of 40 μL. The reactions were incubated at 37°C for 30 min. Further proteolysis was prevented by the addition of 1/5 the volume of a stock solution of protease inhibitors (2.5 mg/mL each of soybean trypsin inhibitor, eggwhite trypsin inhibitor, and chymostatin; purchased individually from Sigma and made up in PBS), followed by a 5 min incubation at room temperature. A volume of 12 μL of 5 X Laemlli loading Buffer was added and the samples were boiled immediately for 5 min. Precipitated material was removed by centrifugation at 16K x g for 1 min at 4°C and the samples were then analyzed by SDS-10% PAGE and autoradiography. A 25 μL fraction of the sample (60 μL) was loaded on a standard polyacrylamide gel.

For the identification of N- and C-terminal tryptic fragments following the 5 min incubation with the protease inhibitors an equal volume of either a 1 in 100 dilution of α-N-terminal specific mAb antiserum or undiluted α-C-terminal specific mAb antiserum (G5) was added to separate 40 μL proteolytic reactions of WT and S325T σ1 proteins. The RIP was then carried out as described below using an equal volume of fixed *Staphylococcus aureus* cell suspension, except that the antigen-antibody complex was dissociated by resuspending the

final washed pellet in 60 μL of 1 X Laemlli loading buffer and boiling for 5 min. A 30 μL fraction was analyzed by SDS-10% PAGE and autoradiography.

Radioimmunoprecipitation Assay

Radioimmunoprecipitation analysis using the neutralizing G5 monoclonal anti-σ1 antibody (kindly provided by Dr. B. N. Fields; Burstin *et al.*, 1982) was performed essentially as described previously (Lee et al., 1981a; Masri et al., 1986), by mixing 20 μL of diluted translation lysate with 30 μL of G5 anti-σ1 antiserum and 10 μL PBS in a 1.7-mL microfuge tube. A negative control was included in all experiments by substituting NSI medium (supernatant from the NSI parent myeloma cell line used to generate the G5 hybridoma) for the G5 anti-σ1 antiserum in a mixture containing WT σ1 lysate. After incubation at room temperature for 1 hr (or overnight at 4°C), 30 μL of a fixed *Staphylococcus aureus* cell suspension (IgGsorb, The Enzyme Centre; prepared according to manufacturer's specifications, except the prepared cells were stored in a solution containing 0.15 M NaCl, 0.05% triton-X 100, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.02% sodium azide at -70°C), containing 5 mg/mL BSA, was added to the mixture and incubation was continued for 15 min, with occasional vortexing. A volume of 0.5 mL of a sarkosyl wash buffer (0.5% sarkosyl, 50 mM Na_2HPO_4, pH 7.4, 0.02% sodium azide) was added and the cell complexes were pelleted by centrifugation in a microfuge at ~5K x g for 10 sec. The pellets were washed 4 more times with 1.0 mL of sarkosyl wash buffer. The antigen-antibody complex was dissociated by the addition of 46.7 μL of a high pH buffer (0.1 % SDS, 2 mM DTT, 6 M urea, 0.1 M H_3PO_4, 50 mM Tris-base, to pH 11.6 with 10 M NaOH) to the final cell pellet, followed by incubation at 37°C for 45 min. The cells and antibody were removed by centrifugation at 16K x g for 10 sec and the supernatant transferred to a fresh tube. The pH of the sample was

neutralized by the addition of 2.3 μL of a phosphoric acid solution (100 mM H_3PO_4, 1 M Tris-HCl, pH 7.4) followed by a 5 min incubation at room temperature. A volume of 12 μL of 5 X Laemlli loading Buffer was added and the samples were analyzed by SDS-10% PAGE and autoradiography. A 40 μL fraction of the sample (60 μL) was loaded on a standard polyacrylamide gel.

CHAPTER III: Results

III.1 Construction of pSE0.9-19 Plasmid

The plasmid pSE0.9-19 (Fig. 1) was designed in order to provide the ssDNA template required in the oligonucleotide-directed mutagenesis procedure and was constructed to meet three criteria: have an Nci I REN site near the 3' end of the S1 insert site, for the rapid isolation of ssDNA template, and to contain the S1 gene sequences to be targeted. Use of the M13 vectors developed by Messing and coworkers (1983), specifically the vector mp19, provided for two of the criteria. An Nci I site located ~600 bp downstream of the polylinker cloning sites (Fig. 1); the necessity for an Nci I site in this location is related to the use of the phosphorothioate-based mutagenesis technique in which the REN Nci I is used to specifically nick the non-mutant strand (non-thionucleotide containing) of the mutant heteroduplex generated, that allows for the subsequent selective removal of the non-mutant strand. In the plasmid pSE0.9-19 an Nci I site was also present at the immediate 3' end of the cloned S1 gene sequence that was introduced from the pES1T3 plasmid. The use of M13 vectors also allows for the simple and rapid isolation of ss template DNA, as the ss form of the recombinant phage DNA is released from the intact host bacterial cell in a filamentous phage particle. Thus, when the non-lytic M13 phage are replicated in host cells grown in suspension broth cultures the progeny phage particles are released into the broth, while the host cells remain intact, allowing for the easy separation of the ssDNA containing phage from all bacterial and intracellular viral components by centrifugation to remove the intact cells. The supernatant recovered contains only progeny phage. A more

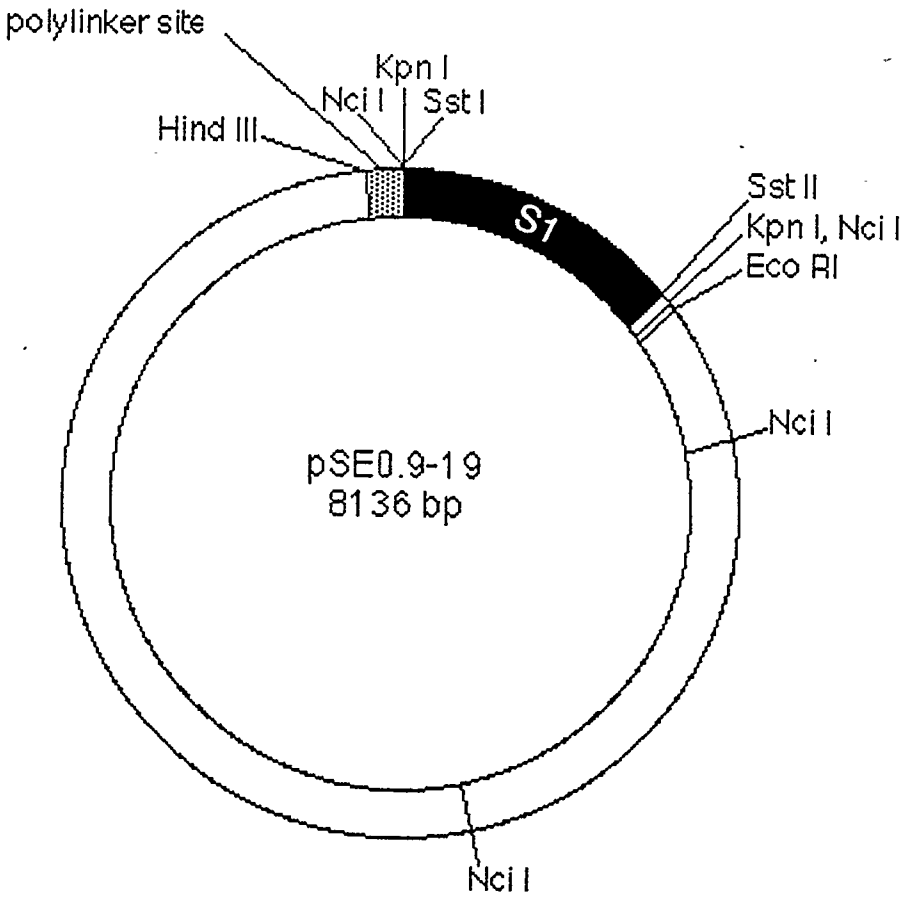


Figure 1: Restriction Map of Plasmid pSE0.9-19. Plasmid pSE0.9-19 was constructed by subcloning the Sst I/EcoRI fragment (from Nt 537 to 1425 of the T3 S1 gene) from pES1T3 (a plasmid containing the entire S1 coding sequence) into M13 mp19 also double digested with Sst I/EcoRI. The plasmid was used as the ssDNA template in the mutagenesis reactions.

detailed discussion of the M13 vectors and their use is given by Messing (1983).

Finally, the plasmid pSE0.9-19 contains the ~890 bp carboxy-terminal portion of the T3 S1 gene that includes all the sites that were targeted for mutagenesis in this project. Plasmid pSE0.9-19 was constructed by subcloning the Sst I/EcoRI fragment (from 537 bp to 1425 bp of the S1 gene) from pES1T3 (a plasmid containing the entire T3 S1 coding sequence plus an authentic T3 S1 3' non-coding sequence cloned into the puc 9 vector; the initiation codon and the first 42 nt of the S1 gene have been replaced by the corresponding lac Z gene sequences that are in frame with the S1 gene) into the M13 mp 19 vector also double digested with Sst I/EcoRI (Fig. 1), as described in Materials and Methods.

III.2 Design of Mutagenic Oligonucleotides

Synthetic mutant oligonucleotides were designed to meet a number of criteria. First, the sequence of the recombinant vector pSE0.9-19 was checked for sites, other than the site targeted, that could possibly compete with the mutant oligonucleotide for priming; this was done by a computer search of the S1 gene sequence and M13 vector sequence using the pattern matching algorithm from the programs of Mount and Conrad (1984a and 1984b), as analyzed on a Zenith AT compatible computer. Oligonucleotide lengths of 16-21 bases long were used for single and double base mismatches; these lengths have been reported to allow hybridization at ambient temperatures and above and result in fewer problems with the recognition of a unique sequence (Gillam and Smith, 1979a; Gillam and Smith, 1979b; Gillam et al., 1980). Longer oligonucleotide lengths are necessary where multiple mismatches are needed for the generation of a given mutant protein and/or when computer

analysis identifies possible competing sites from which priming might also occur. Where possible oligonucleotides were designed with a G/C content of at least ~40 - 50 %, and a G or C base residing at the ends of the oligonucleotide, for added thermal stability. Finally, the position of the mismatch or mismatches were kept towards the middle of the oligonucleotide to protect the mismatched base(s) from the exonuclease activity of polymerases added during DNA synthesis reactions and from possible contaminating enzymes with exonuclease activity. Three nucleotides following the mismatch have been shown to be enough to protect against this problem (Gillam and Smith, 1979a). Since the oligonucleotides were also used as probes to screen for the mutant(s), placement of the mismatch in the middle also allows for the greatest binding differential between a perfectly matched duplex and a mismatched duplex (Zoller and Smith, 1983).

Eight mutagenic oligonucleotides (lengths of 16-21 bases) were used to generate thirteen mutant σ1 proteins (Tables 1 and 2). The mutant oligonucleotides were synthesized as negative strand S1 gene sequences to allow complementation with the positive strand single-stranded template, pSE0.9-19, isolated from the M13 recombinant phage particles. Four of the oligonucleotides were synthesized containing a mixed base combination at the targeted nucleotide sites (Table 1; sites S325, S327, Y450, and P451).

III.3 Mutagenesis on the Cloned S1 Gene

Mutagenesis of the cloned S1 gene was accomplished using the technique described by Nakamaye & Eckstein (1986; Taylor et al., 1985a,b; Sayers et al., 1988), which utilizes phosphorothioate-modified DNA. Briefly, the synthetic oligonucleotide is annealed to the single-stranded wild-type S1 gene (cloned into M13) and used as a primer for polymerization in the presence of ligase

Table 1: Mutagenic Oligonucleotides

Oligonuleotide Sequence/Name [a]	Mixed Base Combination[b]	S1 Sequence (nt) [c]	Length (nt)
dCCAGAATAGX$_2$X$_1$GACAATTCC S325Δ	X$_2$ = G,C,T X$_1$ = T,C	995-976	20
dCCAGAATTCGAGACAA Y326NΔ		995-980	16
dCCACTACCAX$_2$X$_1$ATAGGAGAC S327Δ	X$_2$ = G,C,T X$_1$ = T,C	1001-982	20
dACGATAGATΔTCCACCGTC D365YΔ		1114-1096	19
dGGTAACAAAGTΔCAACGATAG N369YΔ		1128-1108	21
dGGTAACAACGTTCAACGATAG F370VΔ		1128-1108	21
dCTACGCGGGX$_2$X$_1$CGAAACGG Y450Δ	X$_2$ = A,C X$_1$ = A,C	1352-1370	19
dCTACGCX$_2$X$_1$GTACGAAACGG P451Δ	X$_2$ = C,G,T X$_1$ = C,A,T	1352-1370	19

[a] Oligonucleotides listed are given in the 5' to 3' direction as synthesized. These sequences correspond to the negative strand S1 gene sequence.

[b] A nucleotide shown as X indicates a position of multiple base substitutions.

[c] The S1 gene sequence position corresponding to the mutagenic oligonucleotide sequence is given.

Table 2: List of Nucleotide Changes and Respective Amino Acid Substitutions

Conserved Region [a]	Wild Type Sequence & Nucleotide change(s)[b]	Amino Acid Change	Protein Name[c]
	S Y S 325 326 327 5'GGA ATT GTC TCC TAT TCT GGT AGT GG3'		
C	A	S → T	S325T
	AA	S → N	S325N
	G	S → A	S325A
C	G A	Y → N	Y326N
C	A	S → T	S327T
	AA	S → N	S327N
	D N F 365 369 370 5'GAC GGT GGA GAT CTA TCG TTG AAC TTT GTT ACC3'		
D	T	D → Y	D365Y
D	T	N → Y	N369Y
D	G	F → V	F370V
	Y P 450 451 5'CC GTT TCG TAC CCG CGT AG3'		
E	GG	Y → G	Y450G
E	T	P → S	P451S
	A	P → T	P451T
	AA	P → K	P451K

[a] See Figure 12 for conserved regions.

[b] The wild type S1 gene sequence, shown as the positive strand, proceeds the nucleotide changes of a given mutant protein: Region C, Nt 976-1001; Region D, Nt 1096-1128; Region E, 1352-1370.
The nucleotides that were changed are underlined or in boldface type in the wild type S1 sequence; directly below is the nucleotide change(s) of each respective mutant protein.

[c] The protein name represents the format in which a particular protein will be written and addressed throughout the body of the text.

which generates a mutant heteroduplex; unpolymerized ss template DNA is then removed by nitrocellulose filtration. The non-mutant strand is selectively removed due to the incorporation of a thionucleotide (dCTPαS: contains a sulphur atom instead of an oxygen atom at the α-phosphate position of dCTP) into the mutant strand during this synthesis. That is, the heteroduplex is specifically nicked with Nci I to introduce single-stranded nicks in the non-phosphorothioate strand only, followed by exonuclease III digestion which selectively removes part of the non-mutant strand that includes the cloned S1 sequence. The mutant strand is then used as a template for construction of a covalently closed circular homoduplex mutant molecule, followed by transformation.

The specific use of Nci I for nicking in this procedure was not necessary as the inability to cleave thionucleotide containing DNA (phosphorothioate DNA) is observed with other RENs as well (Taylor et al., 1985b; Nakamaye and Eckstein, 1986). However, Eckstein and coworkers have demonstrated that there are variations in mutagenesis efficiency depending on the REN utilized and that of all the RENs used Nci I both nicks completely and does not affect the exonuclease III activity (Nakamaye and Eckstein, 1986). Therefore, Nci I has the properties that produce consistent and high efficiency mutations that approach the theoretical limit and that were desirable for this project. The only stipulation for the use of Nci I is that an Nci I restriction site is not present within the mutagenic oligonucleotide as dCTPαS would not be incorporated at this site during extension.

Analysis of samples removed for agarose gel analysis during the mutagenesis procedure are shown in Fig. 2, as represented by the mutagenesis reaction using the oligonucleotide F370VΔ. Sample 1, S1, was removed after annealing of the oligonucleotide to the ss template and extension and ligation;

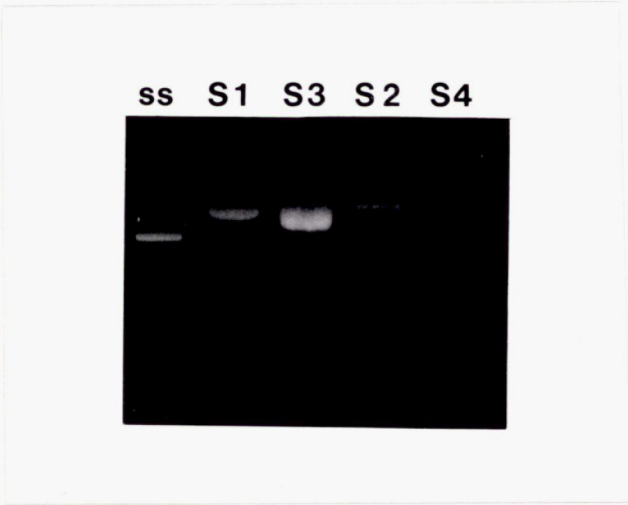


Figure 2: Analysis of the *In Vitro* Mutagenesis Reaction. Samples (S1 - S4) removed at 4 steps in the mutagenesis reaction were analyzed on a 0.8% agarose (TBE) slab gel containing 0.5 μg/mL EtBr. The samples in each lane are as follows: ss, single-stranded pSE0.9-19 plasmid; S1, sample 1, after primer annealing, extension and ligation; S2, sample 2, after nitrocellulose filtration and nicking with Nci I; S3, sample 3, after exonuclease III digestion; S4, sample 4, after repolymerization.

results typically showed greater than 60% of the ssDNA was converted into duplex DNA. Sample 2, S2, was taken after nitrocellulose filtration to remove ssDNA and nicking with Nci I; results show the complete removal of the ss template and nicking to produce a slightly higher migrating nicked DNA band on the gel. Sample 3, S3, was removed after incubation of the nicked DNA with exonuclease III under conditions that would remove ~1500 bases from the non-mutant strand during the incubation time (see Materials and Methods); ~6000 bases total would be removed from the non-mutant strand of the duplex as there are 4 Nci I sites in pSE0.9-19 at which nicking would occur. Fig. 2, S3, shows that Exo III digestion produces a mixed size population of DNA molecules (a smeared band) that migrates between the ligated duplex DNA (S1 lane) and the ss template (ss lane). Sample 4, S4, was removed after repolymerization to obtain the homoduplex mutant DNA; results from Fig. 2 show the recovery of duplex DNA that was used to transform competent *E. coli* cells.

III.4 Screening For Mutant S1 Genes

The screening of putative mutants from mutagenesis reactions using the oligonucleotides Y326NΔ and D365YΔ was done by restriction endonuclease (REN) analysis. Upon transformation of the homoduplex mutant phage DNA synthesized in the mutagenesis reaction, the resulting phage plaques were used to inoculate growth medium. Y326NΔ generated M13 RF DNA was screened for the presence of a new EcoRI site. RF pSE0.9-19 containing a mutated S1 gene sequence (Table 2, Y326: nucleotides 987 and 988 changed from CT to GA, respectively), and consequently an EcoRI site (**GA**ATTC), would be EcoRI digested to two fragments of ~440 bp and 7700 bp in length. WT RF pSE0.9-19 would be linearized to produce a single ~8100 bp fragment. Fig. 3 shows the analysis of 5 EcoRI restricted DNA preparations by agarose gel

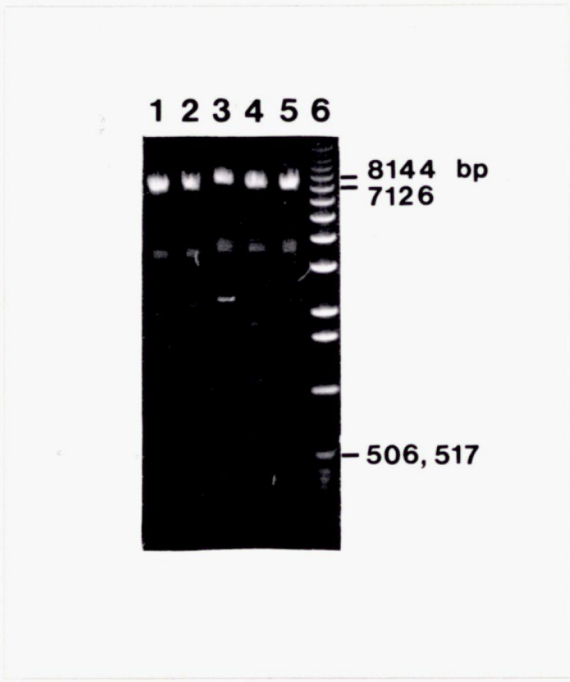


Figure 3: Screening for the Y326N S1 gene by EcoRI Restriction Analysis. Purified RF pSE0.9-19Δ DNA of 5 putative mutant samples were digested with EcoRI and the products analysed on a 0.7% agarose (TBE) slab gel containing 0.5 μg/mL EtBr. Lanes 1 - 5 show the restriction fragments of samples 1 - 5, respectively. Samples 1, 2, 4 and 5 show the restriction fragment pattern expected for a Y326N mutant S1 gene. Lane 6 is the BRL 1kb ladder used for size estimates of the ds DNA fragments.

electrophoresis; preparations 1 - 5 are shown in lanes 1 - 5, respectively. Lanes 1, 2, 4, and 5 show the faint ~440 bp fragment expected for mutant DNA. Furthermore, the distance of migration of the uppermost band in these lanes is that expected for an ~7700 bp fragment and not an 8100 bp fragment as observed in lane 3. It was concluded, therefore, that plasmid preparations 1, 2, 4, and 5 contained the desired S1 gene mutation; this was later confirmed by sequence analysis.

D365YΔ generated DNA was screened for the loss of a Sau3AI site by first isolating the S1 gene fragment from RF pSE0.9-19 DNA and then subjecting this S1 fragment to digestion by Sau3AI. A mutant S1 fragment (Table 2, D365: nucleotide 1105 changed from G to T), lacking one of four Sau3AI sites (GATC), would be cleaved by Sau3AI to yield 4 fragments of lengths 141 bp, 202 bp, 501 bp, and 44 bp. A WT S1 fragment would be cleaved to yield 5 fragments of lengths 141 bp, 202 bp, 224 bp, 277 bp, and 44 bp. Fig. 4 shows the analysis of Sau3AI restricted S1 fragments of 5 DNA preparations by TBE-5% PAGE; preparations 1 -5 are shown in lanes 2 -6, respectively. Lane 4 shows the expected fragment pattern for a mutant S1 gene (although the small 44 bp fragment is not visible) with the loss of the 224 bp and 277 bp fragments (seen in lanes 2, 3, 5, and 6) that form the single 501 bp fragment. Sequence analysis of plasmid preparation 4 later confirmed the exact nucleotide change.

The screening of putative mutants from mutagenesis reactions using the oligonucleotides N369YΔ, F370VΔ, S325Δ, S327Δ, Y450Δ, and P451Δ was done by dot blot hybridization analysis. The procedure involved hybridization of single-stranded DNA, on nylon blots of phage-containing supernatant, with the appropriate ^{32}P-labelled mutagenic oligonucleotide, as described in Materials and Methods. In order to discriminate between mutant and wild type phage dots the nylon filters were washed at increasing temperatures. The first wash

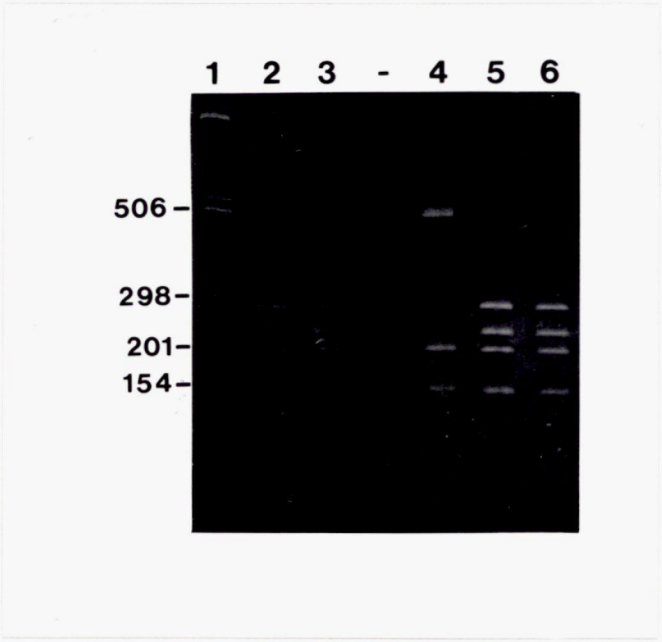


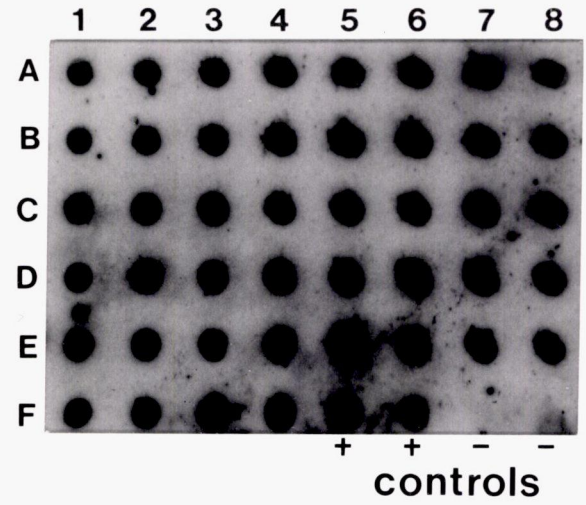
Figure 4: Screening for the D365Y S1 Gene by Sau3AI Restriction Analysis. Isolated S1 gene fragments from the purified pSE0.9-19Δ DNA of 5 putative mutant samples were digested with Sau3AI and the products analyzed by TBE-5% PAGE; the gel was impregnated with EtBr for visualization. Lane 1 is the BRL 1kb ladder used for size estimates of the ds DNA fragments. Lanes 2 - 6 show the restriction fragments of samples 1 - 5, respectively. Sample 4 shows the restriction fragment pattern expected for a D365Y mutant S1 gene.

was done at a temperature 5°C below the calculated temperature of dissociation (T_d); T_d was calculated using the Wallace rule (Wallace et al., 1981), T_d = (4 x number of G, C base pairs) + (2 x number of A, T base pairs).

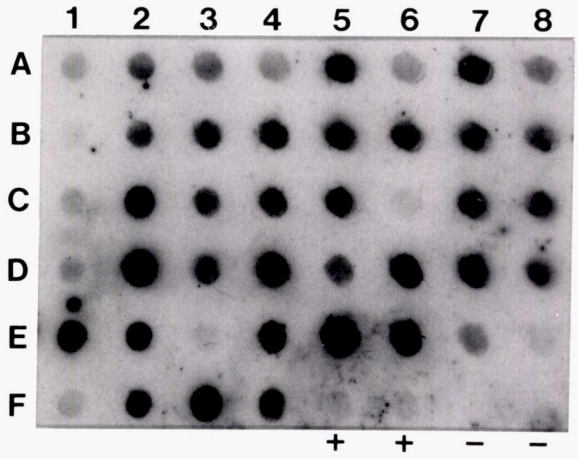
For those mutagenic oligonucleotides containing mixed base sites (Table 1: S325Δ, S327Δ, Y450Δ, and P451Δ) the first wash was done at a temperature of 5°C below the lowest T_d calculated for all possible oligonucleotide combinations of a given oligonucleotide mix. Subsequent wash temperatures were then done at 2°C to 4°C increments until WT and mutant S1 sequences could be distinguished. An example of the hybridization screening method is shown in Fig. 5, which depicts analysis of putative P451 mutant S1 genes. A total of 44 separate phage-containing supernatant preps from the P451Δ mutagenesis reaction were blotted onto the nylon filter. Also blotted were duplicate controls of recombinant pSE0.9-19 (WT S1 gene segment) and M13 mp19 (no S1 segment) phage stocks, indicated in Fig. 5 with plus (+) and minus (-) signs, respectively. The [^{32}P]-labelled oligonucleotide mix P451Δ was used as the probe for mutant S1 gene segments. Diagrams A - E of Fig. 5 are the autoradiographs of the nylon blot after washes at increased temperatures. At a wash temperature of 25°C (room temperature) all ssDNA containing an S1 gene segment hybridize to the labelled P451Δ probe (Fig. 5, A); the negative control, M13 mp19 (spots F7 and F8), containing no S1 gene segment, does not hybridize to the probe, as expected. At a wash temperature of 56°C (Fig. 5, B), 2°C below the lowest T_d calculated for the P451Δ oligonucleotide mix, ~90% of the probe has dissociated from WT containing S1 sequences (for example, control spots F5 and F6). By increasing the wash temperatures to 58°C, 62°C, and 66°C (Fig. 5 C, D, and E, respectively) S1 gene sequences containing different GC contents at the target site could be distinguished. For example, a change from the WT proline 451 codon (CCG),

Figure 5: Screening by Dot-Blot Hybridization. Putative P451 mutant S1 genes (in pSE0.9-19) were screened by hybridization of ss DNA, on nylon blots of phage-containing supernatant, with the [^{32}P]-labelled mutagenic oligonucleotide mix P451Δ. Figures A - E show the autoradiographs of the nylon blot after washes at increased temperatures: **A**, room temperature (25°C); **B**, 56°C; **C**, 58°C; **D**, 62°C; **E**, 66°C; **F**, 70°C. Control phage stocks were also spotted onto the nylon blot and are indicated as (-) for M13 mp19 (no S1 gene segment) or (+) for pSE0.9-19 (containing a WT S1 gene segment).

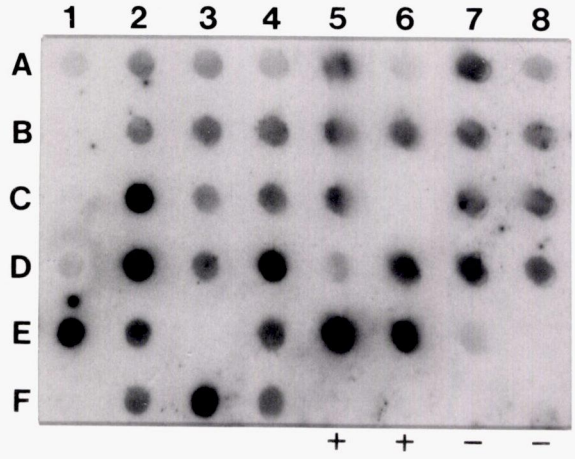
A.


1 2 3 4 5 6 7 8
A
B
C
D
E
F
+ + – –
controls

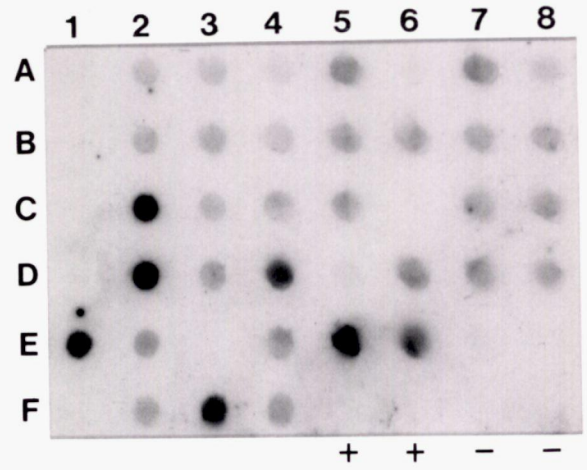
B.


1 2 3 4 5 6 7 8
A
B
C
D
E
F
+ + – –

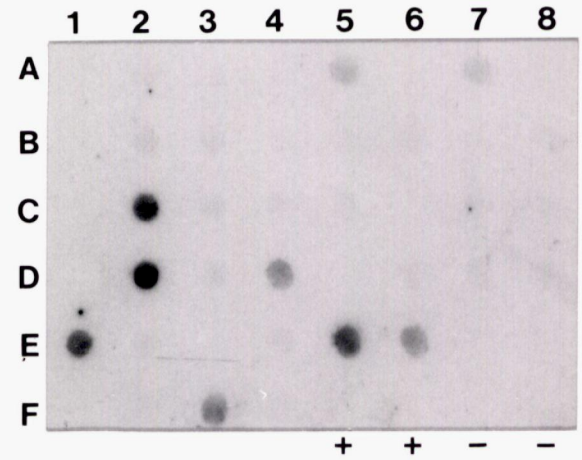
C.


1 2 3 4 5 6 7 8
A
B
C
D
E
F
+ + – –

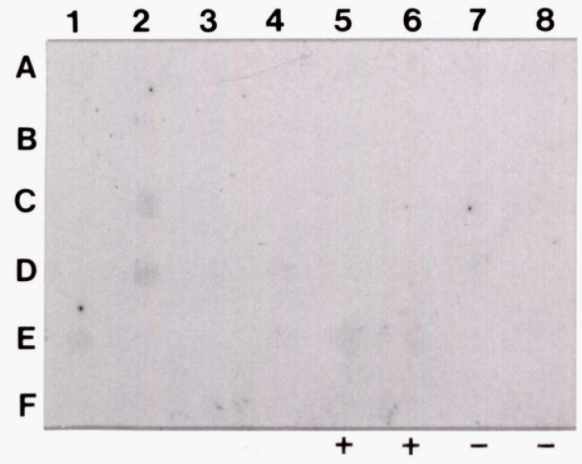
D.



E.



F.



100% GC content, to AAG, 33% GC content, TCG or ACG, both with 67% GC content, or GCG, 100% GC content, are represented in Fig. 5, B, as light, medium and dark spots, respectively:

Spot Shade	Spot Position	Sequence	GC content
light	D1, E7	AAG	33%
medium	C7, F2	ACG, TCG	67%
dark	D2, E5	GCG	100%

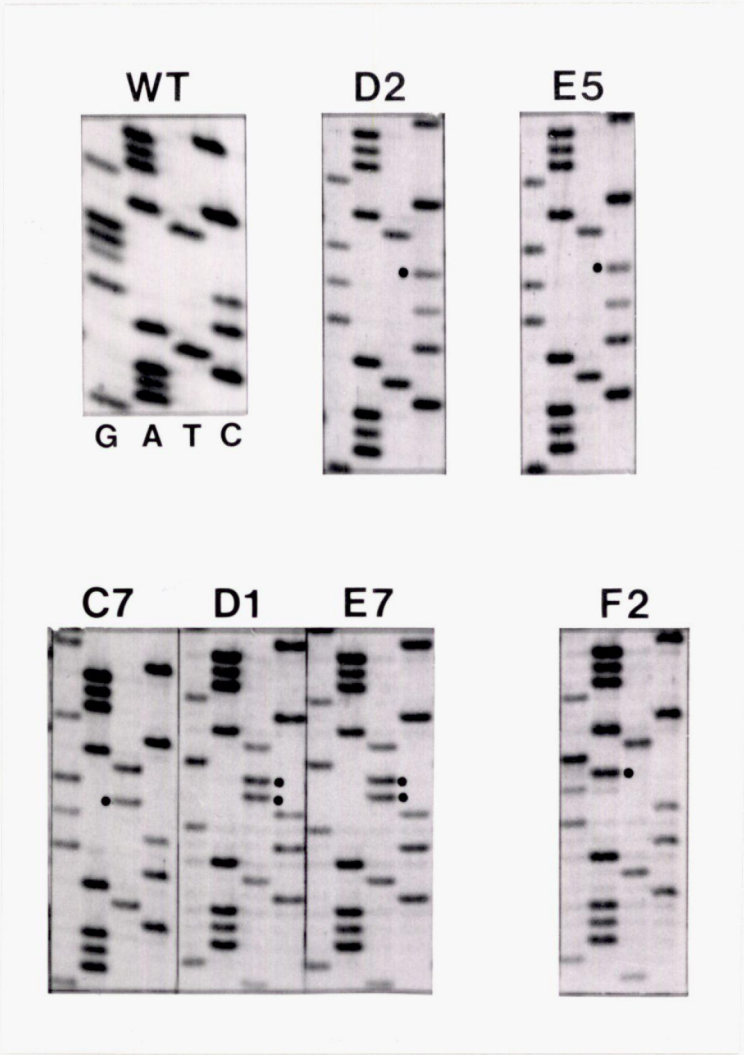
This patterning between GC content and spot shade was also consistently observed when screening for S325, S327, and Y450 mutants using the appropriate [^{32}P]-labelled oligonucleotide mixes as was later confirmed by sequence analysis of the mutant phage DNA (Fig. 6); note, the sequences shown in Fig. 6 represent the negative strand S1 gene sequence, whereas the codons written above for each spot position are in the positive strand sense. Fig 5, F, also shows that at a wash temperature of 70°C essentially all the probe has been dissociated.

All mutant S1 genes were further analyzed by sequencing to determine the exact nucleotide changes. Phage DNA, isolated from the phage stocks corresponding to the mutant spots identified through hybridization analysis, was sequenced using the modified T7 DNA polymerase, Sequenase, as outlined in Materials and Methods. For example, Fig. 6 shows the sequences of the P451 mutants listed above that correspond to the amino acid changes P451K (D1 and E7), P451T (C7), P451S (F2), and P451A (D2 and E5).

III.5 Expression of σ1 Proteins In Vitro

After screening and isolation of RF pSE0.9-19Δ (containing the mutant S1 gene forms) all 13 mutations (Table 2) were subcloned into pG4T3. The mutant S1 gene fragments were excised by Sst I/Kpn I digestion of the RF DNA form of

Figure 6: Sequence analysis of Mutant S1 Genes. Phage ss DNA was sequenced using the dideoxy chain-termination method and Sequenase. The sequencing reactions were analyzed on 8% polyacrylamide gels containing 8 M urea using an electrolyte gradient system, followed by autoradiography. An example of the results obtained is shown here. The sequence is the 5' - 3' direction of the minus strand of the S1 gene (from the bottom of the gel to the top). Reading from left to right the lanes are G - A - T - C, as indicated in lane WT. Only the region of the gel which shows the nucleotide change(s) is presented. The altered bases are indicated by a closed circle. The wild-type S1 gene sequence is shown in the lane marked WT. The titles of the sequences in the other lanes correspond to the spot positions shown in Figure 5 and the mutants identified are as follows: D1 and E7, P451K; C7, P451T; F2, P451S; D2 and E5, P451A.


WT
D2
E5
G A T C
C7
D1
E7
F2

M13 recombinant pSE0.9-19Δ (Fig. 1) and substituted for the corresponding wild-type sequences in pG4T3 for transcription; the plasmid pG4T3 harbours a full-length DNA copy of the serotype 3 S1 gene cloned into the pGEM^{TM}-4Z transcription vector. The site-specific mutation within the pG4T3-Δ clones were reconfirmed by double-stranded DNA sequencing (Materials and Methods).

Purified pG4T3 and pG4T3Δ DNA, linearized with Hind III, were transcribed *in vitro* with SP6 RNA polymerase to produce capped mRNA, as described in Materials and Methods. The plasmid pG4T3, harbouring a WT T3 S1 gene, expresses a biologically functional σ1 product, designated WT (wild-type σ1) in Fig. 7 and 8.

The synthetic transcripts generated *in vitro* were subjected to cell-free translation in the message-dependent rabbit reticulocyte lysate system (Materials and Methods). Aliquots of each translation lysate were analyzed by SDS-10% PAGE and autoradiography. According to findings by Fields and coworkers (Banerjea et al., 1988) the samples were not boiled prior to gel analysis as this group has found this allows multimer forms of σ1 to be detected. The resulting translation products for the WT and pG4T3-Δ directed translations are shown in Fig. 7. The two major translation products are observed to be the monomeric and oligomeric full-length σ1 forms; with the monomeric forms co-migrating with type 3 reovirion σ1, lane R3, and the multimeric forms migrating well above the type 3 reovirion λ proteins. Other translation products are observed, migrating below the monomeric σ1 form, but do not represent full-length functional σ1 products (Fig. 8) and are presumably early translation arrest products. It is also apparent from Fig. 7 that all σ1 proteins are expressed in the *in vitro* translation system at approximately equivalent levels.

III.6 Characterization of σ1 Proteins

In vitro expressed σ1 forms were further analysed by assaying for host cell (mouse L fibroblast) binding activity, stability against limited proteolysis, and the ability to be recognized by a neutralizing monoclonal antibody. All assays were performed using two WT sample controls, prepared separately from two different pG4T3 (WT) DNA preparations, in order to relate the functional and structural integrity of the mutant σ1 forms to that of WT σ1. The data presented in Figures 7 to 11 represent results that were consistently obtained from repeated analysis of the mutant σ1 forms in these assays; with experiments being repeated using different pG4T3Δ DNA, mRNA, and translation preparations.

To determine whether any of the 13 mutant σ1 proteins possessed cell binding capacity, [^{35}S]-labelled translation lysates were applied to monolayers of L cells. After 1 hr incubation at 4°C, the cells were washed and proteins that remained bound were analyzed by SDS-PAGE followed by fluorography. The results are shown in Fig. 8, A. It was consistently observed that all mutant forms of S325 and S327 (region C), and the mutant protein D365Y (region D) bound to L cells at levels equivalent to that observed for WT σ1. However, proteins Y326N (region C), F370V (region D), Y450G (region E), and all mutant forms of P451 (region E) were completely incapable of binding to L cells. The protein N369Y (region D) also exhibits L cell binding activity, although the amounts bound relative to input is considerably less than that for WT. These results show that residues throughout the carboxy-terminus of σ1 have potential significance for the cell binding function of σ1. Of further interest is the

Figure 7: SDS-PAGE analysis of the *in vitro*-Expressed wild-type and mutant σ1 proteins. Synthetic transcripts were generated from the wild-type plasmid pG4T3 and from pG4T3Δ plasmids carrying point mutations affecting single amino acid substitutions in the serotype 3 σ1 protein. Transcripts were translated in the rabbit reticulocyte lysate system in the presence of [^{35}S]methionine, and the radiolabeled products were detected by SDS-10% PAGE (samples were pre-incubated in Laemlli loading buffer at 37°C for 30 min before gel analysis) and autoradiography. The titles of each lane are read vertically from top to bottom; the titles correspond to the 'Protein Name' indicated in Table 2. Lane R3: [^{35}S]-methionine labelled purified virion marker. The σ1 multimer is ~200 KDa and the σ1 monomer is 49 KDa.

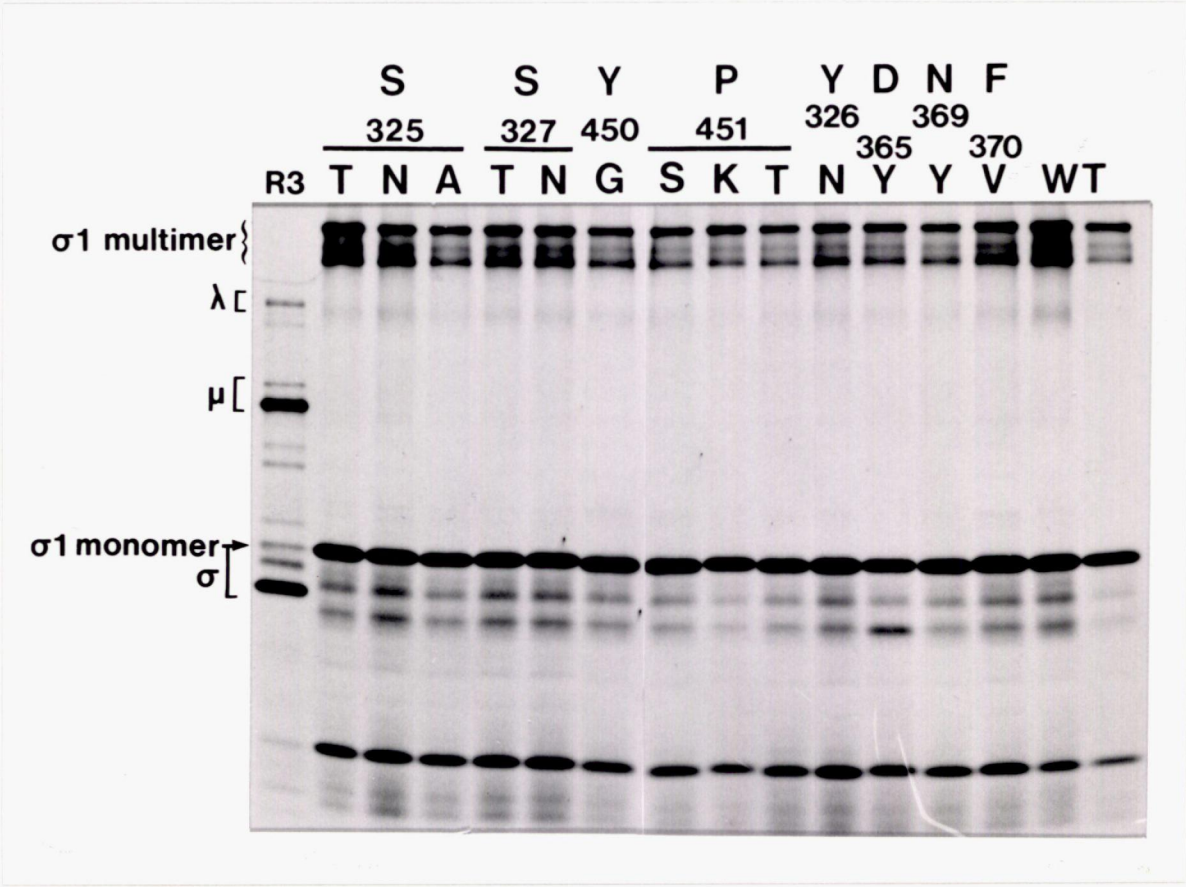
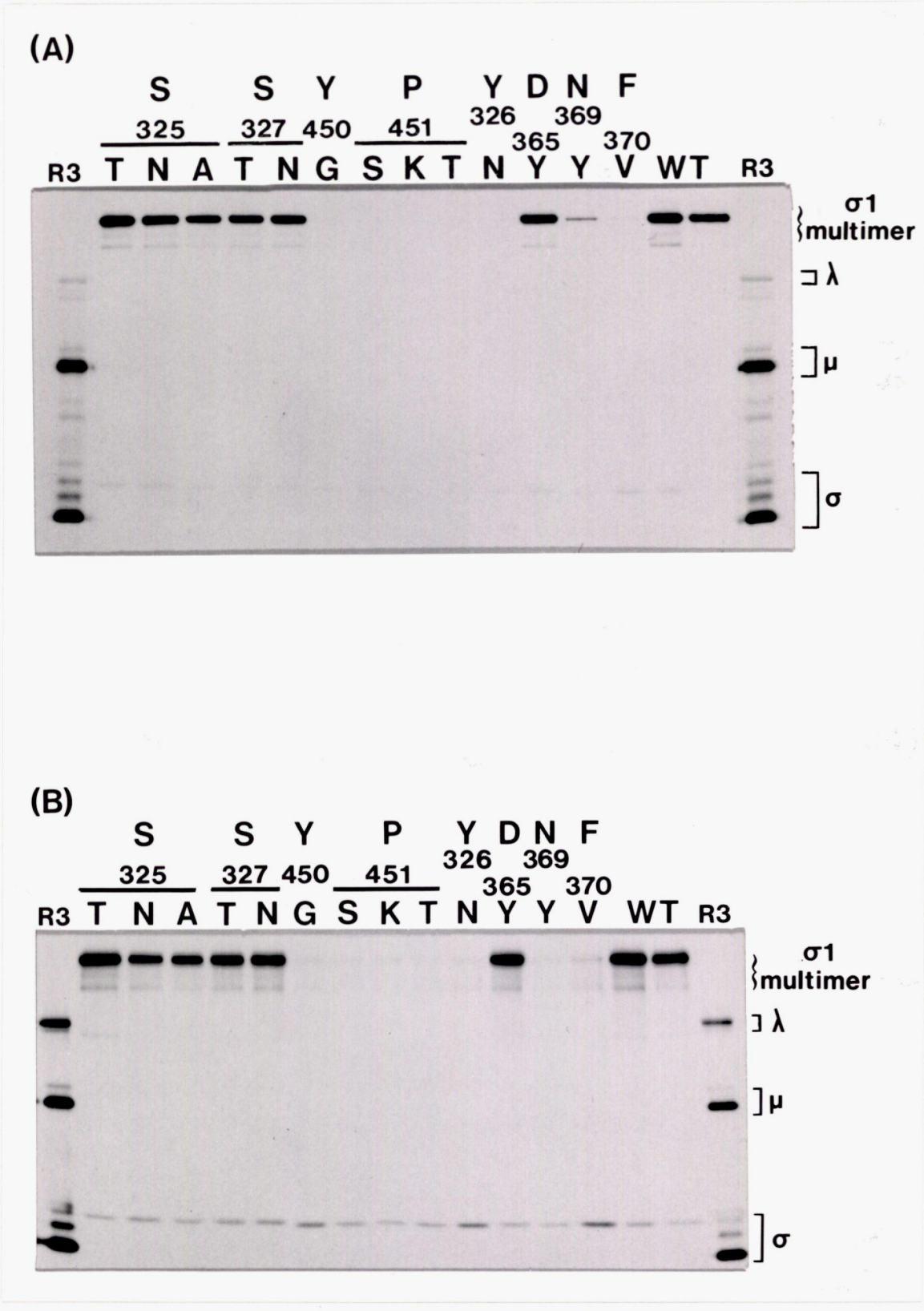

S 325 T
S 327 T
Y 450 G
P 451 S
Y 326 N
D 365 Y
N 369 Y
F 370 V
R3
N A N K T
WT
σ1 multimer
λ
μ
σ1 monomer
σ

Figure 8: Binding of the *in vitro*-Expressed wild-type and mutant σ1 proteins to mouse L fibroblasts. Wild-type and mutant proteins labelled with [^{35}S]methionine in *in vitro* translation reactions were applied as 100 μL aliquots to monolayers of mouse L fibroblasts and incubated at 4°C (A) or 39°C (B) with intermittent rocking for 1 hr. Monolayers were washed extensively with PBS and the cells were lysed with a 1 % Triton X-100 lysis buffer. The nuclei were removed by centrifugation and the lysates analyzed by SDS-10% PAGE (samples were pre-incubated in Laemlli loading buffer at 37°C for 30 min before gel analysis) and autoradiography. Result show that in all three homologous regions, C, D and E, amino acids were targeted that show the complete abrogation of L-cell binding function. The mutant σ1 protein N369Y shows a partial binding ability.


(A)
S S Y P Y D N F
325 327 450 451 326 365 369 370
R3 T N A T N G S K T N Y Y V WT R3
σ1 multimer
λ
μ
σ
(B)
S S Y P Y D N F
325 327 450 451 326 365 369 370
R3 T N A T N G S K T N Y Y V WT R3
σ1 multimer
λ
μ
σ

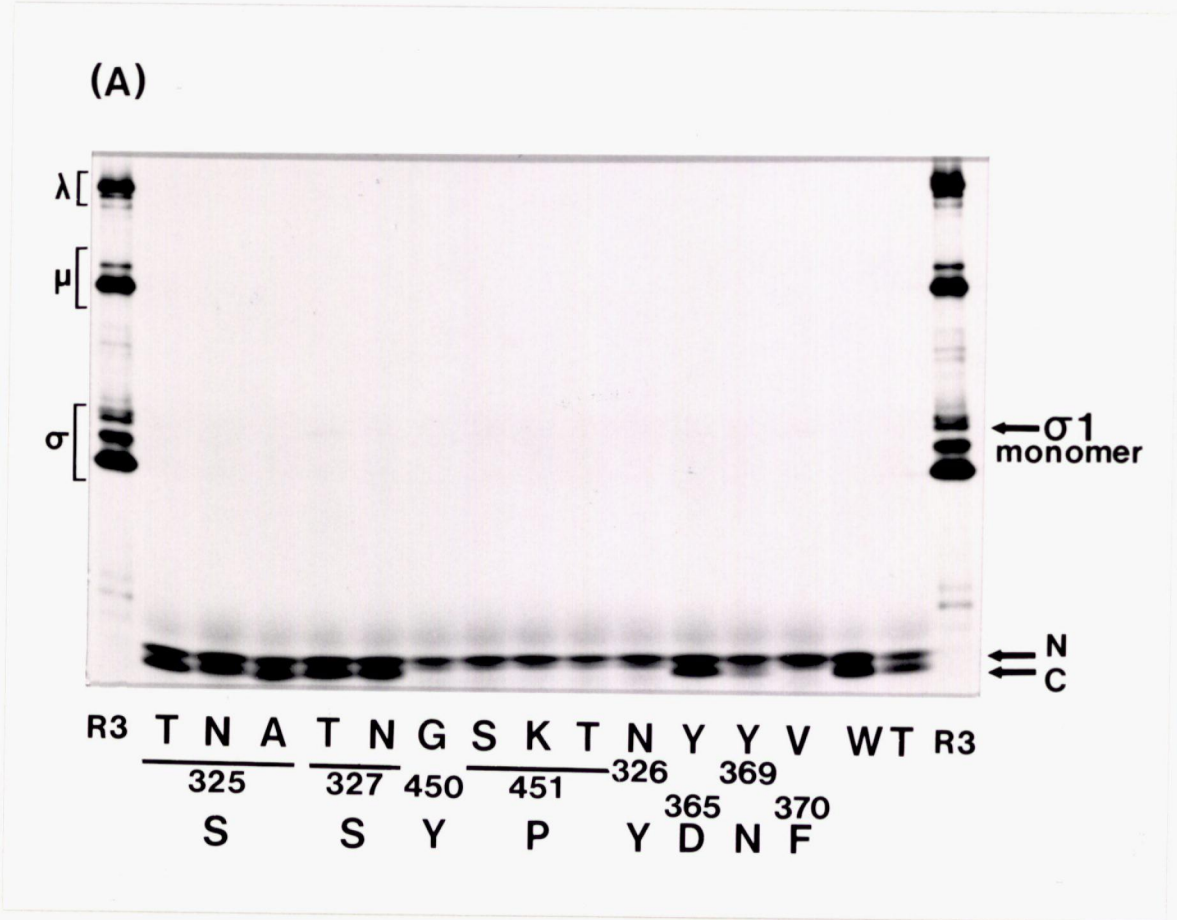
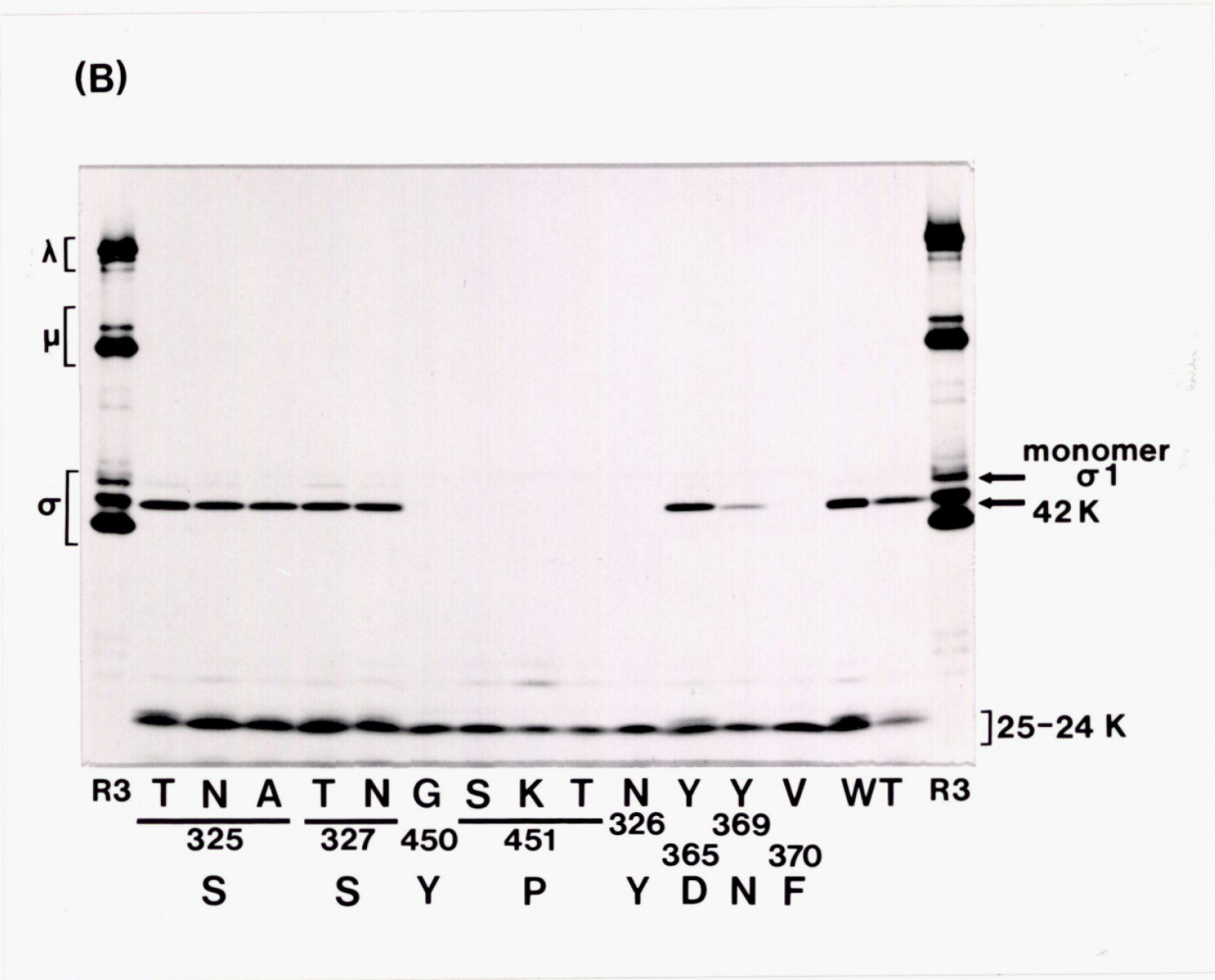


Figure 9: Effect of trypsin (A) or chymotrypsin (B) on the *in vitro*-Expressed wild-type and mutant σ1 proteins. Wild-type and mutant proteins labelled with [^{35}S]-methionine in *in vitro* translation reactions were incubated with TPCK-treated trypsin or TLCK-treated chymotrypsin at 37°C for 30 min. Samples were boiled in Laemlli loading buffer before analysis by SDS-10% PAGE and autoradiography. The tryptic fragments as observed (A) are: N, N-terminal fragments of 26 and 25 KDa; C, C-terminal fragment of ~23 KDa. The chymotryptic fragments as observed (B) are: 42 and 25 KDa, C-terminal fragments; 24 KDa, N-terminal fragment.


(B)
λ
μ
σ
monomer σ1
42 K
]25-24 K
R3 T N A T N G S K T N Y Y V WT R3
325
327
450
451
326
369
365
370
S S Y P Y D N F

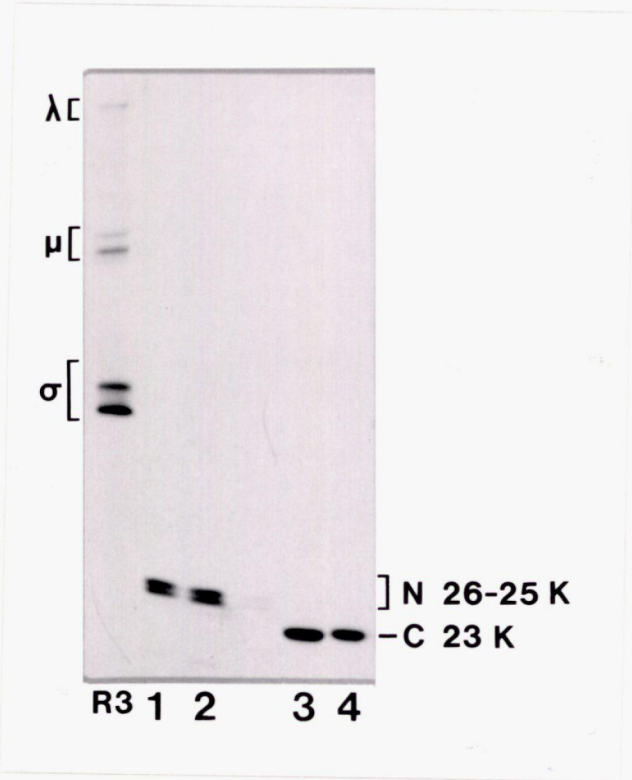


Figure 10: RIP of Trypsin Fragments using α-N and α-C-terminal Specific mAbs. Wild-type and S325T σ1 proteins labelled with [^{35}S]-methionine in *in vitro* translation reactions were incubated with TPCK-treated trypsin (0.01 mg/mL final) at 37°C for 30 min. Trypsin digestion was followed by immunoprecipitation with either an α-N-terminal specific mAb or an α-C-terminal specific mAb. Samples were boiled in Laemlli loading buffer prior to SDS-10% PAGE analysis and autoradiography. Lanes 1 and 2: α-N mAb RIPs. Lanes 3 and 4: α-C mAb RIPs. Tryptic fragments for WT σ1 are shown in lanes 1 and 3 and S325T shown in lanes 2 and 4. Lane R3: [^{35}S]-methionine labelled purified virion marker.

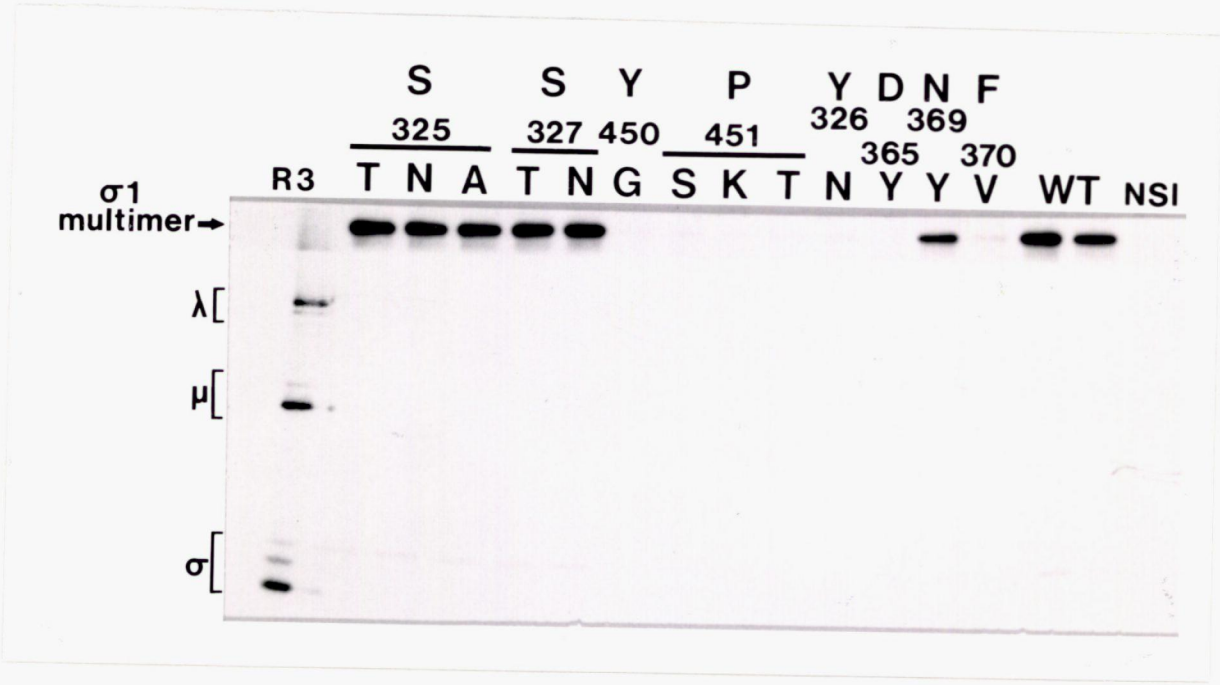


Figure 11: Immunoprecipitation of the *in vitro*-Expressed wild-type and mutant σ1 proteins with the neutralizing G5 monoclonal anti-σ1 antibody. Wild-type and mutant proteins labelled with [^{35}S]methionine in *in vitro* translation reactions were immunoprecipitated with the monoclonal antibody G5. The antigen-antibody complex was dissociated with a high pH buffer (0.1 % SDS, 2 mM DTT, 6 M urea, 0.1 M H_3PO_4, 50 mM Tris-base, pH 11.6) and incubation at 37°C. Staph A and antibody were removed by centrifugation and the pH of the sample adjusted to pH 7.4 before analysis by SDS-10% PAGE and autoradiography. Lane NSI: RIP of WT σ1 in the absence of mAb G5 (negative control).

observation that only the multimeric forms of WT σ1 and mutant σ1 proteins are capable of L cell binding.

To determine if any of the 13 mutant σ1 proteins were temperature sensitive mutants the L-cell binding study was repeated using an incubation temperature of 39°C instead of 4 °C. Analysis by SDS-10% PAGE and autoradiography revealed results that mimic binding of the mutant σ1 proteins at 4°C (Fig. 8, B). However, it can be observed that the protein N369Y which shows partial binding ability at 4°C is completely incapable of binding at 39°C. Also observed in every sample lane in Fig. 8, B, is a faint band that comigrates with monomer reovirion σ1. This band is attributable to non-specific binding as it occurs in each sample lane, whether or not the functional multimeric form of the protein is capable of binding. Alternatively, the presence of this lower migrating band in the 39°C binding study represents binding to internal cellular components, as the incubation of L-cell monolayers at 39°C repeatedly caused partial cell lysis that was not observed when incubations were done at 4°C.

As a means of determining structural integrity, the various mutant proteins were analyzed for stability against limited proteolysis, as described in Materials and Methods. We reasoned that any change in conformation as a consequence of the amino acid change could potentially affect the structure of σ1 in such a way as to expose proteolytic sites otherwise inaccessible on WT σ1. In this way one might observe a proteolytic fragment pattern that significantly differs from that observed for the WT protein. This is indeed the case, as can be seen in Fig. 9, A and B. Limited tryptic digestion, carried out as described under Materials and Methods, results in WT σ1 being cleaved into three fragments; two amino-terminal fragments with molecular weights of approximately 26K and 25K, and a single carboxy-terminal fragment of ~23K molecular weight (Fig. 9, A). Under reaction conditions in which the trypsin

concentration is decreased by half, to 0.005 mg/mL final, only a single amino-terminal fragment of ~25K molecular weight is observed; a result previously reported upon trypsin analysis of σ1 purified from T3 reovirions (Yeung et al., 1989). That the 26K and 25K fragments represent amino-terminal fragments was confirmed by monoclonal antibody analysis using anti-N-terminal specific and anti-C-terminal specific antibodies (Fig. 10). Results from limited tryptic digestion in which the samples are boiled prior to SDS-10% PAGE analysis (Fig. 9, A), shows that for all mutant forms of S325 and S327, and the mutant protein D365Y, the carboxy-terminal tryptic fragment remains intact, as observed for WT σ1. On the other hand, for the mutant proteins Y326N, F370V, Y450G and all forms of P451, the carboxy-terminal tryptic fragment is completely degraded, unlike the WT carboxy-terminal tryptic fragment. The protein N369Y shows a partial degradation of the carboxy-terminal tryptic fragment. For both WT and mutant σ1 proteins the amino-terminal tryptic fragments remain intact. Limited chymotryptic digestion consistently results in WT σ1 being cleaved to show three [^{35}S]-labelled protein bands upon SDS-10% PAGE analysis: A complete analysis of the chymotryptic pattern of WT T3 σ1 has been done in this laboratory and shows that the 42K chymotryptic fragment of T3 σ1 represents a carboxy-terminal fragment, while the lower migrating ~24K and ~25K molecular weight bands represent amino- and carboxy-terminal fragments, respectively. Using the conditions described under Materials and Methods, an ~42K chymotryptic fragment, migrating just below full-length monomeric σ1 (Fig. 9, B, lane R3), and the ~25K and 24K fragments, are observed for all mutant forms of S325 and S327, and for protein D365Y, as well as for WT σ1 (Fig. 9, B). Complete degradation of the 42K and 25K carboxy-terminal fragments is observed for Y326N, F370V, Y450G, and all forms of P451; a partial degradation of these fragments is observed for N369Y

(Fig. 9, B). In all cases the ~24K amino-terminal chymotryptic fragment remains intact. It is of particular interest to note that the loss of binding capability is, in every case, accompanied by the susceptibility of the carboxy-terminal fragments to complete degradation by both trypsin and chymotrypsin.

The mutant proteins have also been characterized by the ability to be recognized by the monoclonal antibody G5 (mAb G5). Several characteristic of this Ab make it an appropriate choice for this analysis. It is a neutralizing Ab, thus having the ability to interfere with the cell binding function of σ1 (Burstin et al., 1982; Spriggs et al., 1983a). The epitope for mAb G5 has been mapped to the carboxy-terminal half of σ1 and in this regard has been shown to specifically recognize only the carboxy-terminal tryptic fragment (Yeung et al., 1989). Furthermore, results in this laboratory have shown that this Ab recognizes only the functional multimer form of σ1. Results of the immunoprecipitation analysis of WT and mutant σ1 proteins with mAb G5 are shown in Fig. 11. All mutant protein forms of S325 and S327, like WT σ1, are recognized by G5. The proteins Y326N, D365Y, F370V, Y450G, and all mutant protein forms of P451 are not recognized by G5. The protein N369Y is observed to be partially recognized by G5. An interesting observation is that, unlike the pattern of protease susceptibility, the pattern of recognizability by G5 does not mirror the pattern of binding function loss displayed by the mutant σ1 proteins. The point in case being protein D365Y, which shows the ability to bind L cells (Fig. 8) but is not recognized by G5.

CHAPTER IV: Discussion

Oligonucleotide-directed mutagenesis has been used to study the reovirus viral attachment protein, σ1, of serotype 3 in order to further fine-map the receptor-binding domain of σ1. To this end eight mutagenic oligonucleotides (Table 1) were synthesized that introduced single amino acid changes in the σ1 protein. The specific nucleotide changes and the resulting amino acid substitutions are indicated in Table 2. The initial four mutagenic oligonucleotides (Y326NΔ, D365YΔ, N369YΔ, and F370VΔ) were synthesized as homogeneous populations such that only a single amino acid change was possible from each mutagenesis reaction with these oligonucleotides. The remaining four mutagenic oligonucleotides were designed with mixed base combinations at the targeted nucleotide sites; this allowed multiple amino acid substitutions to be introduced at a given site and therefore a wider range of change in physical characteristics (for example, size/molecular volume, hydrophobicity, polarity,and charge) of the substituted residues. Nine of the 25 mutant σ1 forms that could be generated from the four mutagenic oligonucleotide mixes were recovered and analyzed. These nine proteins provided a good deal of range in physical characteristics, from moderate to more drastic changes (Taylor, 1986).

Sites throughout the carboxy-terminal (C-terminal) one third of the σ1 protein were targeted based on knowledge from previous studies in which Lee and coworkers using deletion mutagenesis studies (Nagata et al., 1987) and tryptic fragment analysis (Yeung et al., 1989) found that the C-terminal half of σ1 harbours the cell attachment domain. Sections III and IV, shown in Fig. 12 on the amino acid sequence of T3 σ1, represents the region that was alone found

Figure 12: The Amino Acid Sequences of the σ1 Proteins of Reovirus Serotypes 1, 2 and 3. The sequences were optimally aligned using the algorithm of Needleman and Wunsch (1970). Deletions are indicated by (·); amino acids triply conserved are indicated by (*). Regions designated A, B,C, D, and E represent conserved regions. Region D shows the greatest homology, 46%, compared to 32% for the remaining regions. Section divisions of the σ1 protein relating to previous deletion mutagenesis studies (Nagata et al., 1987) are indicated as I-II, II-III, and III-IV.

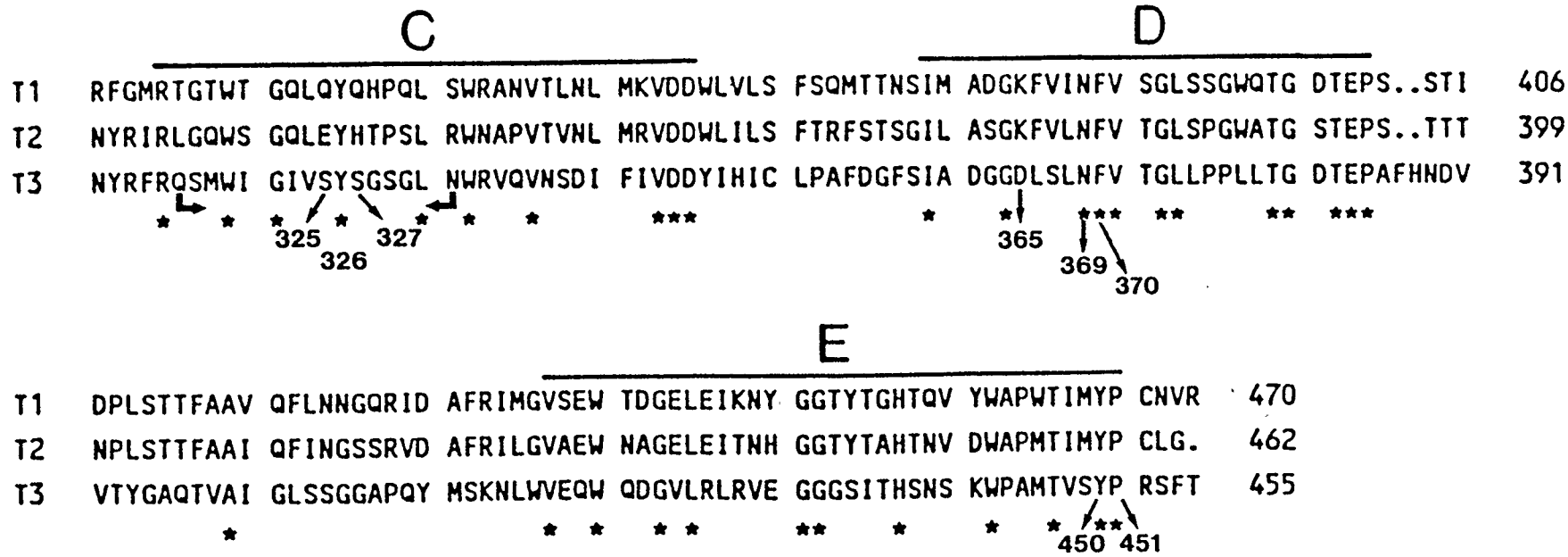
```
T1                                                                              MD ASLITEIRKI     12
T2                                                                              .M SDLVQLIRRE     11
T3                                                                              MD PRLREEVVRL     12
                                                                                   *

T1  VLQLSVSSNG  SQSKEIE...  .EIKKQVQVN VDDIRAANIK LDGLGRQIAD ISNSISTIES RLGEMDNRLV GISSQVTQLS     88
T2  ILLLTGNGES  ANSKH...EI  EEIKKQIKDI SADVNRISNI VDSIQGQLGG LSVRVSAIES GVSENGNRID RLERDVSGIS     88
T3  IIALTSDNGA  SLSKGLESRV  SALEKTSQIH SDTILRITQG LDDANKRIIA LEQSRDDLVA SVSDAQLAIS RLESSIGALQ     92
       *          **          *              *

                                                   A 32%
                                            ___________________
T1  NSVSQNTQSI SSLGDRINAV EPRVDSLDTV TSNLTGRTST LEADVGSLRT ELAALTTRVT TEVTRLDGLI NSGQNSIGEL     168
T2  ASVSGIDSRL SELGDRVNVA EQRIGQLDTV TDNLLERASR LETEVSAITN DLGSLNTRVT TE.......L NDVRQTIAAI     161
T3  TVVNGLDSSV TQLGARVGQL ETGLADVRVD HDNLVARVDT AERNIGSLTT ELSTLTLRVT SIQ....... .......ADF     158
     *          ** *     *       |  ** *        *            *  *  ***
                                 I-II

          B 32%
     _____________
T1  STRLSNVETS MVTTAGRGLQ KNGNTLNVIV GNGMWFNSSN QLQLDLSGQS KGVGFVGTGM VVKIDTNYFA YNSNGEITLV     248
T2  DTRLTTLETD AVTSVGQGLQ KTGNSIKVIV GTGMWFDRNN VLQLFLSNQQ KGLGFIDNGM VVKIDTQYFS FDSNGNITLN     241
T3  ESRISTLERT AVTSAGAPLS IRNNRMTMGL NDGLTL.SGN NLAIRLPGNT .GLNIQNGGL QFRFNTDQFQ IVNNNLTLKT     236
     *    *    **  *  *    *       *     *  *   *     *     *     ↓ *   *
                                                                 II-III

T1  SQINELPSRV STLESAKIDS VLPPLTVREA SGVRTLSFGY DTSDFTIINS VLSLRSRLTL PTYRYPLELD TANNRVQVAD     328
T2  NNISGLPART GSLEASRIDV VAPPLVIQST GSTRLLRLMY EAVDFVVTNN VLTLRNRSVT PTFKFPLELN SADNSVSIHR     321
T3  TVFDSINSRI GATEQSYVAS AVTPLRLNSS TKVLDMLIDS STLE...INS SGQLTVRSTS PNLRYP..IA DVSGGIGMSP     311
           *     *       **                      *   *  *   *   *

            C 32%                                      D 46%
     ______________________                 _______________________
T1  RFGMRTGTWT GQLQYQHPQL SWRANVTLNL MKVDDWLVLS FSQHTTNSIM ADGKFVINFV SGLSSGWQTG DTEPS..STI     406
T2  NYRIRLGQWS GQLEYHTPSL RWNAPVTVNL MRVDDWLILS FTRFSTSGIL ASGKFVLNFV TGLSPGWATG STEPS..TTT     399
T3  NYRFRQSMWI GIVSYSGSGL NWRVQVNSDI FIVDDYIHIC LPAFDGFSIA DGGDLSLNFV TGLLPPLLTG DTEPAFHNDV     391
       *   *   *   *    *  *   *     ***         *   ↓ ***  **   **  ***
                                                    III-IV

                                       E 32%
                          _________________________
T1  DPLSTTFAAV QFLNNGQRID AFRIMGVSEW TDGELEIKNY GGTYTGHTQV YWAPWTIMYP CNVR     470
T2  NPLSTTFAAI QFINGSSRVD AFRILGVAEW NAGELEITNH GGTYTAHTNV DWAPMTIMYP CLG.     462
T3  VTYGAQTVAI GLSSGGAPQY MSKNLWVEQW QDGVLRLRVE GGGSITHSNS KWPAMTVSYP RSFT     455
        *                  *  *  * *   **   *    *  *  **
```

to be sufficient for σ1 binding in these studies.

The choice of residues for mutagenesis was then further narrowed based upon σ1 binding competition studies and sequence analysis. It was reasoned that since the σ1 proteins of all three reovirus serotypes compete for the same receptor on mouse L cells (Lee et al., 1981b), a common recognition mechanism must be involved. Furthermore, it has been shown that carbohydrate moieties on the surface of L cells play an important role in reovirus cell attachment (Armstrong et al., 1984; Gentsch and Pacitti, 1987; Pacitti and Gentsch, 1987). Recent studies in our laboratory have in particular shown the importance of sialic acid from the finding that reovirus is capable of binding to sialic acid alone (Paul et al., 1989). It is, therefore, very plausible that a sialic acid binding region is common to all three σ1 proteins. Indeed, sequence analysis of the σ1 proteins of the 3 serotypes of reovirus in our laboratory (Duncan et al., 1990) has revealed several regions on the σ1 protein that are highly conserved between the serotypes. These regions are designated A - E in Fig. 12 and represent regions in which amino acids conserved between the 3 serotypes are clustered. The percent homology of triply conserved residues in these regions are 32%, except for region D which exhibits the greatest percent homology of 46%. Three of the five conserved regions observed, domains C, D, and E, reside in the carboxy-terminal one-third of σ1 (Fig. 12). The potential evolutionary significance of conserved amino acids located in regions C, D, and E for cell receptor recognition was, therefore, investigated.

The amino acid tyrosine, at position 326 (Y326), and in domain C of the type 3 σ1 protein (Fig. 13), provided a good first target because it was conserved among the three serotypes, and a change to the amino acid

Figure 13: C-terminal Amino Acid Sequences of the σ1 Proteins of Reovirus T1, T2 and T3. Amino acids targeted for mutagenesis and located in conserved regions C, D and E are individually numbered. Residues 317 - 332 of T3 σ1 (indicated by large arrows) encompass a region implicated as the neutralization/cell-attachment epitope of T3 reovirus (Williams et al., 1988).


C
D
T1 RFGMRTGTWT GQLQYQHPQL SWRANVTLNL MKVDDWLVLS FSQMTTNSIM ADGKFVINFV SGLSSGWQTG DTEPS..STI 406
T2 NYRIRLGQWS GQLEYHTPSL RWNAPVTVNL MRVDDWLILS FTRFSTSGIL ASGKFVLNFV TGLSPGWATG STEPS..TTT 399
T3 NYRFRQSMWI GIVSYSGSGL NWRVQVNSDI FIVDDYIHIC LPAFDGFSIA DGGDLSLNFV TGLLPPLLTG DTEPAFHNDV 391
325
326
327
365
369
370
E
T1 DPLSTTFAAV QFLNNGQRID AFRIMGVSEW TDGELEIKNY GGTYTGHTQV YWAPWTIMYP CNVR 470
T2 NPLSTTFAAI QFINGSSRVD AFRILGVAEW NAGELEITNH GGTYTAHTNV DWAPMTIMYP CLG. 462
T3 VTYGAQTVAI GLSSGGAPQY MSKNLWVEQW QDGVLRLRVE GGGSITHSNS KWPAMTVSYP RSFT 455
450
451

asparagine introduced an EcoRI restriction site that was easily screened for. Initial L cell binding studies with the *in vitro* expressed Y326N protein showed the complete abrogation of binding function. This result was of particular interest in consideration of the newly published studies of Greene and coworkers (Williams et al., 1988). Earlier studies by this group (Noseworthy et al., 1983; Kauffman et al., 1983; Gaulton et al., 1985; Bruck et al., 1986) identified an area of amino acid sequence homology shared by T3 σ1 and an anti-idiotypic / antireceptor monoclonal antibody (mAb) that can mimic reovirus T3 by attaching to the same cell-surface receptor. On T3 σ1 this region encompasses amino acids 317 to 332 in domain C and includes the Y326 residue (Fig. 13). Studies using synthetic peptides corresponding to these regions of homology on σ1 and the antireceptor mAb identified this region as the oligopeptide neutralizing / cell-attachment epitope of reovirus T3. Furthermore, computer modeling of this epitope, using sequence similarities of known immunoglobulin hypervariable loop conformations, predicts this region to have a β-turn configuration and that the amino acid side chains in this turn have nearly identical positions when comparing σ1 and the antireceptor mAb. These results lead this group to postulate that this β-turn area is involved in the interaction with the monoclonal antibody G5 (mAb 9BG5) and the reovirus type 3 receptor. My results, showing the protein Y326N to lack cell binding capability (Fig. 8), fits in well with this postulate and provided a good opportunity to further investigate the significance of the side chains in this β-turn region by introducing changes that would disrupt structure and/or function of the hydroxyl side chains of the serine residues located on either side of Y326 (Fig. 13). Thus multiple single amino acids changes were independently introduced at serines 325 (S325) and 327 (S327) using mutagenic oligonucleotide mixes

(Table 1).

Region D is the most highly conserved region of σ1 with 46 % of the amino acid residues being shared by all three proteins. It is likely, therefore, that this amino acid conservation defines an essential site involved in a σ1 function common to all three serotypes. The three residues, aspartate 365 (D365), asparagine 369 (N369), and phenylalanine 370 (F370) of region D (Figure 13) were targeted for mutagenesis, thus taking advantage of the suitability of this region for mutagenesis.

Analysis of region E was based on recent fine structure mapping studies in our laboratory which show that removal of as few as four amino acids from the carboxy-terminus of T3 σ1 completely abrogates L cell binding. This end deletion also leads to a conformational change that upon limited proteolytic analysis with trypsin and chymotrypsin results in the complete degradation of C-terminal fragments that are otherwise stable. Consequently, tyrosine 450 (Y450) and proline 451 (P451), two of three triply conserved residues spanning the terminal twelve amino acids of the σ1 protein (Figure 13), were targeted for mutagenesis in order to analyse conserved amino acids at the C-terminal end of T3 σ1.

Upon identification, all mutant S1 genes were subcloned into the pGEM expression vector (pG4T3) for *in vitro* transcription using SP6 polymerase and cell-free translation in rabbit reticulocyte lysate containing [^{35}S]-methionine. Aliquots of each translation lysate were removed and the expressed products analysed by SDS-10% PAGE and autoradiography (Fig. 7). Analysis of the expressed products allowed the levels of expression of each mutant σ1 form to be equilibrated to the expression level of WT σ1. With input levels of the proteins approximately equivalent, subsequent assays with the proteins would

reveal a relatively quantitative analysis of function and structure relative to WT σ1. With the samples not being boiled prior to PAGE analysis both monomeric and multimeric σ1 forms could be detected. In this way all mutant σ1 forms were observed to be capable of forming stable multimers (Fig. 7), even if the functional multimeric form of the mutant is not capable of binding (Fig. 8, A; for example, the P451 mutant forms). This finding is further evidence for functionally distinct regions on σ1, as demonstrated for the cell binding and hemagglutination functional regions (Nagata et al., 1987; Yeung et al., 1984), and indicates that the region involved in cell binding is also functionally distinct from the region(s) involved in the formation of σ1 oligomers.

After analysis of the *in vitro* expressed products, the mutant σ1 forms were then analysed in three assays for, 1) the ability to bind L-cells, 2) susceptibility to limited proteolysis (using trypsin and chymotrypsin) in order to investigate structural integrity, and 3) the ability to be recognized by the neutralizing mAb G5 as a probe of the structural integrity of the neutralization epitope. The results of the three assays for all mutant σ1 proteins, as described at length in Results, has been summarized in Table 3, with WT σ1 showing the ability to bind L-cells at 4°C and 39°C incubation temperatures (+), to be specifically cleaved by trypsin and chymotrypsin without subsequent degradation of either the amino or carboxy terminal fragments (+), and to be recognized by the neutralizing mAb G5 (+). The summarization of results from the L-cell binding and proteolysis studies in tabular form (Table 3) shows more clearly the pattern observed between binding ability and susceptibility during limited proteolysis. Like WT σ1 all mutant σ1 proteins that are capable of binding are also cleaved to produce N- and C-terminal proteolytic fragments that are not subsequently degraded during limited proteolysis. On the other hand, those mutant σ1

Table 3: Summary of Results for Wild-Type and Mutant σ1 Forms

AMINO ACID TARGET	NEW	L-CELL BINDING 4°C	39°C	PROTEOLYSIS[a] TRP	CHY	mAb G5 RECOGNITION
WT[b]		+	+	+	+	+
S325	T	+	+	+	+	+
	N	+	+	+	+	+
	A	+	+	+	+	+
Y326	N	-	-	-	-	-
S327	T	+	+	+	+	+
	N	+	+	+	+	+
D365	Y	+	+	+	+	-
N369	Y	+/-	-	+/-	+/-	+/-
F370	V	-	-	-	-	-
Y450	G	-	-	-	-	-
P451	S	-	-	-	-	-
	K	-	-	-	-	-
	T	-	-	-	-	-

a + = C-terminal proteolytic fragments not degraded during limited proteolysis.
- = C-terminal proteolytic fragments degraded during limited proteolysis.

b Results are compared to the behavior of the wild-type σ1 protein in all assays. With wild-type σ1 showing the ability to bind mouse L fibroblasts at 4°C (+) and 39°C (+), to be specifically cleavage by trypsin and chymotrypsin without subsequent degradation of either the amino or carboxy terminal fragments (+), and to be recognition by the neutralizing monoclonal antibody G5 (+).

proteins that are incapable of binding are cleaved to produce a stable N-terminal fragment while the C-terminal portion of the protein is completed degraded during limited proteolysis. These results indicate that the loss of binding activity is accompanied by a conformational change in the C-terminus of the σ1 protein. Furthermore, this conformational change in the C-terminus of σ1 apparently does not affect the structural/conformational integrity of the N-terminus of σ1 and, therefore, would not be expected to influence functions associated with the N-terminus, such as anchorage to the virion or oligomerization. This has in fact been demonstrated since, as mentioned earlier, all mutant σ1 forms are capable of forming stable multimers even in the absence of binding ability.

The results as tabulated in Table 3 also clearly show that, unlike susceptibility to limited proteolysis, the ability to be recognized by the neutralizing mAb G5 is not observed to pattern the ability to bind L-cells. This fact is exemplified by the D365Y protein which is capable of binding to L-cells at a level of 100% compared to that of WT σ1 binding (Fig. 8, D365Y), but which is not recognized by the neutralizing mAb G5 (Fig. 11, D365Y). This finding indicates that a conformational change of the σ1 protein can occur without the subsequent loss of binding and that the neutralization and binding epitopes on T3 σ1 are separable.

The potential significance of the results obtained and as summarized in Table 3 will further be analyzed by examining the effects of each modification according to the regions on σ1 in which they are located; that is, in region C, D, or E (Fig. 13). As discussed previously, the residues modified in the conserved region C (Y326, S325, and S327) are also located within an area that includes residues 317 to 332 of T3 σ1 (Fig. 13) that has been identified as the

oligopeptide neutralization / cell-attachment epitope of reovirus T3 (Bruck et al., 1986). This 16 amino acid region was identified as the neutralization / cell-attachment epitope of T3 σ1 by analysing an anti-idiotypic mAb (87.92.6) that is directed against the G5 mAb specific for the virus neutralization epitope on T3 σ1. The functional properties of this anti-receptor mAb 87.92.6 are as follows: attaches to the same cell-surface receptor as T3 σ1, competes with T3 σ1 for binding to specific cellular receptors, immunization of BALB/C mice elicits neutralizing antibodies to T3, and its pattern of binding mimics the tissue tropism of reovirus T3 (Noseworthy et al., 1983; Kauffman et al., 1983; Gaulton et al., 1985). These data were presented to indicate that mAb 87.92.6 biologically resembles the epitope on σ1 that interacts with the cellular receptor for reovirus T3. Amino acid sequence comparison between T3 σ1 and the 87.92.6 mAb revealed an area of significant primary sequence homology corresponding to the 16 amino acid sequence of T3 σ1 encompassing residues 317 to 332 (Bruck et al., 1986). Synthetic peptides were synthesized that correspond to this area of sequence similarity on σ1 (Reo peptide) and on the second complementary determining regions (CDR IIs) of the mAb 87.92.6 heavy- and light-chain variable regions (V_H and V_L peptides, respectively), which correspond to T3 σ1 residues 317 - 324 (V_H) and 323 - 332 (V_L). The V_H and V_L peptides were also linked together using an amino acid terminal cysteine residue (V_H + V_L peptide). The peptides V_H + V_L , V_L, and Reo were shown to bind the mAb G5, with V_H + V_L showing the highest level of mAb G5 binding and V_H showing no binding; V_H + V_L, V_H and V_L were also shown to compete with mAb 87.92.6 for binding by mAb G5 (Williams et al., 1988). Competitive binding assays showed that V_L and V_H + V_L, but not V_H alone, could block binding of reovirus T3 to mAb G5. These results were taken to

indicate that amino acids 323 - 332 of T3 σ1, the area of sequence similarity between the V_L and Reo peptides, specifies the site of interaction between the mAb G5 with reovirus T3 σ1. Further analysis showed that the V_L peptide coupled to BSA (V_L-BSA) and V_H + V_L, but not V_H -BSA or uncoupled V_L, inhibits binding of the antireovirus receptor mAb 87.92.6 to the reovirus T3 cellular receptor. The V_L and V_H + V_L peptides, but not V_H, were also shown to specifically inhibit binding of reovirus T3 to L-cells, R1.1 cells and murine B104 neuroblastoma cells (all of which possess the T3 reovirus receptor; Armstrong et al., 1984; Co et al., 1985a). These results were taken to indicate that the V_L peptide interacts specifically with the reovirus T3 receptor, with the implication that amino acids 323 - 332 of T3 σ1 (region similar to V_L) also directly interacts with the reovirus T3 receptor. Thus it was concluded that the shared primary structure between T3 σ1 and the antireceptor 87.92.6 mAb light chain defines the neutralizing epitope on T3 σ1 and is involved in binding to the reovirus T3 receptor. Analysis in the present mutagenesis studies of the Y326 amino acid located in this epitope, which shows that a substitution of this residue to asparagine results in the complete abrogation of cell binding (Fig. 8), coincides with the evidence that the region represented by amino acids 323 - 332 is involved in cell receptor recognition. However, upon closer examination of the data presented to identify region 323 to 332 as the neutralization / cell-attachment epitope it was noted that the concentration of peptide V_L needed to inhibit reovirus T3 binding by 80% was 250 μM. This equates to 0.45 mg/mL for the 16 amino acid long V_L peptide (taking the average MW of an amino acid to be 112 g/mL) which is equivalent to ~13.4 mg/mL of σ1 protein (T3 σ1 is 455 amino acids long, ~30 times the size of V_L). Since the σ1 protein is ~1% of the total virion proteins (Lee et al., 1981b) the molar equivalent of virus to 13.4

mg/mL of σ1 is 1.3 g/mL. Thus the concentration of V_L peptide needed to inhibit reovirus T3 binding to L-cells by 80% is equivalent to 1.3 g/mL of reovirus T3 of known high-affinity binding (K_D of ~3 x 10^{-9} M) to receptors (Armstrong et al., 1984), suggesting that the V_L peptide exhibits a low affinity binding to the reovirus T3 cellular receptor. This would further suggest then that along with the oligopeptide epitope 323 - 332 other structural components on T3 σ1 are necessary for reovirus T3 binding.

Results from earlier studies also implicate amino acids in regions of T3 σ1 other than the 10 amino acid stretch from 323 to 332 to be important for the structural and/or functional integrity of the reovirus receptor-binding domain. Two antigenic variants of reovirus T3, variants K and A, were developed by growing reovirus T3 in the presence of the G5 neutralizing mAb (Spriggs and Fields, 1982). The K and A variants have reduced virulence and restricted tissue tropism compared to reovirus T3 (Spriggs and Fields, 1982; Spriggs et al., 1983b), functions which are attributed to the σ1 protein - as well as being resistant to neutralization by mAb G5. Sequence analysis demonstrated that the specific changes in the biological properties of these variants were due to a single amino acid substitution in the σ1 protein of each variant (Bassel-Duby et al., 1986). In variant K lysine replaces glutamic acid at position 419 which is located in conserved region E and in variant A valine replaces aspartic acid at position 340 which is located in region C but lies outside the predicted neutralization / cell-attachment epitope (amino acids 323 - 332). It was concluded that amino acids 419 and 340 of σ1 are critical sites that affect virulence and tissue tropism. Data in this report also shows that single amino acid changes throughout the C-terminal one third of T3 σ1, and not just in the confined area of region C (Y326N, Fig 8), results in loss of σ1 binding and

conformational changes. Perturbations of amino acids in region D, the most highly conserved region of σ1, results in proteins with a range of binding capabilities. The protein N369Y shows ~20% the binding capability of WT σ1 (Fig. 8) and a conformational change that results in the partial degradation of proteolytic C-terminal fragments (Fig. 9). F370V shows the complete loss of binding (Fig. 8) and a conformational change that results in the complete degradation of proteolytic C-terminal fragments (Fig. 9). All changes of the conserved amino acids Y450 and P451 located in region E, at the C-terminal end of σ1, also results in the complete loss of cell-binding ability (Fig. 8), with structural changes to the C-terminus that results in its complete degradation during limited proteolysis (Fig. 9). These data show an involvement of residues in regions D and E for the structural/functional integrity of the σ1 cell-binding domain.

Indeed then the importance of amino acids throughout the C-terminal portion of the σ1 protein for the structural and functional integrity of the receptor-binding domain, that was suggested from data obtained in previous studies, has been demonstrated in this report. The implication of this finding is that although the 10 amino acid oligopeptide region from 323 to 332 of T3 σ1 may define the cell-attachment epitope, the T3 σ1 protein appears similar to the viral attachment protein HA (hemagglutinin) of influenza virus in which other highly conserved residues outside of the actual binding site itself are necessary to maintain the structural integrity of the receptor binding site (Weis et al., 1988). Even nonconserved amino acids located in regions outside of these areas have a role in influenza's infectivity - which may also turn out to be the case for reovirus as well - by providing the variation of the antigenic properties and structure around the binding site that allows antigenic variation (Wiley et al.,

1981). Alternately, residues 323 to 332 of T3 σ1 may only define a part of the cell-attachment site which in itself is also composed of residues from other areas of the T3 σ1 primary amino acid sequence. Again this would be reminiscent of the HA of influenza in which the binding site is composed of amino acid side chains from conserved residues that span an area of 131 amino acids on the primary sequence (Tyr 98 to Ser 228; Wiley and Skehel, 1987).

Investigations using the anti-idiotypic approach of studying the T3 reovirus receptor-binding domain defines amino acids 323 to 332 not only as the T3 cell-attachment epitope but also as the mAb G5 neutralization epitope (Williams et al., 1988). Results obtained with the protein D365Y in the present studies, however, indicates that the neutralization and binding epitopes are separable. D365Y binds L-cells at a level equivalent to WT σ1 (Fig. 8) with a structural modification that no longer allows D365Y to be recognized by mAb G5 (Fig. 11) but which does not affect binding. This data shows that a structural modification to the neutralization epitope can occur without a concomitant functional disruption of the receptor-binding epitope. Since a change from aspartic acid to tyrosine is a fairly drastic change - an increase in size as well as a change from a polar, negatively charged side chain to a hydrophobic side chain (Taylor, 1986) - this result would suggest that the G5 neutralization and receptor-binding epitopes are not identical. However, it is very likely that the two epitopes are in close proximity to one another and may even overlap. Particularly when it has been shown that the amino acid changes at residues 419 and 340 in the antigenic variants K and A, respectively - which also are not recognized by the neutralizing mAb G5 - apparently result in altered cell recognition/binding ability, as indicated by their reduced virulence and

restricted tropism.

Williams et al. (1988) have also done computer modeling of the predicted cell-attachment epitope of reovirus T3, amino acids 323 to 332, using two immunoglobulin CDR II sequences of known crystallographically defined three-dimensional structures that have sequence similarity to the T3 σ1 sequence and the antireceptor mAb 87.92.6 V_L CDR II sequence. Computer modeling predicts residues S325 to S329 of T3 σ1 and the corresponding residues of the V_L peptide to have β-turn configurations. The backbone configurations of these turns were observed to differ but the positions of the amino acid side chains were nearly identical. It was postulated that this β-turn area and more specifically the hydroxyl oxygens of the residues in this region (S325, Y326, S327, and S329 of T3 σ1) are directly involved in the interaction with mAb G5 and the reovirus T3 receptor. The potential involvement of the side chain hydroxyl oxygens of residues S325 and S327 of T3 σ1 were analysed in this report. The change of S325 to threonine (S325T), asparagine (S325N), or alanine (S325A) results in moderate to drastic changes in the functional ability of the side chain group at this position. That is, in the ability of the side chains to interact with carbohydrate, an important consideration since it has been shown that carbohydrate moieties on the surface of L cells and erythrocytes play an important role in reovirus cell attachment (Armstrong *et al.*, 1984; Gentsch and Pacitti, 1987; Pacitti and Gentsch, 1987) and that reovirus is capable of binding to sialic acid alone (Paul et al., 1989). Accordingly then, threonine maintains the hydroxy group and both threonine and asparagine maintain the ability to bind carbohydrate (Weis et al., 1988; Fasman, 1989), with the hydrophobic, non-polar nature of alanine being a radical functional change. However, none of these substitutions disrupted the cell binding

function of σ1 (Fig. 8) or the ability to be recognized by the mAb G5 (Fig. 11), even when the postulated functional side chain was completely absent (S325A). These data suggest that the hydroxyl group of S325 is not involved in the interaction with mAb G5 or the reovirus T3 receptor. S327 was also changed to threonine (S327T) and asparagine (S327N), and again there was no disruption of either the cell binding function or the ability to be recognized by mAb G5 (Fig. 8 and 11). That the S327 hydroxyl group is not involved in the interaction with the reovirus T3 cellular receptor, however, cannot be concluded since both threonine and asparagine, like serine, have the potential to bind carbohydrate moieties through hydrogen-bond interactions (Weis et al., 1988; Fasman, 1989). Isolation and analysis of the S327A protein is, however, presently underway in order to determine the potential importance of the S327 side chain group.

The analysis of the two conserved residues Y450 and P451 in region E, at the C-terminal end of T3 σ1 (Fig. 13), was prompted by end deletion studies in this laboratory. Data from these studies show that the removal of as few as four amino acids from the C-terminus of T3 σ1 completely abrogates L-cell binding, results in the complete degradation of C-terminal proteolytic fragments during limited proteolysis using trypsin and chymotrypsin while the N-terminal fragments remain stable, and also affects σ1 such that it is no longer recognized by an anti-C-terminal specific mAb (mAb G5) but is recognized by an anti-N-terminal specific mAb. Similarly the change of Y450 to glycine (Y450G) or P451 to serine (P451S), threonine (P451T) or lysine (P451K) completely abrogates L-cell binding (Fig. 8), results in the complete degradation of C-terminal proteolytic fragments during limited proteolysis using trypsin and chymotrypsin (Fig. 9), and also results in σ1 forms that are no longer recognized

by the mAb G5 (Fig. 11). It is interesting to note in this regard that the removal of a few residues from either end of a polypeptide has often been observed to result in large changes in the overall stability of the protein (Wetlaufer and Ristow, 1973; Janin and Wodak, 1983; Fischer and Schmid, 1990). For example, studies of ribonuclease A have shown that removal of either 20 residues from the N-terminus or four residues from the C-terminus results both in the loss of ~80% enzymatic activity and in the inability of the truncated protein chains to re-form all correct disulfide bonds after denaturation and reduction (Haber and Anfinsen, 1961; Taniuchi, 1970). However, greater than 90% enzymatic activity can be regained by adding back the S-peptide (1-20) or the C-terminal 110-124 peptide, respectively, to the reoxidation mixture (Kato and Anfinsen, 1969; Andria and Taniuchi, 1978). In other studies, large fragments of staphylococcal nuclease were found to lack specific structure, however, when overlapping peptides were incubated together folding was possible (Andria et al., 1971). Studies of such small single domain proteins suggest that in at least some cases essentially the entire polypeptide chain is necessary for stability and folding, as implicated for σ1. However, it is also interesting to note that the 4 amino acid end deletion of σ1 apparently does not affect the N-terminal domain of T3 σ1 since it is recognized by an N-terminal specific mAb and N-terminal proteolytic fragments are stable during limited proteolysis using trypsin and chymotrypsin. This finding is not unusual since there are many cases of larger proteins that show stable and autonomously folding domains, such as σ1. Examples include the domains of BSA (Johanson et al., 1981), of the immunoglubulin light chain (Tsunenaga et al., 1987), and of tryptophan synthase (Zetina and Goldberg, 1980; Matthews and Crisanti, 1981). Taken together, findings such as these lend credence to the possibility that Y450 and

P451 may serve a critical function for the correct folding of the σ1 C-terminal receptor-binding domain. Although it certainly does not rule out other possibilities such as Y450 and P451 being directly involved in cell receptor recognition or serving to directly stabilize the binding site.

Although the exact nature of the σ1-receptor interaction is still unclear, several lines of evidence, including the data obtained in this study, suggest then that the reovirus cell recognition structure is not simply a single linear epitope on the σ1 molecule; as has been indicated by the studies using an anti-idiotypic antibody approach that localizes the cell-attachment site of reovirus T3 to an ~10 amino acid linear region (residues 323 - 332). Indeed, evidence suggests it is more likely that the ability of reovirus to recognize and bind to host cells involves either a single but highly complex structural binding site (conformational epitope), such as the influenza hemagglutinin receptor binding pocket (Wiley and Shekel, 1987), or possibly even involves two separable synergistic binding sites, as demonstrated for the cell-binding domain of human fibronectin (Obara et al., 1988).

The observation that the three reovirus serotypes compete for binding to mouse L cell receptors (Lee et al., 1981b) suggests that a common recognition mechanism is involved. Through the use of a series of synthetic sialosides, it has also been shown in this laboratory that the minimal receptor determinant recognized by reovirus is the α-anomeric form of sialic acid (Paul et al., 1989). The potential number of receptor candidates for reovirus, therefore, is large due to the large number of sialic acid-containing membrane components on a variety of cell types. The relatively nonstringent requirement for reovirus receptor recognition may account for the ubiquitous nature of this virus and its ability to bind to and infect a variety of cell types *in vitro*. A corollary of this

observation would be that reovirus recognizes not one, but many cell surface macromolecules, as long as the sialic acid component is both accessible to the virus and in the α-anomeric form, linked in either an α-2,4 or α-2,6 configuration (Paul et al., 1989). In fact, it has been shown in this laboratory that reovirus recognizes multiple cell surface proteins on Western blots of L-cell plasma membrane proteins (Choi et al., 1990). However, in order to rationalize the protein σ1-mediated tissue tropism exhibited by the various reovirus serotypes in *in vivo* model systems, clearly additional elements must be involved in the specific interaction of reovirus with a receptor, beyond the simple recognition of NeuNAc residues.

The involvement of additional parameters is also evident from the observation that although sialic acid is an absolute requirement in reovirus binding to both erythrocytes and L-cells (Armstrong et al., 1984; Gentsch and Pacitti, 1985; Paul and Lee, 1987; Gentsch and Pacitti, 1987), the two attachment mechanisms apparently involve both common and distinct regions of σ1 (Spriggs et al., 1983a; Nagata et al., 1987), as discussed previously. Furthermore, although T1 and T3 reoviruses were found to recognize the same set of multiple proteins on Western blots, quantitative differences between the two serotypes in binding to some of the proteins were also observed (Choi et al., 1990).

Additionaly, in the present studies an inability or reduced ability to bind L-cells is observed to occur for mutant σ1 forms that have single amino acid changes in any of the highly conserved regions C, D, or E, throughout the C-terminus of σ1. While other single amino acid changes in regions C and D produce mutant σ1 forms that are capable of binding L-cells at WT levels. Limited proteolysis using trypsin and chymotrypsin reveals that those mutant σ1

forms incapable of binding L-cells also show proteolytic fragment patterns that differ from WT T3 σ1 in that the C-terminal fragments are completely degraded; WT T3 σ1 being cleaved to produce stable C-terminal and N-terminal fragment populations. Those mutant σ1 forms that are incapable of binding are also incapable of interacting with the neutralizing mAb G5 that specifically interacts with an epitope in the C-terminal half of the functional multimer form of T3 σ1 that subsequently prevents binding of reovirus T3 to host cells. These data indicate that the loss of σ1 binding is accompanied by a conformational change of the σ1 protein and suggests that conserved residues throughout the C-terminus of T3 σ1 are involved in the structural and/or functional integrity of the receptor-binding domain.

Conclusions and Future Prospects

The amino acids selected for substitutions in this study are all located in regions on σ1 highly conserved between the three reovirus serotypes, although all are not conserved amino acids. These regions are suggestive of structures conserved between the three reovirus serotypes that are involved in functions common to the serotypes, such as the recognition of the common L-cell receptor and possibly represent a sialic acid-binding site. In addition to the protein-sialic acid interaction, however, it is likely that another type of interaction, one more specific in nature, is also involved and that this σ1-receptor interaction is responsible for the different σ1-mediated tissue tropisms exhibited by the three serotypes.

The results obtained from this study demonstrate that residues located in all three conserved regions C, D and E are important structural and/or functional components of the T3 σ1 receptor-binding domain. Furthermore, it has been demonstrated that the neutralization and cell-attachment epitopes of T3 σ1 are not identical, although they may overlap.

There is potentially a greater significance of at least some of residues studied in this project to the interactions at the receptor-binding site itself. Since here is a protein that is highly diverged between the reovirus serotypes and , therefore, seems capable of accommodating a great deal of various amino acid substitutions throughout its structure and even to retain features and functions common among the three serotypes. Yet this is the same protein that could be grossly affected by the amino acid substitutions of this study. That at least some of these amino acids are not themselves directly involved at the receptor-binding site as structural or functional components, then, is difficult to believe. In this regard, it is also interesting to remember that all the amino acid

substitutions produced in this study were of residues located in regions highly conserved between the reovirus serotypes. However, a greater interpretation of the data from this study than that already given is not feasible, given the present levels of understanding in all relevant fields.

So the questions remain. How could some of the amino acid substitutions made in this study have such gross conformational and functional effects in such an apparently highly accommodating protein without themselves being directly involved in the structural and/or functional integrity of the receptor-binding site itself? Even more importantly, what are the residues involved in the interaction of σ1 with its host cell receptor and indeed what are the specific interactions? The answers await the three-dimensional structural determination of the σ1 protein by X-ray crystallography of the purified σ1 protein and/or through the improving conformational prediction methods, followed by further mutagenesis studies of the σ1 proteins of all three reovirus serotypes; and in general for a more complete understanding of how the structure of proteins relate to their functions.

Together with the rapidly growing body of knowledge concerning the σ1 proteins structure and functions, I believe the information ascertained in this study provides important information for future studies aimed at fully understanding the structure/function relationship of this protein at the molecular level.

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Appendix

Reagent Sources

General chemicals and reagents were purchase from the Fisher Scientific Co. and Sigma Chemical Co., and were reagent grade. The following reagents were purchased from the indicated suppliers: all proteases, protease inhibitors, antibiotics, BSA, and detergents were from the Sigma Chemical Co.; reagents for gel electrophoresis (electrophoresis grade) were from Bio-Rad and the Sigma Chemical Co.; methylmercuric hydroxide was from Alfa Ventron, Danvers, MA; nucleotides, deoxynucleotides, ribonucleotides, and RNA cap structure analog [m7G(5')ppp(5')G] were from Pharmacia; Hind III λ DNA and 1 Kbp ladder were from BRL. The following enzymes were purchased from the indicated suppliers: restriction endonucleases were purchased from BRL, Pharmacia, Bio/Can Scientific, or New England BioLabs; SP6 RNA polymerase and RNase-free DNase I were from BRL; T4 PNK, Biolabs; T4 DNA ligase, Klenow fragment of *E. coli* DNA polymerase I, and Exo III were from BRL; RNase A, Sigma.

Radioactive chemicals were purchased from New England Nuclear (NEN), ICN Biomedicals Inc., or Amersham.

Materials for the mutagenesis protocol were obtained as follows: syringe filter holder - Polycarbonate, 13mm, Sartorius (SM 16514), from CANLAB; nitrocellulose membrane discs - ultra pure, 13mm, Sartorius (11336013N), from CANLAB; α-thio-2'-deoxyribosylcytosine 5'-triphosphate (dCTPαS), from NEN Dupont Canada.

Mutagenesis Buffers

The preparation of buffers for the mutagenesis protocol was as follows (NB: all buffers were made from sterile stock solutions):

1. Buffer 1

1X	5X	Stocks	1.5 ml of 5X
120 mM NaCl	600mM	5 M	0.18 mL
120 mM Tris-Cl, pH 8.0	600mM	1 M	0.90 mL

Add 0.42 mL of H_2O.

2. 1.0 M DTT (dithiothreitol) in water. Filter sterilized and stored at -20°C.
3. Prepare a sterile 100mM $MgCl_2$ solution. This is a 10X stock solution.
4. Nucleotide Mix 1 (Nt. mix 1)

1X	10X	Stocks	40 µL of 10X
1.0mM ATP	10mM	20mM	20 µL
250uM dCTPαS	2.5mM	16.5mM	6.1 µL
250uM dATP	2.5mM	100mM	1.0 µL
250uM dGTP	2.5mM	100mM	1.0 µL
250uM dTTP	2.5mM	100mM	1.0 µL

Add 10.9 µL of H_2O.

Aliquot into 10 µL volumes & store frozen at -70°C.
The nucleotide dCTPαS was stored at -70°C.

5. Buffer 2: 5mM Tris-HCl, 0.5mM EDTA, pH 8.0 (ie. TE, pH 8.0, diluted 1:1 with H_2O).
6. Buffer 3

1X	10X	Stocks	1.0mL of 10X
30mM NaCl	300mM	5.0M	60 µL
12mM Tris-HCl, pH 8.0	120mM	1.0M	120 µL
6mM $MgCl_2$	60mM	1.0M	60 µL
10mM DTT	100mM	1.0M	*

Add 660 µL of H_2O.

*Just before use added 1.0 M DTT to a final concentration of 100 mM (ie. 1.0 µL of 1.0 M DTT added to 9.0 µL 10 X incomplete Buffer 3).

7. Buffer 4

1X	10X	Stocks	100 µL of 10X
52.2mM Tris-HCl, pH 8.0	522mM	1.0M	52.2 µL
2.1mM $MgCl_2$	21mM	1.0M	2.1 µL
3.5mM DTT	35mM	1.0M	3.5 µL
			Add 42.2 µL of H_2O.

8. Nt. Mix 2

1X	10X	Stocks	100 µL of 10X
250uM dATP	2.5mM	100mM	2.5 µL
250uM dCTP	2.5mM	100mM	2.5 µL
250uM dGTP	2.5mM	100mM	2.5 µL
250uM dTTP	2.5mM	100mM	2.5 µL
350uM ATP	3.5mM	20mM	17.5 µL
			Add 72.5 µL of H_2O.

Aliquoted into 50 µL volumes & stored at -70°C.

Media

	LB (Luria-Bertani) Medium	2xYT Medium
bacto-tryptone	10 g	16 g
bacto-yeast extract	5 g	10 g
NaCl	10 g	5 g

Volume to 1 L with dH_2O and autoclave. For agar plates add 20 g of bacto-agar/L of medium prior to autoclaving, cool the medium to 50 - 60°C and pour 25 - 30 mL per standard petri dish.

M9 Medium

10 X Salts:	Na_2HPO_4	60 g
	KH_2PO_4	30 g
	NaCl	5 g
	NH_4Cl	10 g

To 1 L with dH_2O, autoclave and store at room temperature. The following solutions were also prepared separately, autoclaved and stored at room temperature: 1 M $MgSO_4$-$7H_2O$; 20% glucose (w/v); 1% thiamine (w/v); 0.1 M $CaCl_2$. To prepare 1 L of M9 medium add together the following:

10 X salts	100 mL
1 M $MgSO_4$-$7H_2O$	1 mL
20% glucose	10 mL
1% thiamine	1 mL
0.1 M $CaCl_2$	1 mL
dH_2O	887 mL

For agar plates, add 20 g of bacto-agar to 900 mL of dH_2O, autoclave, let cool to 55 - 60°C and add the remaining sterile solutions.

B-broth Top Agar

bacto-tryptone	10 g
NaCl	8 g
bacto-agar	8 g

Volume to 1 L and autoclave. Let cool and added 1 mL of a sterile 1% thiamine (Vit B_1) solution. Aliquoted into sterile 50 mL bottles and stored at room temperature.

SOB & SOC Medium

bacto-tryptone	20 g
bacto-yeast extract	5 g
NaCl	0.58 g
KCl	0.19 g

Volume to 1 L and autoclave. Prepared a solution of 1 M $MgCl_2$-$6H_2O$, 1 M $MgSO_4$-$7H_2O$ (2 M Mg^{2+}) and sterilized by filtration through a 0.22 µm filter. The 2 M Mg^{2+} solution is diluted 1 in 100 (20 mM final) into the medium just before use. For SOC medium, a 2 M glucose solution is also diluted 1 in 100 (20 mM final) into the medium.

Stock Buffer Solutions

10 X TBE (0.9 M Tris, 0.9 M boric acid, 25 mM EDTA)	
Tris base	108 g
boric acid	55 g
glacial acetic acid	-
EDTA	9.3 g
dH_2O to	1 L

50 X TAE (2 M Tris, 0.1 M EDTA)
242 g
-
57.1 mL
37.2 g
1 L

10 X PBS (1.4 M NaCl, 27 mM KCl, 80 mM Na_2HPO_4, 15 mM KH_2PO_4)	
NaCl	81.82 g
KCl	2.01 g
Na_2HPO_4	11.36 g
KH_2PO_4	2.04 g
dH_2O to	1 L

20 X SCC (3 M NaCl, 0.3 M sodium tricitrate)	
NaCl	175.3 g
sodium tricitrate	88.2 g
pH to 7.0 with 10 N NaOH, to 1 L with dH_2O.	

Stock Solutions

1000 X ampicillin	- 1 g of ampicillin in 10 mL of dH_2O, filter sterilized through a 0.22 µm filter and stored at -20°C.
100 mM IPTG	- 0.125 g of IPTG in 5 mL of dH_2O, filter sterilized and stored in 200 µL aliquots at -20°C.
2% Xgal	- 0.2 g of Xgal in 10 mL of dimethyformamide, stored at -20°C in the dark.
DNase-free RNase	- a 1 mg/mL stock solution made in dH_2O, boiled for 10 min and stored in 500 µL aliquots at -20°C.
EtBr	- a 10 mg/mL stock of ethidium bromide made in dH_2O, heated to 68°C for 30 min, and stored in the dark.

phenol - all phenol used was saturated with Tris-HCl (pH 8.0); done by repeated extractions with 1.0 M Tris-HCl (pH 8.0), until the pH of the aqueous phase was >7.6, and stored in 1 mL aliquots at -20°C in the dark.

chloroform - prepared as a mixture of chloroform:isoamyl alcohol in a ratio of 24:1 (v/v).

Bacterial Strains

DH5αF' - F' φ80dlacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r_k-, m_k+) supE44 λ- thi-1 gyrA relA1

JM109 - F' lacI^{q}ZΔM15 Δ(lac-proAB) recA1 endA1 hsdR17 supE44 λ- thi gyrA96 relA1 traD36 proAB

Ethanol Precipitation

Nucleic acid was precipitated from aqueous solutions by adding one-tenth the volume of 3.5 M sodium acetate (pH 5.2), mixing the solution, and then adding 95% ethanol at 2 to 2.5 times this volume. The solution was briefly vortexed, and depending upon the expected concentration of DNA or RNA, was left at room temperature for 30 min or at 4°C overnight. The nucleic acid precipitate was recovered by centrifugation at 16K x g for 15 min at 4°C. The supernatant was decanted and the pellet washed once with 70% ethanol to remove any residual salt. The tube was inverted to allow excess liquid to drain away from the pellet followed by drying of the pellet in a vacuum desiccator. The pellet was resuspended in an appropriate buffer for subsequent use.

Preparation of Plasmid and Phage RF DNA

Covalently closed circular plasmid and phage RF DNA were prepared using a modified alkaline-SDS lysis procedure (Birnboim & Doly, 1979) as outlined by Maniatis et al. (1982); DNA was isolated from large scale, 250 mL (pp. 90), or small scale, 5 mL (pp. 368) cultures grown at 37°C overnight with shaking in 2xYT broth containing the appropriate antibiotic for selection of plasmid-containing cells.

For large scale preparation of DNA, the 250 mL overnight culture was pelleted at 10K x g for 10 min at 4°C and the supernatant discarded. The pellet was washed by resuspending in 40 mL of cold TE (pH 7.5), transferring to a 50-mL nalgene centrifuge tube, followed by centrifugation at 10K x g for 10 min at 4°C to pellet the cells. The supernatant was discarded and the cell pellet was resuspended in 6 mL of ice cold solution I (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA) and incubated at room temperature for 5 min followed by the addition of 12 mL of freshly prepared solution II (0.2 N NaOH, 1 % SDS). The contents were mixed by gently inverting the tube several times, and the tube was place on ice for 10 min. A 7.5 mL volume of ice cold solution III was added (5 M potassium acetate solution, pH 4.8, prepared as follows: to 60 mL of 5 M potassium acetate was added 11.5 mL of glacial acetic acid and 28.5 mL of H_2O) and the contents mixed by inversion of the tube. After a 20 min incubation on ice, the solution was centrifuged at 12.5K x g for 25 min at 4°C, the supernatant poured into a sterile 50-mL nalgene centrifuge tube and recentrifuged at 12.5K x g for 25 min at 4°C. A 22 mL volume of the supernatant was transferred to a sterile 50-mL nalgene centrifuge tube and the nucleic acid was precipitated by the addition of 13.5 mL (0.6 volumes) of isopropanol and incubation at room temperature for 15 min. The precipitate was recovered by centrifugation at 12.5K x g for 15 min at room temperature, the supernatant was

decanted, and the pellet was washed two times with 20 mL of 70 % ethanol. The ethanol was carefully poured off and the pellet was dried in a vacuum desiccator for 10 min. The pellet was dissovled in 3 mL of TE (pH 8.0) and DNase-free RNase A was added to a concentration of 20 µg/mL. The solution was incubated at 42°C for 30 min, followed by a phenol:chloroform extraction to remove proteins. The aqueous phase was transferred to a 15-mL polyethalene centrfuge tube and DNA ethanol-precipitated. To further purify the DNA a PEG-precipitation was done as follows. The DNA pellet was dissolved in 1.6 mL of H_2O and 0.4 mL of 4 M NaCl was added and the solution mixed by vortexing briefly. A volume of 2 mL of 13 % PEG 8000 was added, the solution again mixed by vortexing and the solution incubated on ice for 30 min and then at room temperature for 30 min. The precipitate was collected by centrifugation at 12.5K x g for 15 min at room temperature, the supernatant decanted and the pellet washed with 70 % ethanol. The DNA pellet was dissolved in 500 µL of TE (pH 8.0) and the DNA concentration was determined by measuring the A_{260}. Typically 1 to 3 mg of DNA from a 250 mL culture were recovered using this procedure.

For small scale preparation of DNA, a 1.5 mL sample of a 5 mL overnight culture was transferred to a 1.7-mL microfuge tube and the cells were pelleted in a microfuge at 16K x g for 30 sec at room temperature. The supernatant was removed, an additional 1.5 mL of culture added, and the tubes were again spun for 30 sec. The supernatant was removed and the tube vortexed to loosen the pellet. A volume of 100 µL of ice cold solution I (see large scale DNA procedure) was added to the pellet, the cells resuspended by vortexing and the solution incubated for 5 min at room temperature. A volume of 200 µL of freshly prepared solution II was added, the samples mixed by inversion and the tubes incubated for 5 min on ice. A volume of 150 µL of solution III was then added,

the contents mixed briefly by vortexing, and the tube incubated for 5 min on ice. Following centrifugation in a microfuge at 16K x g for 5 min at 4°C, the supernatants were transferred to a fresh 1.7-mL microfuge tube and recentrifuged, if necessary, to remove any white precipitate carried over with the supernatant. The supernatant was extracted once with phenol:chloroform and the nucleic acid precipitated by the addition of 2 volumes of ethanol at room temperature and incubation for 2 mim. The precipitate was collected by centrifugation at room temperature for 5 min, the pellet washed once with 1 mL of 70 % ethanol and dried in a vacuum desiccator. The dried pellet was resuspended in 50 µL of TE (pH 7.5) containing 20 µg/mL of DNase-free RNase A and incubated at 37°C for 15 min or overnight at 4°.

Nucleic Acid Quantitation

(A) Absorbance: The nucleic acid sample was diluted with TE (pH 7.5) to a final volume of 300 µL. Measurements were done at a 260 nm wavelength light source in a Bausch and Lomb UV spectrophotometer. Samples were added to quartz microcuvettes (300 µL capacity), using TE (pH 7.5) to first zero the spectrophotometer. The nucleic acid concentration was calculated assuming 1 A_{260} = 50 µg/mL of double-stranded nucleic acid and 40 µg/mL of single-strnaded nucleic acid (Maniatis et al., 1982, pp. 468).

(B) Estimation of Ethidium Bromide Fluorescence from Agarose Gels: Relatively small amounts of DNA were quantitated by visually comparing EtBr fluorescence intensity of the nucleic acid sample to that of a standard DNA sample in a horizontal slab agarose gel. Standards of known DNA concentration were prepared by REN digestion of 100 ng of plasmid pES1T3, as estimated by A_{260} measurements, with Sst I and Kpn I to give an ~78 ng 3176 bp fragment and an ~22 ng 900 bp fragment).

Ligations and Transformations

Ligations: All DNA fragments to be cloned were gel purified as described and ligated into the complementary sites present in the polycloning region of the vector using T4 DNA ligase. All ligation reactions were set up as 25 µL ligation mixtures containing 5 µL of 5X ligation buffer (250 mMTris-Cl, pH 7.5, 50 mM $MgCl_2$, 25 % (w/v) PEG 8000, 5 mM ATP, 5 mM DTT; filter sterilized using a 0.22µm filter and stored at -20°C), 250 - 500 ng of DNA/25 µL (typically 200 - 300 ng of M13 vector and 300 - 400 ng of puc vector, plus insert at vector:insert molar ratios of 1:2 or 1:3), and 0.1 - 1.0 units of T4 DNA ligase. The samples were ligated for 12 to 16 hr at 4°C and then 2 to 3 hr at room temperature. The reactions were diluted to 100 µL with H_2O, to decrease the PEG concentration, and 5 to 10 µL was added to 200 to 300 µL of competent cells.

Transformation: The competent cell-DNA mixtures were incubated on ice for 30 min, heat shocked at 42°C for 90 sec and then placed on ice for 2 to 3 min. For plasmid cloning, 800 µL of SOC medium was then added and the cells were incubated at 37°C for 1 hr with shaking. After incubation, 100 and 200 µL samples were spread onto 2xYT agar plates (containing the appropriate antibiotic for selection of plasmid containing cells; 100 µg of ampicillin/mL for Gemini vectors) using a flame-bent sterile pasteur pipette. The plates were allowed to dry at room temperature and then incubated at 37°C overnight. For transformation of phage DNA see section II.3. All ligation and transformation experiments included the following controls: (1) 1 ng of supercoiled or phage RF DNA, (2) linearized vector not ligated and (3) linearized vector self-ligated.

Restriction Endonuclease (REN) Digestions

REN digestions were done in 0.5-mL microfuge tubes in a reaction volume of 10 to 100 µL containing the following reagents: 1/10 the volume of the appropriate 10 X REN buffer (BRL REact™ Buffers were used as supplied or made and used with the appropriate REN according to the BRL REact™ Buffer Selection guide), 0.5 - 2.0 units of REN / µg DNA, and dH_2O to the final reaction volume. The DNA sample was added and the mixture incubated at 37°C for 30 min to 1 hr. Samples were analyzed by gel eletrophoresis.

Isolation of REN Fragments

REN DNA fragments were purified from agarose gels using low melting point (LMP) agarose as follows. The REN DNA was separated on a standard 1% agarose prep gel (using TAE buffer) containing 0.25 µg of ethidium bromide/mL of gel solution. Immediately in front of the DNA fragment to be isolated a small gel slab was cut out to form a trough. This trough was then filled with a melted 1% LMP agarose solution made up in TAE buffer and the solution allowed to solidify. Electrophoresis was carried out at 12 volts/cm until the DNA fragment had migrated into the LMP agarose slab. The DNA fragment was then excised from the gel and placed in a 1.5-mL microfuge tube. The microfuge tube and TE buffered phenol were heated separately in a 68°C water bath for 5 min (or until the LMP agarose was melted) an equal volume of the heated phenol was then added to the DNA sample. The microfuge tube was vortexed vigorously for 15 - 20 sec and the two solvent phases separated by centrifugation in a microfuge at 16K x g for 10 min. The upper aqueous phase was transferred to a fresh microfuge tube and extracted 2 times with an equal volume of ether. When necessary the volume of the DNA sample was reduced by repeated extractions with equal volumes of sec-butanol. The DNA was

ethanol precipitated, collected by centrifugation, dried under vacuum and resuspended in an appropriate volume of TE (pH 8.0).

Agarose Gel Electrophoresis

(A) For DNA. Horizontal, submarine, agarose slab gels were used for the analysis of DNA (analytical gel) and for the purification of DNA fragments (prep gel). Gels were prepared by boiling the required amount of agarose in either TBE (analytical gels) or TAE (prep gels) buffer. The solution was cooled to 55°C, EtBr was added (0.5 µg/mL final) and the solution was poured into a removable plastic tray. A comb was inserted and the gel was allowed to solidify (typically gels were between 3 to 10 mm thick). DNA samples were prepared by adding 1/6 the volume of 6 X DNA sample buffer (60 mM Tris-HCl, pH 8.0, 120 mM EDTA, 15 % ficoll-400, 0.25 % bromophenol blue, 0.25 % xylene cyanol). Electrophoresis was at 6 to 12 volts/cm.

(B) For RNA. Horizontal, submarine, agarose slab gels were used as described above except the gel apparatus was scrubed with a detergent, rinsed with DEPC-treated H_2O, soaked in 3 % hydrogen peroxide for 3 hr, rinsed with DEPC-treated H_2O and used only for RNA analysis. RNA samples were prepared by adding an equal volume of 20 mM CH_3HgOH and incubating for 10 to 15 min, for denaturation of the RNA. The samples, containing 1/6 the volume of 6 X RNA sample buffer (DSB made with DEPC-treated H_2O), were run on an RNase free 1 % TAE agarose gel containing EtBr (all solutions were made with DEPC-treated H_2O).

Agarose gels were photographed by placing the EtBr containing gel on a UV transilluminator and taking a polaroid picture using Polaroid Type 57 (positive) or Type 55 (positive-negative) film and a 23A gelatin filter (orange).

Preparation of Rnase-free Solutions

Containers for Rnase-free work were decontaminated by first cleaning with soap and water, followed by an ethanol rinse. Containers were then soaked in 3% hydrogen peroxide for 10 min at room temperature, followed by rinsing with DEPC-treated water. All glassware was then baked at 200°C for 3 hr prior to use.

All Rnase-free buffers and solutions were made in pure DEPC-treated water. Reagents, containers, and solutions for Rnase-free work were stored separately from general lab supplies.

DEPC dH_2O - 2 to 3 drops (from a pasteur capillary pipet) of DEPC (Sigma) were added to 500 mL of dH_2O. Shaken well, let stand at room temperature for 30 min, and autoclaved.

SDS-PAGE and Autoradiography

SDS-PAGE: Polypeptides produced *in vitro* and assayed as described in section II.9 were analyzed on discontinuous SDS-polyacrylamide slab gels as described by Laemmli and Favre (1973). Standard gels (10 cm x 15 cm x 1.5 mm) were run on a homemade gel apparatus, similar to that described by Studier (1973), at a constant current of 35 mAmp for 2.5 - 3 hr. Also used was the Biorad Mini Protean II apparatus (gels were 5 cm x 8.5 cm x 1.5 mm); gels were run at a constant voltage of 150 V for 1 - 1.5 hr. All reagents were purchased from Bio-Rad (electrophoresis grade), except for boric acid and EDTA (Sigma Chemical Co.) and glycerol (Fisher Scientific Co.).

The stacking gel was composed of 5% acrylamide:N,N'-methylenebisacrylamide (30:0.8), 0.125 M Tris-HCl (pH 6.8) and 0.2% SDS, and was polymerized by the addition of 0.1% TEMED (N, N, N', N'-tetramethylethylenediamine) and 0.05% ammonium persulfate [fresh 10% stock

solutions (w/v) were prepared weekly]. For SDS-10% PAGE, the resolving gel was composed of 10% acrylamide:N,N'-methylenebisacrylamide (30:0.8), 0.375 M Tris-HCl (pH 8.8), 0.2% SDS, and 5% glycerol and was polymerized by the addition of 0.05% TEMED and 0.05% ammonium persulfate. The Tris-glycine electrophoresis buffer (running buffer) contained 25 mM Tris base, 192 mM glycine, and 0.1% SDS, and was prepared as a 10 X stock. Prior to loading, all samples were made 40 mM in Tris-HCl (pH 6.8), 1% in SDS, 10% in glycerol, 2% in β-mercaptoethanol, 0.1% in bromophenol blue by the addition of a 1/5 volume of 5 X Laemlli loading buffer (0.2 M Tris-HCl, pH 6.8, 5% SDS, 50% glycerol, 10% β-mercaptoethanol, 0.5% bromophenol blue).

Fluorography: Performed essentially as described by Laskey and Mills (1975) using PPO (2, 5-diphenyloxazole, Fisher Scientific Co.). After electrophoresis was complete, when the bromophenol blue marker dye had reached the bottom of the gel, the gels were fixed at room temperature for 30 min (or overnight) in 5 - 10 volumes of methanol: acetic acid:dH_2O (15:10:75). The gels were then sequentially soaked, while gently rocking, in 3 - 5 volumes of the following solutions at room temperature: 7% acetic acid for 30 min, 2 times in DMSO (Fisher Scientific Co.) for 30 min each, DMSO-20% PPO for 3 hr, the gel rinsed with water, and finally soaked in dH_2O for 1 hr.

Autoradiography: Radioactively-labelled samples in gels were detected by autoradiography as follows. The gels were placed onto a sheet of 3 MM Whatman filter paper, covered with plastic wrap, and dried at 80°C for 1.5 - 2 hr under vacuum. The plastic wrap was removed and the dried gels subjected to autoradiography using Kodak XAR-5 X-ray film exposed at -70°C.

Radiolabelled Reovirus Marker

A total of 5 x 10^7 L-cells in a suspension culture were pelleted by centrifugation in a Sorval GLC-2B centrifuge at 1500 rpm and an angular velocity of 5 for 10 min. The pellet was washed once with JMEM, resuspended in 30 mL of JMEM and infected with a T3 reovirus inoculum at an MOI of 10 - 100 pfu/cell. The infected cell suspension was gently rocked for 1 hr at room temperature, followed by a 2 hr incubation at 37°C in a 5% CO_2 incubator. A volume of 30 mL of JMEM+5% fetal calf serum (FCS) was added and the cell suspension plated on a 150 cm^2 tissue culture flask. The infected cell monolayers were incubated a further 10 - 12 hr. The medium was then replaced with 5 mL of methionine-free JMEM+5% FCS, containing 0.29 mg of L-glutamine/mL. After a 1 hr incubation period, the medium was replaced with 5 mL of methionine-free JMEM+5% dialyzed FCS, containing Trans [^{35}S]-methionine label at a final activity of 0.02 mCi/mL. The cells were incubated for 2 - 3 hr, washed 2 times with PBS solution and the L-cells harvested by scrapping off the monolayer into 10 mL of PBS solution using a rubber policeman. The cells were pelleted by centrifugation at 2500 rpm, at an angular velocity of 5, for 5 min. The supernatant was decanted and the pellet stored at -70°C until needed.

The pellet was thawed and the cells lysed with 1 mL of solubilization buffer [50 mM Tris-HCl, pH 7.9, 25% sucrose, 1% nonidet P-40 (NP 40), 0.5% sodium desoxycholate (DOC), 2 mM DTT, 5 mM EDTA, 0.1% Aprotinin (protease inhibitor), 1 mM PMSF]. The nuclei were removed by centrifugation at 2500 rpm, at an angular velocity of 5, for 5 min. The supernatant was futher cleared by centrifugation at 16K x g for 5 min. The supernatant was preadsorbed with 500 µL of a 10% fixed *Staphylococcus aureus* cell suspension (IgGsorb) for 15 min at 4°C. The cells were removed by centrifugation at 16K x g for 10 sec and

the supernatant transfered to a fresh tube. Polyclonal anti-reovirus antiserum was added to a final dilution of 1:100 and the mixture incubated on ice for 1 hr. A volume of 500 µL of 10% fixed *Staphylococcus aureus* cell suspension was added and the mixture incubated on ice for 15 min. The cells were pelleted by centrifugation at 16K x g for 10 sec and washed 3 times with 1 mL of 0.5 mL of a sarkosyl wash buffer (0.5% sarkosyl, 50 mM $NaPO_4$, pH 7.4, 0.02% sodium azide). The final pellet was resuspended in 2 mL of 1 X Laemlli loading buffer and the solution was boiled for 5 min. Aliquots of this solution were diluted appropriately and used as a reovirion protein marker on polyacrylamide gels.

Competant Cells

Competant cells were prepared according to the method of Hanahan (1985). An overnight culture of DH5αF' or JM109 was diluted 1 in 100 into 100 mL of SOB medium in a 1L flask. The culture incubated at 37°C, with rapid agitation in a gyrotory shaker (> 200 rpm), until the density of the culture had reached an Abs_{550} of 0.45 - 0.55 (~2 - 3.5 hr). The culture was then added to 2 sterile 50 mL centrifuge tubes and set on ice for 15 min. The cells were pelleted by centrifugation at 1500 x g for 10 min at 4°C, the supernatant discarded, and the pellet resuspended in a 1/3 volume (16.5 mL/tube) of transformation buffer [TFB: 100 mM KCl, 45 mM MnCl-4 H_2O, 10 mM $CaCl_2$, 3mM hexamine-cobalt (III) chloride, 10 mM K-MES (2[N-morpholino]ethone sulfonic acid)] by gentle vortexing. The cells were chilled on ice for 15 min, collected by by centrifugation at 1500 x g for 10 min at 4°C, and resuspended in a 1/12.5 volume (4 mL/tube) of TFB. Dimethysulfoxide (DMSO) was added (140 µL/4 mL of cells), on ice for 5 min, followed by the addition of 2.25 M DTT (140 µL/4 mL of cells) and on ice for 10 min. A final volume of 140 µL of DMSO/4 mL of cells was added to the cells followed by a 5 min incubation on ice. Aliquots of 200 -

300 µL of the competent cells were added to prechilled tubes and kept on ice until use. DNA in volumes of 20 µL or less was added to a tube for DNA transformation (see above).

TFB: A 1 M stock solution of MES is made, the pH adjusted to 6.3 with concentrated KOH (K-MES), the solution sterilized by filtration through a 0.22 µm filter and stored at -20°C. A 10 mM K-MES solution is prepared with pure water and the salts are added as solids. This TFB solution is sterilized by filtration through a 0.22 µm filter and stored at 4°C for up to a year.

Frozen Storage of Bacteria

Bacterial cells were stored on sealed agar plates for up to one month at 4°C. However, for long term storage, up to a year, an isolated colony from an agar plate was innoculated into 5 mL of SOB medium and grown at 37°C, with shaking, for 6 hr, until mid-late log phase was reached. The culture was diluted 1 to 1 in a solution of 60% growth medium, 40% sterile glycerol. Aliquots of 1 mL were added to sterile 1.5-mL sterile microfuge tubes, chilled on ice for 30 min, flash frozen in liquid nitrogen, and immediately placed at -20°C and -70°C. To retrieve cells, a part of the frozen cell suspension was scraped off and added to 5 mL of growth medium and incubated overnight at 37°C with rapid agitaion.