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IDENTIFICATION OF SEQUENCE ELEMENTS REQUIRED FOR REPLICATION AND ASSEMBLY OF REOVIRUS M1 GENE AND EXPRESSION OF THE M1 GENE IN MAMMALIAN CELLS

A Thesis Submitted to the

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ABSTRACT

The M1 genome segment of mammalian reovirus was characterized with respect to replication and expression.

Reoviruses are double-stranded (ds) RNA viruses containing a genome of 10 dsRNA segments. A unique feature of reovirus replication is that each progeny virus contains one, and only one, of each of the 10 segments of dsRNA. However, the genetic elements that control the sorting and assembly of genome segments were not known. The M1 gene encodes the $\mu 2$ protein which is a minor component of the virus core. Genetic studies have associated the M1 gene with cytopathic effect in cultured cells and pathogenesis in infected animals. The genetic basis for this function was not clear either.

Because of the segmented nature of reovirus genome, coinfection of cells with two different serotypes can produce progeny viruses that contain genome segments from both serotypes (reassortants). Type 1 and type 3 reassortants that possess the type 3 L2 segment and type 1 M3 segment generate M1 segment deletion (smaller M1 segments) on serial passage (Brown et al., 1983). Since the smaller M1 segments can still undergo replication and be encapsidated into progeny viruses they must have maintained the genetic elements or signals controlling the replication and encapsidation of the M1 gene. By analyzing reovirus M1 deletion mutants, it was found that both termini of the M1 gene were conserved. The consensus sequences were the 132-135 nucleotides of the M1 5' terminus and the 183-185 nucleotides of the 3' end. The shortest M1 segment

identified was 344 nucleotides long. The results indicate that these two terminal regions contain all the genetic signals sufficient for the replication and assembly of the M1 gene and that 344 nucleotides could be the minimum length required for packaging into virions.

The full-size type 1 M1 and type 3 M1 segments were also cloned and the nucleotide sequence of the type 1 M1 segment was compared with the type 3 M1 segment previously reported. The M1 segments of the two serotypes share a high degree of homology both at the nucleotide sequence level (97.79%) and at the predicted amino acid sequence level (98.64%), indicating that this gene is structurally conserved and is likely to play several critical roles in the virus. Fifty-one nucleotide substitutions and 10 amino acid substitutions were identified between type 1 and type 3 M1 segments. These substitutions must be responsible for the phenotypic differences in cytopathic effect and pathogenesis that map to the M1 gene by genetic analyses.

The M1 gene was further characterized by expression in mammalian cells. Because the antibody induced by purified reovirus reacts very poorly with μ2 protein, μ2-specific antibody was first generated by immunizing rabbits with Trp-E-μ2 fusion protein expressed in *E. coli*. Reovirus μ2 protein was then stably expressed in mouse L cells by transfection with M1 gene-containing dicistronic constructs containing the neomycin resistance gene driven by the mouse phosphoglycerate kinase promoter. The expression of type 1 μ2 protein was usually higher than that of type 3 μ2 protein and the expression pattern varied in the different host cells transfected. These results suggest interactions of M1 gene or μ2 protein with host factors. In addition to these findings, one μ2-

expressing L929 cell line was tested for its ability to support the growth of a reovirus temperature-sensitive (ts) mutant with a defect in its M1 gene. The restricted replication of the ts mutant at nonpermissive temperature was complemented by the μ2-expressing cell line, demonstrating the feasibility of cultivating reoviruses with defective M1 genes.

Mutagenesis of the M1 gene-containing DNA constructs and expression studies also clarified the translation initiation site of the M1 gene. It was found that translation of the M1 gene initiates from the first AUG codon starting at nucleotide 14 and that if there is initiation from AUG_{161} as in 5'-terminus-truncated constructs, a protein about 5 kDa smaller than μ2 protein is produced. Deletion of the 5'-terminal region or the 3' untranslated region of the M1 gene did not improve the expression of μ2 or truncated μ2 protein.

With the information about the M1 gene obtained from this study, preliminary reverse genetics work was initiated in attempts to further characterize the genetic signals within the conserved M1 termini and to develop reovirus as an expression vector. Although rescue of the M1 RNA analogue into reovirus was not achieved, the results suggest that differences between viral M1 RNA and the M1 analogue, such as methylation may be recognized by the packaging machinery of reovirus.

Identification of the consensus M1 termini not only helps our understanding of the sorting and assembly mechanism in reovirus replication but also provides the basis for further reverse genetics and mutagenesis studies. Stable expression of μ2 protein in mammalian cells can be used for further investigation of the function of μ2 protein, for example, its role in virus stability and interactions with the host, as well as for the

reverse genetics approach to complement the growth of defective reoviruses containing a rescued M1 gene analogue.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	adenosine
bp	base pair
C	cytidine
CITE	cap-independent translation initiation element
diam	diameter
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleoside 5'-triphosphate
DTT	dithiothreitol
dsRNA	double-stranded ribonucleic acid
G	guanosine
h (hr)	hour
IPTG	isopropylthio-ß-D-galactoside
ISVP	intermediate subviral particle
kb	kilobase
kDa	kilodalton
M1	the M1 genome segment
MEM	minimum essential medium
min	minute
ml	millilitre
moi	multiplicity of infection

mRNA	messenger ribonucleic acid
MW	molecular weight
μg	microgram
μl	microliter
Neo	neomycin resistant gene
ng	nanogram
nm	nanometre
nt	nucleotide
PAAP	protein A-alkaline phosphatase
PAGE	polyacrymide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCS	polycloning site
PFU	plaque-forming unit
pgk	phosphoglycerate kinase promoter
RIPA	radioimmunoprecipitation
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription followed by PCR amplification
SDS	sodium dodecyl sulfate
ssRNA	single-stranded ribonucleic acid
T	thymidine

T1	reovirus serotype 1
T2	reovirus serotype 2
T3	reovirus serotype 3
ts	temperature-sensitive
U	uridine, unit
UTR	untranslated region
v	volume
w	weight

CHAPTER ONE

GENERAL INTRODUCTION

1.1 The Family Reoviridae

Reovirus is a member of the Orthoreovirus genus, one of the six genera of the family Reoviridae (reviewed by Tylor and Fields, 1990). Three of the genera - Reovirus, Orbivirus, and Rotavirus - infect animals, including humans whereas the remaining three genera - Cypovirus, Phytoreovirus, Fijivirus - infect only plants and insects. The viruses belonging to these genera are classified together because they have similar structural features, nucleic acid type and composition, and replication strategies.

The general characteristics of the Reoviridae are the following. The viral particles are approximately 70 nm in diameter, nearly spherical icosahedrous, nonenveloped, and possess a double or triple protein capsid shell. The viral genome consists of 10-12 segments of double-stranded RNA (dsRNA). Viral replication is fully cytoplasmic. Following penetration into host cells, the virus undergoes partial uncoating to activate transcription where full-length, capped (5'), nonpolyadenylated mRNA transcripts are synthesized. The virions possess all the enzymes required for transcription of dsRNA into mRNA (Joklik, 1983; Tyler and Fields, 1990).

1.2 The Reovirus Genus (Orthoreovirus)

Mammalian reovirus is the prototype of the Orthoreovirus genus (reviewed by Schiff & Fields, 1990). The name reovirus was derived from *r*espiratory, *e*nteric, *o*rphan *virus*.

The viruses in this genus all have a genome of 10 segments of dsRNA (L1, L2, L3, M1, M2, M3, S1, S2, S3, S4). The mammalian reoviruses all share a common complement-fixing antigen though neutralization and hemagglutination-inhibition tests can identify three distinct serotypes. The original ECHO 10 virus (strain "Lang") became the prototype for reovirus type 1. An isolate from a child with diarrhoea (strain "Jones") became the prototype for reovirus type 2. The prototypes for reovirus type 3 were the "Dearing" isolate from a child with a diarrhoeal illness and the "Abney" isolate from a child with an upper respiratory illness. Viruses of the three serotypes are morphologically almost identical but the homologous genes and several proteins from the prototype viruses of the serotypes can be identified by differences in their mobility on polyacrymide gels (Schiff and Fields, 1990).

The avian reoviruses show varying degrees of serologic relatedness to the three mammalian virus serotypes. At least 77 strains of avian reoviruses have been isolated. The avian reoviruses differ from their mammalian counterparts in that most isolates produce cell fusion, lack hemagglutinating activity and are unable to grow in mammalian cell lines. Nelson Bay virus, a reovirus isolated from a flying fox (Petropus poliocephalus), shares the group-specific antigen of mammalian reoviruses and is able to grow in mammalian tissue culture but possesses the cell-fusion activity characteristic of avian reoviruses (Schiff and Fields, 1990).

1.3 The Structure of Reoviruses

Reoviruses have a double capsid shell structure (reviewed by Schiff & Fields, 1990).

Reovirus particles consist of an inner protein shell ("core"), which contains the 10 dsRNA genome segments, surrounded by an outer protein shell ("outer capsid"). The core, which contains the viral transcriptase and replicase activities, is composed of three major proteins (λ1, λ2, σ2 encoded by L3, L2, and S2, respectively) and the minor proteins λ3 and μ2 (Xu et al., 1993). λ2 forms projections (spikes) that extend from the core to the outer surface of the virion. The outer capsid, composed predominantly of complexes of the proteins σ3 and μ1c (encoded by S4 and M2 respectively), forms a nearly spherical triangulated icosahedron, with 12 vertices. The presence of a spike at each of the vertices is a prominent feature of the reovirus core. The viral hemagglutinin, σ1 (encoded by S1), is a relatively minor component of the outer capsid. σ1 is located in close promixity to the λ2 core spikes and can extend from the surface of the virion. Figure 1.1 is a schematic representation of reovirus and Table 1.1 shows the reovirus proteins and their location in the virion.

The three dimensional structure of reovirus has been obtained by cryo-electron microscopy and image processing (Metcalf et al., 1991). It was proposed that the outer shell of reovirus is organized into 200 triangles each containing three μ1c and three σ3 molecules and that there are 12 penton craters each containing five σ1 molecules. Further analysis (Dryden et al., 1993) indicated that the intact virion (~850-Å diam) is designed for environmental stability in which the dsRNA genome is protected not only by tight σ3-μ1c, λ2-σ3, and λ2-μ1c interactions in the outer capsid but also by a densely packed core shell formed primarily by λ1 and σ2. The segmented genome appears to be packed in a liquid crystalline fashion at radii <240 Å (Dryden et al., 1993).

Fig. 1.1 Schematic representation of reovirus. The length of the 10 genome segments of dsRNA (L1 to S4) is not shown strictly in scale.

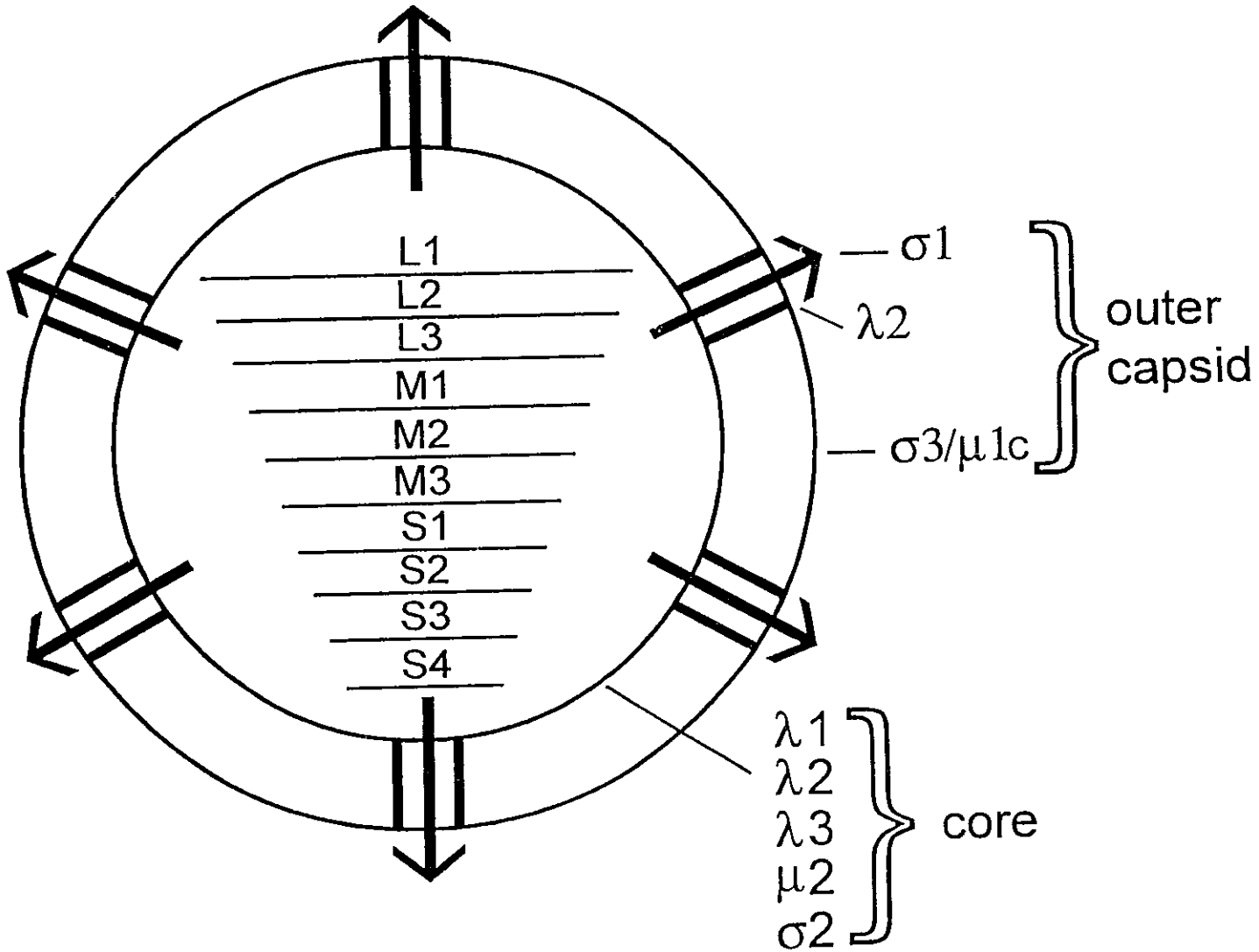

L1
L2
L3
M1
M2
M3
S1
S2
S3
S4
σ1
λ2
σ3/μ1c
outer capsid
λ1
λ2
λ3
μ2
σ2
core

Table 1.1 Reovirus proteins and their location in the virion*

Species	Genome Segment	Location
λ1	L3	Core
λ2	L2	Core spike
λ3	L1	Core
μ1/μ1c	M2	Outer capsid
μ2	M1	Core
σ1	S1	Outer capsid
σ2	S2	Core
σ3	S4	Outer capsid
μNs	M3	Nonstructural
σNs	S3	Nonstructural
σ1s	S1	Nonstructural

* extracted from Schiff and Fields, 1990

1.4 The Genome of Reoviruses

Studies of Gomatos and collaborators in the early 1960s that indicated that the RNA of reovirus particles was double-stranded created a great deal of interest, since they provided the first demonstration of the occurrence of dsRNA in nature (Joklik, 1983). It was then shown that reovirus genomic RNA comprises three size classes of molecules, large (L), medium (M), and small (S), and that these molecules are discrete segments rather than random fragments of larger molecules. These three size classes were further separated by polyacrymide gel electrophoresis into ten unique molecular species that are present in equimolar amounts and possess an aggregate molecular weight of about $15x10^6$. Examination of the genome segment patterns of reovirus isolates from human, cattle, and mice or from widely different geographic origins showed extensive

electrophoretic variability.

Because of the segmented nature of reovirus, coinfection of cells with two different serotypes can produce progeny viruses that contain genome segments from both serotypes (reassortment). This variation was found in all three serotypes and involved all ten genome segments. The homologous RNA segments from the 3 prototype reovirus serotypes differ slightly in their mobility on polyacrymide gel and thus the electrophoretic migration rate as a genetic marker has been exploited in a variety of genetic and biological studies (Schiff and Fields 1990). The analysis of reassortant viruses made between reoviruses with distinct phenotypes has allowed the determination of the genetic basis for many biological properties of reoviruses. Variation in electrophoretic migration rate of RNA and some proteins has facilitated the coding assignment of genes in reassortant reoviruses.

Recently, all of the genes of reovirus type 3 Dearing were cloned into the plasmid pBR322 and the complete sequences have been determined (Cashdollar et al., 1984; Cashdollar et al., 1985; Wiener and Joklik, 1987; Wiener et al., 1989a; 1989b; Wiener and Joklik, 1989).

A single protein product has been identified for each of the reovirus gene segments with the exception of the S1 gene segment. The S1 mRNA is functionally dicistronic, and it encodes two proteins in separate reading frames (Ernst and Shatkin, 1985; Jacobs and Samuel, 1985; Jacobs et al., 1985; Sarkar et al., 1985; reviewed by Both, 1988; Schiff and Fields, 1990).

1.5 Proteins of Reovirus

Early studies of the protein composition of reovirus particles identified three size classes of proteins. The large (L) dsRNA segments encode three large (λ) proteins that are components of the viral core. The three medium-sized (M) segments encode two structural proteins (μ1, μ2) and one nonstructural protein (μNs). The four small (S) dsRNA segments encode three structural (σ1, σ2, σ3) and two nonstructural (σNs, σ1s) proteins (Schiff & Fields, 1990). The nature of the polypeptide encoded by each of the 10 dsRNA segments were identified by analysis of the in vitro translation products of purified individual reovirus segments (McCrae and Joklik, 1978) and by analysis of reassortants (Mustoe et al., 1978).

The λ1 protein is a major component of the reovirus core. The predicted amino acid sequence contains a nucleotide-binding site and a potential metal-binding site, suggesting a role in transcription (Bartlett and Joklik, 1988). It has also been shown to bind reovirus genomic dsRNA in a blotting assay (Schiff et al., 1988).

The λ2 is also a major component of the core with five molecules of λ2 protein associate to form the spikes that are located at the 12 vertices of the icosahedral viral core. Sequence of λ2 revealed two consensus elements for GTP-binding sites and showed a region of similarity to the catalytic subunit of the vaccinia virus mRNA capping enzyme (Seliger et al., 1987). Biochemical studies have shown that λ2 is the reovirus guanylyltransferase and thus is the reovirus capping enzyme but it provides neither the required 5'-ppG-terminated substrate nor does it methylate the cap structure (Mao and Joklik, 1991). It is proposed that those functions may be performed by λ2 pentamers or

other individual or complexed components of reovirus cores. A computer-assisted analysis identified a putative methyltransferase domain in λ2 protein (Koonin, 1993).

The λ3 encoded by L1 was suggested to be a component of the viral transcriptase as a result of genetic studies (Schiff and Fields, 1990). It was recently shown to have poly C dependent poly G polymerase activity (Starnes and Joklik, 1993).

The primary translation product of the M2 gene segment is the μ1 protein with a relative molecular weight (MW) of 80 kDa. However, in the virion outer capsid, the majority of μ1 exists in the form of a 72-kDa cleavage product μ1C, that appears to be generated during virion assembly. M2 gene is associated with chromium release from mouse L cells and it is suggested that the M2 gene product (in its cleaved form) plays a role in interacting with cell membranes (Lucia-Jandris et al., 1993). A carboxy-terminal cleavage fragment of protein μ1/μ1C is also described as present in infectious subviral particles of mammalian reoviruses and has been proposed to have a role in penetration of virus into host cells (Nibert and Fields, 1992).

The μ2 encoded by M1 is a minor component of the viral core, with only about 12 copies per viral particle (Smith et al., 1969; Schiff and Fields, 1990). Analysis of this protein has been lacking presumably because of the low amounts in the virus. Genetic studies have associated μ2 with cytopathic effect in cell culture (Moody and Joklik, 1989), pathogenesis (Sherry and Fields, 1989; Sherry and Blum, 1994), and virulence in the liver of infected animals (Haller et al., 1995).

The μNs protein encoded by the M3 gene exists in infected cells in the form of (a) the 75-kDa primary translation product and (b) a related protein (μNSc) that is 5 kDa

smaller (Schiff and Fields, 1990). μNs has been found to be associated with the cytoskeleton of infected cells (Mora et al., 1987), suggesting that it might serve to anchor the viral structures involved in genome synthesis and assembly to the cell matrix.

The σ1 is the cell attachment protein of reovirus and has hemagglutinating activity (Nibert et al., 1990; Dermody et al., 1990; Duncan et al., 1990; Turner et al., 1992). Other functions of σ1 include tissue tropism and specific injury, pathways of spread and growth in intestine. σ1 is also the antigen for neutralizing antibody induction. Translation from the second open reading frame (ORF) in the S1 gene produces a nonstructural protein σ1s (also called p14 or σ1NS)(Cashdollar et al., 1989; Fajardo and Shatkin, 1990; Doohan and Samuel, 1992; Doohan and Samuel, 1993). A region of the S1 mRNA immediately downstream of the σ1 1st ORF initiation codon AUG_{14} but well upstream of the σ1s 2nd ORF initiation codon AUG_{75} is indicated to play a major role in determining the relative efficiency of synthesis of σ1 and σ1s from the reovirus bicistronic S1 mRNA (Bell and Samuel, 1993).

σ2 is a major protein in the viral core. It has also been shown to bind reovirus genomic dsRNA in a blotting assay (Dermody et al., 1991). Recently, a study of revertants of the assembly-defective mutant tsC447 identified a region of σ2 which may be important in mediating assembly of the core particle (Coombs et al., 1994).

The σ3 protein forms complexes with μ1c and together they form the bulk of the reovirus outer capsid. It was shown that σ3 and N-terminal myristoylation of μ1 are required for site-specific cleavage of μ1 to μ1C in transfected cells (Tillotson and Shatkin, 1992). Early biochemical studies indicated that σ3 has affinity for dsRNA

(Huismans and Joklik, 1976). A well-documented function of σ3 is translational stimulation (Giantini and Shatkin, 1989; Seliger et al., 1992; Lloyd and Shatkin, 1992; Martin and NcCrae, 1993; Beattle et al., 1995). By binding and sequestering dsRNA through its dsRNA binding ability, σ3 can inhibit the interferon-induced, dsRNA-dependent protein kinase, PKR, and thus prevent phosphorylation of translation initiation factor eIF2 α subunit (Lloyd and Shatkin, 1992).

The σNs encoded by S3 segment has been shown to possess strong affinity for ssRNA. The S3 genome segments of three serotypes have been sequenced (Wiener and Joklik, 1987). σNs is implicated with genome assortment in viral replication (Antczak and Joklik, 1992).

1.6 The Reovirus Replication Cycle

Reovirus can infect a variety of cell lines derived from different species and different tissue types (Zarbl & Millward 1983). As a result of this extensive host range, several aspects of the reovirus replication cycle have been studied at one time or another in different mammalian cells. However, most of the recent studies concerning the molecular biology of the reovirus replication cycle have been carried out on mouse L929 fibroblasts (Schiff & Fields, 1990).

Reovirus adsorbs efficiently to L cells both in monolayer and in suspension culture, at temperatures between 4°C and 37°C, with 60-80% of the viral inoculum being cell-associated by 1 hr. Adsorption occurs via specific receptors on the cell surface that bind to the cell attachment protein, σ1, of the virus. It is suggested that the erythrocyte

surface receptor for reovirus is glycophorin A (Paul and Lee, 1987) and reovirus type 3 binds to antagonist domains of the ß-adrenergic receptor (Donta and Shanley, 1990). It was reported that the α-anomeric form of sialic acid is the minimal receptor determinant recognized by reovirus (Paul et al., 1989). The nature of the receptor remains a controversial issue. Recently it was shown that reovirus can recognize the epidermal growth factor receptor (Tang et al., 1993). Multiple receptors on different cells may be used by reoviruses. It was shown that reovirus is capable of binding to at least 30 membrane proteins (Choi, 1994).

Virions bound to cell surface receptors are taken into the cell probably by receptor-mediated endocytosis. Uncoating of reovirus particles within lysosomes begins 20-30 min after penetration and is complete within 2-3 hours. Uncoating involves proteolytic digestion of the outer capsid and generates intermediate subviral particles (ISVP) (Borsa et al., 1981; Drayna and Fields, 1982). Cleavage of μ1c during uncoating results in the generation of a fragment referred to as δ. Studies with reovirus type 3 have also shown that the ISVP derived from digestion of virion particles by chymotrypsin can directly penetrate L cells, without appearing to enter coated vesicles or lysosomes typical of the endocytotic pathway. However, others were unable to detect direct penetration of L cells by reovirus serotype 1 ISVPs (Sturzenbecker et al., 1987). Instead, it was found that in the intestines of infected neonatal mice intraluminal proteolytic digestion plays an important role in replication of type 1 reovirus (Bodkin et al., 1989) and it was suggested that digestion of type 1 virus to ISVPs enhances interaction between virus and the cellular receptors (Bass et al., 1990). The infectious subviral particles were reported to induce

the formation of anion-selective, multisized channels in planar lipid bilayers (Tosteson et al., 1993). Protective antibodies act by inhibiting internalization of virus-antibody complexes bound to the cell surface and inhibiting intracellular proteolytic uncoating of the virion (Virgin IV et al., 1994).

After penetration and uncoating, transcription of viral mRNAs is mediated by core particles generated from the ISVP. The transition from ISVP to core involves release of the σ1 proteins and the remaining outer capsid layer formed by 200 trimers of μ1c subunits. In the ISVP-to-core transition, domains of λ2 subunits undergo conformational change to form a central channel which allows the potential diffusion of substrates for transcription and exit of newly synthesized mRNA segments (Dryden et al., 1993).

The transcription reaction is fully conservative, with both strands of the parental dsRNA remaining within the uncoated core particle and neither strand appearing among the transcription products. The minus strand from each of the 10 parental dsRNA segments is the only strand transcribed. Transcription is end to end, producing full-length complete copies of each genome segment. The enzymes involved in capping the 5'-terminal end of mRNA transcripts are activated at the same time as the transcriptase.

The capped transcripts then serve as mRNAs for protein synthesis. The viral proteins and the capped transcripts are assembled together to form immature progeny viral particles. Replication occurs within these nascent progeny particles via synthesis of a minus strand of RNA using the previously synthesized capped plus strand as template. Synthesis of the minus strand proceeds in the 5' to 3' direction from a single initiation point. Once the complementary minus strand is synthesized, it remains

associated with the plus strand (Sakuma and Watanabe, 1972).

Monoclonal antibodies directed against reovirus proteins were used to study the reovirus genome assortment into progeny genomes (Antczak and Joklik, 1992). It was found that (1) μNS, σNS and σ3 rapidly become associated with mRNA molecules to form ssRNA-containing complexes (ssRCCs) and each ssRCC contains one RNA molecule and 10-30 protein molecules; (2) dsRNA-containing RCCs have not only μNS, σNS and σ3 but also λ2; (3) the relative proportions of the 10 genome segments in dsRCCs are equimolar. The results suggest that genome segment assortment into progeny genomes is linked to the transcription of plus strands into minus strands.

Once dsRNA synthesis is complete, these particles appear to undergo some structural rearrangement, become RNase-resistant, and begin to actively synthesize the mRNAs that encode viral polypeptides. The structural polypeptides probably self-assemble to form the viral core and outer capsid. Finally, cell lysis results in the release of mature virions.

1.7 Genetics of Reovirus

The genetics of reovirus have been comprehensively reviewed (Ramig and Fields, 1983; Schiff and Fields, 1990). The early genetic analyses were directed toward the isolation and physiological and genetic characterization of a collection of genetic markers such as temperature-sensitive (ts) conditional lethal mutants (Fields and Joklik, 1969; Ito and Joklik, 1972; Fields et al., 1972). More recent studies have emphasized the use of

recombinant viruses generated during mixed infection of tissue-culture cells with two different reovirus serotypes and the cloning and sequence analysis of the genome segments.

The ts mutants of reovirus were isolated following chemical mutagenesis, serial high moi passage of wild-type virus and from "pseudorevertants" obtained from persistently infected L cells. Ts mutants have been mapped to discrete dsRNA gene segments based on SDS-PAGE analysis of the migration of gene segments of ts+ reassortants derived from crosses between ts mutants and wild-type reovirus. The ts mutant gene groups tsA, tsB, tsC, tsD, tsE, tsF, tsG, tsH, tsI, or tsJ refer to ts mutations mapped to segments M2, L2, S2, L1, S3, M3, S4, M1, L3, or S1, respectively (Schiff and Fields, 1990). Specific genomic mutations have been identified for group C ts mutant tsC447 by sequence analysis (Wiener et al., 1989b). Study of revertants identified one substitution in tsC447 responsible for the ts defect in its S2 gene (Coombs et al., 1994).

Deletion mutants have been generated by serial passage of reovirus at high moi. These mutants exhibit a variety of phenotypes including defective interference and the ability to establish persistent infection. L1 is the gene segment most frequently deleted but deletions involving the L3 and M1 genes are also relatively common. Type 1 strain Lang and type 3 strain Dearing reassortants that possess the type 3 L2 segment and type 1 M3 segment generate M1 segment deletion on serial passage (Brown et al., 1983).

1.8 The M1 Genome Segment and μ2 Protein

The M1 genome segment encodes protein μ2. It appears to be an inner core

component, but its precise location is not known, primarily because reovirus particles contain so few molecules of μ2, probably 12 copies per virion (Schiff and Fields, 1990).

The M1 genome segment is 2304 nucleotides long containing a single large open reading frame from nucleotide 14 to 2224 with two in-frame initiation codons starting at nucleotides 14 and 161 (Wiener et al., 1989a). It was reported that in reovirus-infected cells AUG_{161} is the initiation codon used for translation of the M1 gene (Roner et al., 1993). M1 messenger RNA is translated very inefficiently in infected cells, only about 1% of the translation efficiency of the most efficiently translated S4 gene (Gaillard & Joklik, 1985). However, the M1 mRNA is translated very efficiently *in vitro* (Roner et al., 1989).

In studies using genetic reassortants, the μ2 protein controls the extent of cytopathic effects and plaque size in L929 cells (Moody & Joklik, 1989), myocarditis in the mouse (Sherry & Fields, 1989; Sherry and Blum, 1994) as well as heart cell replication *in vitro* (Matoba et al., 1991) and bovine aortic endothelial cells (Matoba et al., 1993).

The M1 gene segment and μ2 protein are poorly characterized for reovirus presumably because of the low amount of μ2 protein in virus particles, poor reactivity of μ2 with the anti-reovirus antibody and consequently the difficulty in the detection of μ2 protein.

1.9 Objectives and General Approach of This Study

One characteristic of reovirus replication is that each progeny virion contains one, but only one, of each of the 10 dsRNA genome segments. The signals that control the

replication of each genome segment and the mechanism that ensures that 10 unique RNA segments are assembled together in each nascent viral particle were not known when this study was initiated. Identification of the signals required for replication and assembly would not only help to understand the mechanism of viral replication but also pave the way for development of reovirus as an expression vector. The reovirus vector would contain the identified signals and the foreign gene to be expressed. The signals would assure the replication and assembly of the foreign gene and the expression product of the foreign gene would be useful for variety of purposes. One application of the reoviral vector would be for vaccine development with the gene encoding the immunogen inserted into the reoviral vector. Because reovirus is not a human pathogen the vaccine would be very safe for recipients. Secondly, reovirus can infect a broad range of cell types, which would be of unique significance. Thirdly, reovirus vaccines could be used orally which would stimulate mucosal immunity as well as systemic immune responses. The segmented nature of this virus and the potential of reassortment would confer versatility to the reovirus vaccine. Another application would be expression of proteins which could then be used for vaccination or for research.

The M1 genome segment is implicated in pathogenesis, suggesting its potential importance in the interaction of reovirus with host cells. However, this is the gene we know the least about. In particular, why is the M1 gene expressed so poorly in infected cells as early studies reported and what is the possible relation between the poor expression and its role in pathogenesis?

1.9.1 Objectives

This study was aimed at:

I. Characterization of signals in reovirus M1 genome segment required for its replication and assembly. This information would also help to pinpoint the signals in other genome segments.

II. Characterization of elements of reovirus M1 genome segment that control its expression in host cells.

1.9.2 General Approach

The general approach involved

a. analysis of M1 deletion mutants to identify conserved consensus sequences.

b. cloning and sequencing of the full length M1 genome segment of reovirus.

c. development of a helper virus-free system to support the growth of reoviruses containing an M1 gene defective in encoding functional viral $\mu 2$ protein.

d. generation of a system for introduction of a foreign gene attached to the identified consensus M1 sequence elements into reoviruses.

CHAPTER TWO

GENERAL MATERIAL AND METHODS

2.1 Viruses and Cells

Reovirus T1 Lang and T3 Dearing, and T2 Jones were obtained from Dr. Bernard N. Fields. T1xT3 reassortants and their high passage stocks were produced previously by Dr. Earl G. Brown in Dr. Fields' laboratory for the genetic analysis of the phenomenon of defective interference (Brown et al., 1983).

Reoviruses were cultivated in mouse fibroblast L929 cells grown in monolayer culture at 37°C with 3.5 % CO_2 or in suspension at 34°C in Joklik-modified MEM (minimum essential medium) supplemented with fetal bovine serum to 5%.

Viruses grown in monolayer cells were released by freeze-and-thaw three times. Viruses grown in L929 suspension culture were harvested by centrifugation and purified by freon extraction at 4°C. The freon extraction began with suspension of the infected cell pellet in HO buffer (10 mM Tris, 250 mM NaCl, 10 mM 2-mercaptoethanol) followed by the addition of sodium desoxycholate to 0.5% and 1/2 volume of freon, sonication to mix the phases and then centrifugation at 9k rpm for 10 min in a Beckman JA20 rotor to separate the aqueous phase from the organic phase. The aqueous phase was reextracted with freon and the resulting aqueous phase containing the virus was further purified by CsCl gradient (1.2-1.4 g/ml) centrifugation. The purified virus was dialysed overnight against dialysis buffer (0.15 M NaCl, 0.01 M $MgCl_2$, 0.01 M Tris pH 7.4) and quantitated by spectrophotometry at 260nm. 1 $OD_{260} \approx 184.5 \mu g$ of reovirus

and 1 mg of reovirus $\approx 1.13 \times 10^{13}$ reovirus particles (Smith et al., 1969). Purified virus was kept at 4°C or at -70°C for long term storage.

Plaque Assay Viruses were titrated by plaque assay in L929 cells in 6-well plates (Costar). 1.2×10^6 cells were seeded in each well and allowed to sit overnight at 37°C in a CO_2 incubator. For plaque assay, medium was poured off and cell sheets washed once with PBS (phosphate buffered saline) followed by inoculation with virus diluted in PBS containing 0.2% gelatin. Plates were incubated at 37°C or 4°C for 1 hour with agitation every 15 minutes followed by overlay with 1x199 medium containing 1% agar, 5% or 2.5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and mycostatin at 100 U/ml. For T1 Lang and T3 Dearing, plates were overlaid once more with the same medium at day 3 p.i. (postinfection) and with serum-free 1x199 containing 0.015% neutral red at day 6 p.i. For T2 Jones, a second overlay at day 6 p.i. was followed by neutral red overlay at day 9 p.i. Plaques were counted the day after neutral red overlay.

To keep a lasting record of the plaque assay, Plates were fixed with Carnoy's fixative (3 volume of 100% methanol plus 1 volume of acetic acid) followed by flushing with tap water.

2.2 Cloning of dsRNA Genome Segments

Viral dsRNA was isolated from purified virus by treatment with 1% SDS and 10μg/ml proteinase K at 37°C for 30 min, followed by phenol/chloroform extraction and precipitation with sodium acetate and ethanol. The dsRNA was dissolved in TE (10 mM

Tris-HCl, pH 7.4, 1 mM EDTA).

Cytoplasmic extracts of infected monolayer cells were also used as a source of dsRNA for cloning and amplification by reverse transcription and polymerase chain reaction (PCR). The cytoplasmic fraction of infected cells was extracted when CPE was detectable by scraping cells from the flask, centrifugation, lysis of cell pellets in a buffer containing 0.5% NP40, 159 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$, and by removal of cell debris and precipitation with 0.1 vol of 5M NaCl and 3 vol of 100% ethanol. The extracts were dissolved in 100 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1% SDS, and 100 μg/ml proteinase K, followed by incubation at 37^{0}C for 30 min before phenol/chloroform extraction and ethanol precipitation with 0.1 vol of 2.5 M sodium acetate, pH 5.4, and 2.5 vol of ethanol (Sambrook et al., 1989, pp.E.10-11).

The cloning of reovirus dsRNA was carried out by a modification of methods of Cashdollar et al. (1982) and Schmid et al. (1987). The viral dsRNA was melted at 50^{0}C in 90% DMSO plus 10% 10 mM Tris-HCl, pH 7.4, for 45 min prior to reverse transcription. The samples were added to a reverse transcription mix containing 100 mM Tris-HCl, pH8.3, 130 mM KCl, 10 mM $MgCl_2$, 25 mM dithiothreitol, 1 mM each dNTP, 100 ng of each of the two primers complementary to either strand (M1 5'+ and M1 5'-, see appendix two), and AMV reverse transcriptase, and incubated at 42^{0}C for 1 hr. For cloning without PCR amplification, the RNA/cDNA (complementary DNA) hybrids were treated with NaOH, 75 mM, at 60^{0}C for 1 hr, neutralized, precipitated and dissolved in annealing solution (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 50% formamide) to allow dsDNA to form. After purification through a Sephacryl 400 spun

column and precipitation, the dsDNA underwent end-repair in a 8-μl reaction mix containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.1 mM of each of the 4 dNTP, and 1.65 units of DNA polymerase I Klenow fragment, for 30 min at 21^{0}C. The reaction was stopped by heating at 70^{0}C for 5 min followed by phosphorylation of 5' termini by supplementing the reaction with 5% polyethylene glycol 8000, 1 mM ATP, 1 mM DTT, 0.2 mg/ml of BSA, and 2 units of T4 polynucleotide kinase; reaction was for 30 min at 37^{0}C before heating at 70^{0}C for 5 min. The dsDNA was blunt-end ligated into the Sma I site of dephosphorylated pGEM-7Zf(+) by adding appropriate amount of vector and T4 DNA ligase and incubating at 21^{0}C for 4 hr. Escherichia coli DH5α were transformed by electroporation with ligation mix and screened by α complementation. The RNA/cDNA hybrid could be directly amplified by PCR before cloning into a vector such as pGEM7Zf(+).

2.3 Polymerase Chain Reaction (PCR)

DNA template or cDNA/RNA hybrid products derived from reverse transcription could be amplified by PCR using automated thermal cyclers. Each 100-μl PCR reaction contained 1 ng of DNA template or 1 μl of reaction product from reverse transcription, 100 ng each of the two primers pointing in opposite directions (M1 5'+ and M1 5'-, see appendix two), 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 mM of each dNTP, and 2 units of Taq DNA polymerase. For some reactions, 0.1% NP40 was included. Between 25-40 cycles were used for different

reactions (94°C 30 sec; 48°C 30 sec; 72°C 1 min). For amplification of longer fragments (up to 8 kb), the time for denaturation was extended to as much as 1 min and the time for elongation up to 6 min. For amplification of short fragment (100-400 bp), the time for elongation was as short as 15 sec. The temperature for annealing varied from 48°C to 55°C, depending on the length of primer sequence used and on whether mutations were introduced into the primers. When necessary, annealing temperatures as low as 35°C were used for the first two cycles. Occasionally, the first one or two cycles were carried out manually using the Klenow fragment instead of Taq DNA polymerase. In this case, the reaction mix without polymerase was heated to 94°C for 5 min followed by incubation at 37°C for 10 min and then at 22°C for 5 min. The elongation was initiated by addition of Klenow fragment and the reaction was for 30 min. Taq DNA polymerase was then added to the reaction mix with the remaining cycles performed using an automated thermal cycler.

Reaction products (dsDNA) were checked by agarose gel electrophoresis and then extracted with chloroform, phenol/chloroform and precipitated with ammonium acetate, ethanol, and glycogen (for shorter fragments). For cloning, the dsDNA was purified by spun column chromatography using Sephacryl 400 (for fragments >400 bp) or Sephadex G-50, before it was precipitated, and dissolved in water.

To improve the specificity of amplification, nested PCR was used to amplify low amounts of template. The PCR reaction was first carried out with two primers at standard or low annealing temperature and then the reaction product was amplified again at higher annealing temperature with a second pair of primers complementary to

sequences internal to the first pair of primers.

2.4 Transformation of E. coli and Screening of Transformants

2.4.1 Transformation by electroporation

To prepare cells for transformation, a fresh overnight culture of E. coli (10 ml) was diluted into 1 litre of L-broth (1% Bacto tryptone, 0.5% Bact yeast extract, 0.5% NaCl) and incubated at 37^{0}C with vigorous shaking to an A_{600} of 0.5-1.0 (2-3 hrs). Cells were transferred to centrifuge bottles and chilled on ice for 15 to 30 min before pelleting in a cold rotor at 4000xg for 15 min. The medium was then removed and the pellet was resuspended in a total of 1 litre of cold water and centrifuged as above. The pellet was resuspended in 0.5 litre of cold water and centrifuged again. The pellet was resuspended in 10-20 ml of cold 10% glycerol, centrifuged, resuspended to a final volume of 2-3 ml in cold 10% glycerol and frozen in aliquots on dry ice. For transformation, cells were thawed on ice, 20 μl of cell suspension was mixed with 1 μl of diluted ligation mix (1:4 with 0.5xTE buffer or water). The DNA-cell mix was then suspended between the bosses of a BRL microelectroporation chamber and pulsed at 2000 volts. Cells were diluted with 2 ml of SOC (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and allowed to recover at 37^{0}C for 1 hr followed by plating on LB plates (L-Broth plus 1.5% Bacto agar) containing ampicillin.

2.4.2 Transformation by $CaCl_2$ method

To prepare cells for $CaCl_2$ transformation, E. coli cells were grown overnight in 10 ml 2YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) at 37°C without shaking followed by dilution into 100 ml of P medium (15.9 mM K_2HPO_4, 6.3mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 1.8 μM $FeSO_4$, 1% casamino acids, 0.25% glucose) and grown at 37°C with shaking until the A_{600}=0.3-0.4. Cells were chilled on ice, pelleted, washed with 100 ml ice cold 10 mM NaCl, pelleted again, resuspended in 50 ml of 50 mM $CaCl_2$, placed on ice for 15 min, centrifuged and then resuspended in 10 ml of 50 mM $CaCl_2$ containing 16% glycerol. Aliquots were frozen in a ethanol/dry ice bath and stored at -70°C. For transformation, 50 μl of competent cells were mixed with 1-2 μl of diluted ligation mix (1:4 in 1xTE), incubated on ice for 30 min, heat shocked at 42°C for 45 seconds, chilled on ice for 2 min, diluted with 400 μl of SOC prewarmed at room temperature, shaken at 37°C for 1 hr, and plated on L-Broth plates containing ampicillin.

2.4.3 Screening for transformants

Transformants were screened by either colour selection (α-complementation), hybridization, cracking gel electrophoresis or restriction digestion of plasmid DNA preparations.

For α-complementation, 20 μl of Bluo-gal or X-gal (50 mg/ml in dimethylformamide), 100 μl of IPTG (isopropylthio-ß-D-galactoside, 100 mM) and 25 μl of ampicillin (100 mg/ml) were spread over LB agar plates before the plates were inoculated with the transformed bacteria. Colonies that contained active ß-galactosidase

were blue whereas those transformed by a plasmid into which the gene had been inserted were white since the reading frame for the amino-terminal fragment of ß-galactosidase was interrupted.

The white colonies were selected for screening or all the ampicillin-resistant colonies from the transformation could be screened by hybridization using ^{32}P-labelled oligonucleotide or dsDNA as probe. White colonies were first transferred to a master plate and a duplicate nylon membrane and grown overnight. For screening of all colonies, they were transferred to a nylon membrane by lifting. The membranes (colonies side up) were then transferred to 3 MM paper saturated with 10% SDS for 3 min, then to paper saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 min, then to paper saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 min, followed by transfer to paper saturated with 2xSSC (0.3 M NaCl, 30 mM Sodium Citrate, pH7.0) for 5 min followed by washing in 2xSSC for a few seconds. The nylon membranes were dried at room temperature or in a vaccum oven, prehybridized at 65^{0}C for a minimum of 30 min in modified Denhardt's reagent, namely, 1 vol of 20xSSC (3 M NaCl, 0.3 M Sodium Citrate, pH7.0) and 3 vol of 50x Denhardt's reagent (1%(w/v) bovine serum albumin, 1%(w/v) Ficoll, 1%(w/v) polyvinylpyrrolidone (pvp), 1%(w/v) SDS), and then hybridized in fresh buffer (1 vol of 20xSSC, 3 vol of 50X Denhardt's reagent) containing radioactive probe at 42^{0}C-68^{0}C for 2 hrs to overnight. Oligonucleotide probes were labelled in a kinase reaction containing gamma-^{32}P ATP and polynucleotide kinase (Maniatis et al., 1982, pp.125) followed by purification with G-50 spun column chromatography (Maniatis et al., 1982, pp.466).

Purified oligo probe was directly added to the hybridization buffer. dsDNA was labelled by random priming or nick translation. dsDNA probes were boiled for 5 min and then chilled on ice before being added to the hybridization solution. The hybridized membranes were washed in SSC-SDS buffer and exposed to X-ray film (Maniatis et al., 1982, pp.326-328).

Although screening by hybridization is the first choice when the ratio of positive colonies is very low, cracking gel screening can be a simple, fast, yet efficient method when the number of colonies is small and/or the ratio of positive colonies is high. Each colony was transferred with a toothpick to a master plate and the toothpick was then washed in a freshly made buffer containing 0.2 M NaOH, 0.5% SDS and 20%(w/v) sucrose. The samples were incubated at 70°C for 5 min, cooled at RT, followed by addition of 1.5 μl of 4 M KCl and 0.5 μl of 10x DNA sample buffer, incubation on ice for 5 min and centrifugation in a microfuge at 4°C for 3 min. 25 μl of the supernatant was run on 0.8% agarose gel together with control plasmid and molecular weight marker. 3 to 5 colonies could be pooled together in the first round of screening of large numbers of colonies.

Restriction digestion is usually an essential step in screening of transformants. By choosing different restriction enzymes, plasmids with cloned genes as well as the orientation of the cloned genes were identified.

Occasionally, PCR was used for screening of transformants, especially when there was no appropriate restriction enzyme site available.

2.5 Preparation of Plasmid DNA

2.5.1 Small-scale preparation (miniprep)

Two methods were used for plasmid minipreps, the alkaline method developed by Dr. Birnboim (Birnboim and Doly, 1979) and the boiling method (Holmes and Quigley, 1981). In the alkaline method, the bacterial pellet of a 1.5 ml overnight culture was resuspended in 200 μl solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) plus 4 mg/ml of lysozyme, incubated at RT for 5 min followed by addition of 400 μl of 0.2 M NaOH, 1% SDS and incubation at RT for 5 min. 300 μl of 7.5 M NH_4Ac (ammonium acetate) was then added and the sample was incubated on ice for 10 min before being centrifuged at 10,000 rpm in a microfuge for 3 min (4^0C). The supernatant was then transferred to a fresh tube and 500 μl of isopropanol was added. Following a 10 min incubation at RT, the plasmid DNA was pelleted in a microfuge for 10 min at 4^0C and the pellet was washed with 70% cold ethanol, dried, dissolved in 100 μl TE and centrifuged in a microfuge for 2 min. The plasmid DNA obtained was used for restriction digestion. For further purification, the DNA sample was treated with RNase (final concentration 20 μg/ml) at 37^0C for 30 min, followed by phenol/chloroform extraction, Sephacryl S-400 spun column chromatography and ethanol precipitation.

In the boiling method (Holmes and Quigley, 1981), the bacterial pellet was resuspended in 370 μl of STET (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 5% Triton X-100) followed by addition of 5 μl of lysozyme (50 mg/ml in 10 mM Tris-HCl pH 8.0). Samples were boiled for 40 seconds, followed by centrifugation

at 12,000g for 10 min at RT. The pellet of bacterial debris was then removed from the centrifuge tube with a toothpick and the DNA was precipitated with 40 μl of 3M sodium acetate (pH 5.2) and 420 μl of isopropanol. The DNA pellet was dissolved in 50 μl TE (pH 7.6) containing DNase-free pancreatic RNase (20 mg/ml) and used for restriction digestion. Further purification of the DNA was as stated above for DNA prepared by the alkaline method.

2.5.2 Medium-scale preparation (midiprep)

This was carried out by using a commercial kit from Qiagen, according to the manufacturer's protocol. About 100 μg of plasmid DNA could be obtained from 50 to 100 ml of overnight bacterial culture and the DNA could be used directly for most types of work.

2.5.3 Large-scale preparation (maxiprep)

The protocol described in Molecular Cloning (Sambrook et al., 1989, pp. 1.38-1.52) was used with modifications. 200 μl of overnight culture was inoculated into 25 ml of 2YT containing ampicillin at 100 μM and incubated at 37^{0}C for 2-3 hrs with shaking at 200 rpm. The 25 ml culture was then inoculated into 1 litre 2YT with ampicillin and incubated at 37^{0}C for 3-4 hrs with shaking at 150-200 rpm. 170 mg of chloramphenicol (final concentration 170 μg/ml) was then added to the culture and the bacteria were grown at 37^{0}C overnight with shaking at 150-200 rpm. The bacteria were centrifuged

and the pellet was resuspended in total of 8 ml Solution I and separated into two 30 ml polyallomer centrifuge tubes followed by addition of 1 ml Solution I containing 20 mg/ml lysozyme to each tube, mixing and incubation at RT for 10 min. 10 ml of freshly prepared Solution II (0.2 M NaOH, 1% SDS) was then added to each tube, and the samples were mixed by inversion and incubated at RT for 10 min before addition of 15 ml Solution III (7.5 M NH_4Ac), mixing by inversion, incubation on ice for 20 min and centrifugation in a Beckman SW28 rotor at 20,000 rpm for 25 min (4^0C). The supernatant from each tube was transferred to two cold 50 ml tubes and 10 ml isopropanol was added to each tube. After incubation at RT for 15 min, the DNA was pelleted in a Beckman JA20 rotor at 10k rpm for 30 min (4^0C) before dissolving the two pellets in 4.0 ml of TE (pH 7.6)(final volume 4.5 ml) followed by addition of 4.95 g of CsCl, mixing, addition of 80 μl 10 mg/ml ethidium bromide (in water) per ml of solution and centrifugation in a JA20 rotor at 10k rpm for 10 min at 4^0C. The supernatant was transferred into two quick sealing 5 ml ultracentrifuge tubes and centrifuged in a Beckman VTi65 rotor at 55k rpm for 9-18 hrs at 20^0C. The band was collected from the gradient with a 22 gauge needle on a 1 ml syringe, transferred to a 5 ml polystyrene tube, and the sample was extracted with n-butanol saturated with water until it was colourless (usually three times). The aqueous phase was transferred to a 15 ml tube, precipitated with two volumes of cold ethanol on ice for 30 min and centrifuged in a JA20 rotor at 12k rpm for 30 min at 4^0C. The pellet was dissolved in 300 μl TE, precipitated with 150 μl 7.5 M NH_4Ac and 900 μl cold ethanol, followed by centrifugation, resuspension and 2 more precipitations. The purified DNA was dissolved

in TE and could be used for all purposes.

2.6 Mutagenesis by in vivo Recombination

Jones & Howard (1991) developed a novel PCR method that permits the rapid generation of site-specific mutants and recombinant DNA constructs with a minimum number of steps and primers. DNA segments are modified by using amplifying primers that add homologous ends to the polymerase chain reaction product(s). These homologous ends undergo recombination in vivo following transformation of recA- E.coli strains used routinely in cloning. In this method, in vivo circularization of PCR products containing plasmid sequences with a selectable marker permits the rapid cloning of the desired mutant or recombinant.

For generation of a point mutation, the plasmid, containing the insert of interest, was simutaneously mutated and amplified using PCR. In addition to the site-specific mutation to be incorporated into the PCR product, the two amplifying primers also had 5' ends complementary to each other. The linear PCR products were purified as stated above for ordinary PCR products, dissolved in water and directly transformed into E. coli DH5α (about 300 ng DNA per 100 μl competent cells) by the $CaCl_2$ transformation method.

This method was also used to amplify and mutate a segment from one plasmid for insertion into another plasmid at a specific location with a defined orientation. The segment to be inserted into the recipient plasmid was amplified with two primers that contained the mutations as well as terminal sequences that overlapped with the ends of

the linearized recipient plasmid. About 50 ng of linearized recipient plasmid was mixed with 100-250 ng of amplified insert and the mix was transformed into 100 μl of competent DH5α using the $CaCl_2$ method.

2.7 Sequencing

DNA sequencing was performed using the Sequenase kit and protocol (United States Biochemical Corp.). Briefly, 2-4 μg of DNA from a maxiprep, midiprep or purified from miniprep was first denatured with 8μl of 2M NaOH in 40 μl reaction for 10 min at RT followed by precipitation with 7 μl of 3 M sodium acetate (pH 4.8) and 100 μl of ethanol. The denatured DNA was dissolved in 10 μl H_2O and added to an annealing reaction containing 2 μl of primer (10 ng/μl). After heating at 65^{0}C for 5 min, the sample was quickly transferred to 37^{0}C water bath for 10 min followed by incubation at RT for 5-10 min. The annealed template-primer was then added to the labelling reaction containing 12.5 μCi of α-^{35}S dATP and Sequenase (modified T7 DNA polymerase) and incubated at RT for 5 min. The labelling reaction mix was then transferred to each of the four termination reactions and incubation at 37^{0}C continued for 5 min before stop solution was added. The samples were heated at 75^{0}C-80^{0}C for 2 min and loaded on to a 6% sequencing gel (for each gel, H_2O 17.6 ml, 5x TBE 8.8 ml, urea 18.48 g, acrylamide 2.5 g, bis-acrylamide 0.14 g, 10% ammonium persulfate 66 μl, and TEMED 22 μl) and electrophoresed in 1x TBE buffer. The inner sequencing plate was coated with adhesive silane (A-174 240 μl, glacial acetic acid 240 μl, ethanol to 80 ml) and the outer plate was coated with repellant (2 ml of dimethyldichlorosilane in 98 ml of 1,1,1-

chloroethane). After electrophoresis, the gel was fixed to the inner plate with fixer (7% glacial acetic acid plus 25% methanol), dried and exposed to X-ray film. The sequencing data were analyzed by the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package and the IBI DNA/Protein Sequence Analysis program.

2.8 Antibodies Induced by Fusion Proteins Expressed in E. coli.

The cloned DNA of the gene encoding the protein, to which antibody was needed, was first subcloned into vector pATH according to the manufacturer's instructions (Oncogen Science, Inc.). pATH are trp E-containing expression vectors that encode the amino-half of component I of anthranilate synthetase, approximately 33 kDa. Insertion of a cloned gene in frame with the trp E gene of the E. coli trp operon results in high level expression of a fusion protein in E. coli RR1 after induction with indoleacrylic acid (IAA). For screening transformed RR1, a colony was grown overnight at 37°C in 1 ml of ampicillin-containing M9 medium (for 1 litre: 6 g of $Na_2HPO_4 \cdot H_2O$, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 5 g of casamino acids (CA), 1 ml of 1 M $MgSO_4$, 0.2 ml of 0.5 M $CaCl_2$, 5 ml of 40% glucose, 10 ml of 1 mg/ml thiamine B1, 2 ml of 10 mg/ml tryptophan (W)). 0.5 ml of the overnight culture was then diluted into 5 ml of M9 medium without W and grown for 1 hr at 37°C with good aeration followed by addition of 25 μl (5 μl/ml) of 1 mg/ml IAA in ethanol and growth at 37°C for 2 hrs. 1 ml of culture was pelleted, the pellet was resuspended in 50 μl cracking buffer (0.01 M sodium phosphate pH7.2, 1% 2-ME, 1% SDS and 6 M urea), incubated at 37°C for 0.5-3 hrs and 10 μl was analyzed by SDS-PAGE (Laemmli, 1970) and staining with Coomassie

Brilliant Blue R250 (Sambrook et al., 1989, pp.18.55). After screening, scale-up cultures of the positive clones were grown and the fusion protein was then separated by SDS-PAGE (Laemmli system), cut out from the gels, eluted and then dialysed against distilled deionized water (Bell et al., 1984). About 100 μg of the dialysed fusion protein was mixed with an equal volume of complete Freund's adjuvant to form an emulsion and injected into a rabbit at four sites, 2 subcutaneous injections on the back and into both hind leg muscles. The rabbits were boosted once after three weeks with the same fusion protein mixed with incomplete Freund's adjuvant and then bled every week to test for antibody by radioimmunoprecipitation (RIPA).

2.9 Transfection of Mammalian Cells & Selection of Transfectants

Mammalian cell lines L929, NIH/3T3, C127-I, MDCK, and COS-1 were transfected with DNA by lipofection using reagents lipofectin or lipofectamine and protocols from GIBCO-BRL. Taking transfection of L929 cells in 60 mm tissue culture dishes as an example, 80-85% confluent (for transient expression) or 60-65% confluent cell monolayers were washed with PBS twice and then loaded with 2 ml of MEM (serum free and antibiotics free) and 100 μl of DNA-lipofectin mix (5-10 μg of DNA plus 20 μl of lipofectin which had been incubated at RT for 15-45 min), followed by incubation at 37^{0}C with 3.5% CO_2 for 5-8 hrs. Complete MEM was then added (10% fetal calf serum final concentration) and cells were incubated for 40-48 hrs before testing for transient expression or split into 4-5 T25 flasks for selection of stable transfectants. If the cells were deleteriously affected after incubation in lipofection mix, the mix was removed,

complete MEM was added, and the medium was changed again next day. When lipofectamine was used for transfection, 10 μl of lipofectamine plus 5 μg of DNA was used per one 60 mm dish. As the selectable marker in transfections was the neomycin resistance gene or the puromycin resistance gene, geneticin (G418)(500 μg/ml) or puromycin (5 μg/ml) was used for selection of stable transfectants. The medium was changed every seven days for G-418 and every three days for puromycin selection. After most cells died, 1/3 volume of conditioned medium was included in the medium. Generally, colonies of resistant cells were visible following two weeks of selection. Individual colonies were picked using a pasteur pipette, transferred to fresh flasks and grown up for testing of expression. At this stage, the selective pressure was removed but 1/3 volume of conditioned medium was still included.

2.10 Western Blot & Radioimmunoprecipitation Assay (RIPA)

Western blot and RIPA were used to detect proteins expressed in infected or transfected cells. For RIPA, cell monolayers were washed twice with PBS before labelling with ^{35}S-methionine at 50 mCi/ml for 2 hrs (infected cells) or 4-8 hrs (for transfectants expressing reovirus μ2 proteins).

To harvest proteins for both RIPA and western blot, cell sheets in 60 mm or 35 mm dishes were washed with cold PBS twice and lysed in 0.5 ml (for a 60 mm dish) of lysing buffer (50 mM Tris pH8-9, 150 mM NaCl, 1% NP40, 10% glycerol, and 1 mM PMSF (phenylmethylsulfonyl fluoride, 100 mM stock in isopropanol, freshly added) on

ice for 20 min. The lysed cells were then scraped into centrifuge tubes and pelleted to remove the cell debris (Schaffhausen et al., 1978). For western blot, cells were lysed in a smaller volume (0.2 ml for a 60 mm dish) than for RIPA. The cell lysate could be tested immediately or stored at -20^{0}C for future use.

For western blot (Sambrook et al., 1989, pp.18.60-18.75), cell lysate was mixed with an equal volume of 2x protein sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.1% bromophenol blue, 10% 2-mercaptoethanol freshly added) and proteins were separated by SDS-PAGE (Laemmli gel). The separated proteins were transferred to nylon membrane at 25 volts for 8-16 hrs or at 220 mA for 2 hrs at 4^{0}C. The membrane was dried on 3MM paper at RT and then either stored in a plastic bag at 4^{0}C for future use or blocked in 5% milk (nonfat dried milk in PBS) for 1 hr at RT followed by the addition of primary antibody in fresh milk, and incubated at 4^{0}C for 2 hrs with gentle shaking. The membrane was then washed 3 times in PBS, once in a buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.5 to remove phosphate before it was incubated in 2% milk (in 150 mM NaCl, 50 mM Tris pH7.5) containing 1:1000 diluted PAAP (protein A-alkaline phosphatase conjugate, 1 mg/ml stock in water) at RT for 1 hr. This was followed by 4 washes (10 min each) at RT in 150 mM NaCl, 50 mM Tris-HCl pH 7.5, and incubation with the chromogenic substrates. Alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl pH 9.5) was first mixed well with nitro blue tetrazolium (NBT) stock (5% in 70% dimethylformamide) at 66μl NBT stock per 10 ml buffer before addition of 33 μl (per 10 ml buffer) of 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The membrane was then incubated with the

chromogenic substrates at RT with gentle shaking and the colour reaction was monitored carefully. When the bands were of the desired intensity (about 20 min), the reaction was stopped with 50 ml of PBS containing 200 μl of 0.5 M EDTA (pH 8.0).

For RIPA (Schaffhausen et al., 1978), the harvested cell lysate was mixed with antibody (1.5 to 5 μl) and 200 μl of 10% protein-A agarose slurry that had been equilibrated in lysing buffer without PMSF (100 mg in 5 ml buffer) at 4^{0}C for 1-16 hrs. The agarose beads were then pelleted in a microfuge for 20 seconds followed by 2-3 washes, each time with 1 ml of cold washing buffer (100 mM Tris-HCl pH 8-9, 500 mM LiCl, 1% 2ME) and rotating the tube at 4^{0}C for 20 min. The final wash was with TE (10 mM Tris-HCl pH 7.6, 1 mM EDTA) and 1x protein sample buffer (dilution of 2X buffer with water) was added to the washed beads after removal of TE. The precipitated proteins were then separated by SDS-PAGE and the gel was fixed in acetic acid/methanol, dried and exposed to X-ray film.

2.11 Extraction of RNA and DNA from cultured cells

There were several ways to extract RNA from cultured cell monolayers. The one described by Sambrook et al. (1989, pp. 7.6-7.9) isolates cytoplasmic RNA by using NP40 to disrupt cells and vanadyl complex to inhibit RNases. Briefly, after medium was removed and cells washed with cold PBS twice, cells were scraped into microfuge tubes and pelleted at 14 k rpm in a microfuge for 30 seconds at 4^{0}C, followed by resuspension in lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.6, 0.5% NP40, 1 mM DTT (dithiothreitol), 20 mM vanadyl-ribonucleoside complexes (200 mM stock,

Maniatis et al., 1982). After vortexing for 15 seconds, the suspension was kept on ice for 5 min before centrifugation for 2 min at 4^0C. The nuclei-containing pellet could be used for extraction of DNA and the supernatant was transferred to a fresh tube and proteinase digestion buffer was added (0.2 M Tris-HCl pH 8.0, 25 mM EDTA, 0.3 M NaCl, 2% SDS), followed by addition of proteinase K to a final concentration of 50 μg/ml and digestion at 37^0C for 30 min. After phenol/chloroform extraction, the RNA was precipitated from the aqueous phase with cold isopropanol and dissolved in TE. The DNA in the extract was removed by RNase-free DNase I if necessary in a reaction containing 10 mM $MgCl_2$, 0.1 mM DTT, 1000 units/ml placental RNase inhibitor or 10 mM vanadyl-ribonucleoside complexes, 2 μg/ml DNase I at 37^0 for 60 min (Sambrook et al., 1989, pp.7.8). For storage of RNA, 2.5 volumes of ethanol were added and samples were kept at -70^0C. To recover the RNA, 1/10 volume of 3 M NaAc was added and RNA was pelleted in a microfuge and dissolved in H_2O treated with DEPC (diethyl pyrocarbonate)(Sambrook et al., 1989 pp.7.4).

Another method for extraction of RNA from mammalian cells in monolayer was described by Chomczynski and Sacchi (1987) in which guanidinium thiocyanate was used to disrupt cells.

To isolate DNA from cultured cells, a method modified from Blin and Stafford (Sambrook et al., 1989, pp.9.16-9.23) was used. Cell monolayers were washed twice with cold PBS, scraped, pelleted, washed again and resuspended in TE (pH 8.0)($5x10^7$ cells/ml). For each ml of cell suspension, 10 ml of extraction buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA, 20 μg/ml pancreatic RNase, 0.5% SDS) was added and the

solution was incubated at 37°C for 1 hr. Following addition of proteinase K to 100 μg/ml and gentle mixing, the suspension of lysed cells was incubated at 50°C for 3 hrs and swirled periodically. After extraction with an equal volume of phenol, the viscous aqueous phase was transferred with a wide-bore pipette and the extraction was repeated twice. The DNA was then precipitated with 0.2 volume of 10 M NH_4Ac and 2 volume of ethanol at 5000g for 5 min at RT, followed by washing of the pellet with 70% ethanol, draining and addition of TE (1 ml for each $5x10^6$ cells). The DNA was dissolved at 4°C on a rocking platform overnight and then stored at 4°C.

2.12 Northern Blot and Southern Blot Hybridization

2.12.1 Northern analysis

RNA samples were separated on 4.5% polyacrylamide gels in 1x TBE, pH 8.3 (Ogden and Adams, 1987). Gels were stained and equilibrated in 1x TBE containing ethidium bromide to remove urea and then RNA was electroblotted to nylon membrane in 0.5x TBE at 15 volts for 16 hrs or at 65 volts for 2 hrs at 4°C (Kramer, 1990). Membranes were baked and prehybridized for 1-2 hrs at 68°C in modified Denhardt's reagent as stated in section 2.4.3 for screening of transformants. Denatured dsDNA probe or oligonucleotide probe was added and hybridization was carried out for 16-24 hrs at 60°C (for dsDNA probe), followed by one wash at RT in 2x SSC, 0.1% SDS and 3 washes at 60°C in 0.2x SSC, 0.1% SDS.

2.12.2 Southern analysis

DNA (50 μg in 200 μl of TE, from 10^7 cells) was first digested with a restriction enzyme (e.g. EcoRI 100 units) overnight, precipitated, dissolved in water and separated by electrophoresis on 0.8% agarose gels at 1 volt/cm in 1x TBE. After staining and photographing, gels were immersed in 0.2 M HCl for 10 min, rinsed with water and DNA was denatured in several volumes of 0.5 M NaCl, 0.2 M NaOH for 30 min with constant, gentle agitation. The DNA was then transferred from the gel to nylon membrane (Zeta-probe) in 0.5x TBE at 25 volts overnight or at 80 volts for 4 hrs at 4^0C (Wahl et al., 1987). After transfer, membranes were soaked in 1x TBE briefly to remove pieces of agarose, placed on 3MM paper saturated with 0.4 M NaOH for 10 min, rinsed in 2x SSC, dried in a vaccum oven at 80^0C for 30 min to 1 hr and then hybridized with probe as described above for northern blot analysis.

2.13 In vitro Transcription of DNA and in vitro Translation

The protocol for transcription was from Promega Notes No. 39, 1992 pp.12-16 and from Sambrook et al., 1989. For run-off transcription, plasmid DNA was first cut with a restriction enzyme and then treated with T4 DNA polymerase if a 3' overhang was generated, followed by phenol/chloroform extraction and precipitation. The pelleted DNA was dissolved in DEPC-treated H_2O for transcription. For run-on transcription, plasmid DNA was extracted with phenol/chloroform to remove RNase, precipitated and dissolved in DEPC-H_2O. Transcription reactions (50 μl) contained 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1 mM spermidine, 10 mM NaCl, 10 mM DTT, 1 mg/ml BSA, 0.5

mM each of rATP, rCTP and rUTP, 0.05 mM rGTP, 0.5 mM m^7GpppG (cap analog), 10 μCi $\alpha^{32}P$ rGTP, 30-60 units RNasin or RNA guard (Pharmacia), 0.1 μg DNA template and 40 units T7 or SP6 RNA polymerase. Transcription was allowed for 60 min at 37°C for T7 RNA polymerase or at 40°C for SP6 RNA polymerase, followed by addition of RNase-free DNase I at 1 unit per μg of DNA, incubation at 37°C for 15 min, phenol/chloroform extraction and NaAc/ethanol precipitation. The integrity and size of transcripts were checked by electrophoresis through 4.5% polyacrylamide gels containing 7 M urea (Sambrook et al., 1989, pp.7.76).

In vitro translation of the transcripts was performed with a kit and protocol from GIBCO-BRL. Briefly, RNA was pelleted, dissolved in DEPC-H_2O, heated at 67°C for 10 min and immediately cooled on ice before being added to the 30 μl reaction containing 10 μl of nuclease-treated rabbit reticulocyte lysate, 30 units of RNasin, 3 μl of translation mixture without methionine, 2.4 μl of 1 M potassium acetate, and 2.5 μl of ^{35}S-methionine (1200 Ci/mmole, 10 mCi/ml). The reaction was incubated at 30°C for 60 min and the product was then separated by SDS-PAGE or used in RIPA. If required, pancreatic RNase A could be added to the translation product to degrade the mRNA. When labelling was not required the radioactive methionine was replaced with cold methionine.

2.14 Digestion of reovirus and in vitro transcription from activated cores

Digestion and transcriptase reactions were performed as previously described (Skehel and Joklik, 1969; Drayna and Fields, 1982). 100 μg of purified reovirus was digested

at 37°C for 90 min with chymotrypsin at 100 μg/ml in a 100 μl reaction in 1x SSC (0.15 M NaCl, 0.015 M sodium citrate) and stopped by adding 1 μl of 0.1 M phenylmethylsulfonyl fluoride and 100 μl of 1x SSC. 100 μl of the digestion mixture was then added to a 250 μl transcription reaction containing 0.1 M Tris-HCl pH 8.0, 0.01 M $MgCl_2$, 0.01 M DTT, 3.3 mM phosphoenolpyruvate, 100 μg/ml of pyruvate kinase, 2 mM each of ATP, CTP, GTP, UTP, and 250 units of RNasin. The reaction was incubated at 40°C from 90 min to 13 hrs. The RNA transcripts were purified by centrifugation to remove virus cores, treatment with SDS, extraction with phenol/chloroform, and precipitation with PEG 8000. After resuspension in 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) the RNA was further extracted with phenol/chloroform three times, chloroform twice and precipitated with sodium acetate and ethanol. The purified RNA was translated in rabbit reticulocyte lysate using the kit and protocol from GIBCO-BRL.

2.15 RNase Protection & Primer Extension

2.15.1 RNase protection

The method from Current Protocols in Molecular Biology (Ausubel et al., 1989) was followed. In vitro transcription of ssRNA was carried out for 60 min at 37°C or 40°C, followed by treatment with DNase I, phenol/chloroform extraction, and NH_4Ac/ethanol precipitation. The probe was then dissolved in annealing buffer (hybridization buffer) and 30 μl of probe was used to dissolve each RNA pellet collected from the alcoholic RNA suspension. After heating at 85°C for 5 min the reaction was incubated at 45°C

overnight followed by digestion at 30°C for 45-60 min with 350 μl of RNase digestion buffer containing 40 μg/ml of RNase A, 2 μg/ml of RNase T1, 300 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA. The samples were then precipitated with 1/2 vol of 7.5 M NH_4Ac, 3 vol of ethanol and separated by electrophoresis 6% polyacrylamide gels containing 7 M urea.

2.15.2 Primer extension

The method described by Boorstein and Craig (1989) was used. Oligonucleotide primers were labelled with ^{32}P by T4 polynucleotide kinase, purified through G-50 spun columns, precipitated and dissolved in DEPC-H_2O. RNA sample (in DEPC-H_2O) was combined with a labelled primer (total volume 8 μl or less) followed by addition of 5x ss hybridization buffer (1.5 M NaCl, 50 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0)(2 μl for a 10-μl reaction). The reaction was heated at 80°C for 4 min and then allowed to anneal at 48°C for 2-3 hrs. The reaction (10 μl) was diluted with 40 μl of 1.25x reverse transcriptase buffer (1.25 mM each of dATP, dCTP, dGTP, dTTP, 12.5 mM DTT, 12.5 mM Tris-HCl pH 8.4, 7.5 mM $MgCl_2$) or with the 4x reverse transcriptase buffer (as used for standard reverse transcription) plus 2 μl of 4 dNTPs (25 mM each). Avian reverse transcriptase (12 unit/50 μl reaction) was added and the reaction was incubated at 48°C for 30 min and stopped by adding EDTA to a final concentration of 10 mM. The products were precipitated with 0.1 vol of 3 M NaAc and 2 vol of ethanol, resuspended in sequencing loading buffer (formamide loading buffer) and separated on sequencing gels. Reaction(s) of the corresponding DNA were included as controls.

CHAPTER THREE

ANALYSIS OF M1 DELETION MUTANTS TO IDENTIFY CONSERVED CONSENSUS SEQUENCES REQUIRED FOR REPLICATION AND ENCAPSIDATION OF THE M1 GENOME SEGMENT

3.1 Introduction

Although it is known that each reovirion contains one of each of the 10 genome segments, the signals that control the replication of each genome segment and the mechanism that ensures that 10 unique RNA segments are assembled together in each nascent particle remain unknown. The terminal regions of all 10 genome segments share GCUA at their 5' end and UCAUC at their 3' end (Antczak et al., 1982). Adjacent to these common end-specific structures is a segment-specific stretch of nucleotides that is complementary to a sequence at the opposite end of the segment (Gaillard et al., 1982). Although these short terminal regions are expected to be important for specific functions required in replication and assembly they are probably not sufficient in themselves to signal all the events in replication and assembly.

Studies with wound tumor virus (WTV), the type member of the genus Phytoreovirus of reoviridae, suggested that each genome segment must contain at least two operational sequence domains for genome packaging: one that specifies that it is a viral and not a cellular RNA (perhaps the conserved terminal sequences) and a second that specifies that it is a particular RNA genome segment (perhaps the segment-specific terminal inverted

repeat sequences (Anzola et al., 1987). It was further proposed that the signals required for replication of the WTV genome reside within the terminal domains of the individual segments due to their location and their conservation in deletion mutants (Xu et al., 1989; Dall et al., 1990).

Serial passage of viruses at high multiplicity of infection can generate deletion mutants (reviewed by Perrault, 1981). Specific T1xT3 reassortants containing the L2 genome segment of T3 and the M3 genome segment of T1 can produce M1 deletion mutants on serial undiluted passage (Brown et al., 1983). Since genome segments with deletions can still replicate and be assembled into progeny virus, it was reasoned that the M1 genome segments with deletions must have maintained the signals essential for the replication and assembly of the M1 segment. The smallest fragments would consist primarily of these sequences.

From northern blot analysis using probes complementary to the 3' end of the plus sense and to the 3' end of the minus sense, it was further found in this laboratory that the short M1 genome segments all maintained the termini of the M1 segment (Zou and Brown, 1992b).

3.2 Methods

Cloning and sequencing of the smallest M1 segments with deletions from high passage reassortants were carried out to identify the conserved sequences.

High passage reassortant viruses were harvested from infected L929 monolayers and viral dsRNA was extracted and purified for reverse transcription. cDNA was synthesized

and then amplified by PCR and the PCR product was separated by agarose gel electrophoresis. The primers used in reverse transcription and PCR reactions were M1 5'+ and M1 5'-, complementary to the 3' ends of the minus strand and the plus strand, respectively. After staining with ethidium bromide and visualization with UV light, the smallest PCR products were isolated and the DNA was recovered from the gel slice by electroelution, followed by n-butanol extraction to remove ethidium bromide, phenol/chloroform extraction, Sephacryl S-400 spun column chromatography, and ethanol precipitation. Amplified M1 deletion fragments were then end-repaired using DNA polymerase I Klenow fragment, phosphorylated and ligated into plasmid pGEM-7Zf(+). The cloned M1 deletion fragments were then sequenced and the sequences were compared with that of the intact T1 Lang M1 segment.

3.3 Results

3.3.1 Sequences of the smallest M1 deletion fragments

Twenty fragments of the T1 M1 segment were derived from nine serially passaged stocks of reassortants. Some of the fragments sequenced turned out to have the same sequence except for a few substitutions and as a result thirteen different sequences with variable deletions emerged. Comparison of sequences of these fragments with the intact M1 segment showed that (1) the smallest fragment was 344 nucleotides in length; (2) the minimum consensus sequence from the 5' end was 132-135 nucleotides long; (3) the minimum consensus sequence from the 3' end was 183-185 nucleotides long; and (4) all the fragments had the internal region of the M1 segment deleted and could have been the

result of single internal deletion events. Table 3.1 shows the thirteen M1 segments with different internal deletions.

Table 3.1

Terminal Regions of the M1 Segment Found in Deleted Fragments

Fragment	No. bases	5' end[a]	3' end[a]
1A1-8	390	162-163	227-228
1B3-3	392	176-177	215-216
47A3-2	378	161-162	216-217
47A2-4	367	150	217
47B5-2	370	146-147	223-224
47B4-3	411	177-178	233-234
47B4-5	377	152-154	223-225
98A10-1	373	188-190	183-185
98A10-4	393	171	222
98B11-1	367	132-135	232-235
140B8-19	401	147	254
G2A9-1	344	140	204
G2B11-2	403	173-174	229-230

[a] The 5' and 3' end sequences of each fragment are coterminal with the intact M1 segment for the sizes indicated; where there was ambiguity as to the origin of nucleotides surrounding the joining region, the terminal regions are shown as ranges.

Products of the PCR reaction that were the result of mispriming events were also cloned and sequenced. A 154 nucleotide-long product containing the 3' end of the M1 gene was cloned from the PCR products of the unpassaged T1 control. This was presumably the product of mispriming by the M1 5'+ primer on 11 nucleotides of the minus strand of the M1 segment that were complementary to the 3' end of the primer. DNA molecules that contained both primers at their termini flanking sequences that were

totally unrelated to the M1 gene were obtained from several stocks. None of these PCR products contained regions of homology with each other or the M1 genome segment.

Mutations were observed in the primer binding regions of 2 clones, in the 5' end of 98A10-4 and the 3' end of G2B11-17, presumably representing errors in primer synthesis. Some of the point mutations outside of the primer binding sites may have been created during the PCR step but many of them were probably produced during viral replication since the mutations were nonrandomly distributed among the 20 M1 fragments sequenced and 11 of them did not have substitutions. One substitution was seen in 4 of the 20 fragments, 2 substitutions in 2, and single fragments had 3, 4, and 5 substitutions. The small M1 fragments detected by Northern blot of both of the passaged stocks of reassortant EB86 were not amplified by PCR. This may have been due to extensive or specific substitutions in the primer binding region that inhibited primer-dependent reverse transcription or DNA amplification.

3.3.2. Comparison of the sequence at the joining sites and their location in the M1 segment

Alignment of the M1 deletion mutants according to the site of deletion (where the terminal regions are joined) did not indicate a common sequence motif on either side of the junction of the terminal regions (Fig. 3.1). It was not possible to unambiguously assign the deletion site since 9 out of 13 deletion fragments had 1 to 3 nucleotides at the junction of the terminal sequences that could have been derived from either the 5' or 3' ends of the M1 sequence (Fig. 3.2). These short regions of homology at the junctions

Fig. 3.1 The break/joining points in the M1 deletion fragments. The arrows indicate the nucleotides that are common to the 5' and 3' ends of the M1 sequence.

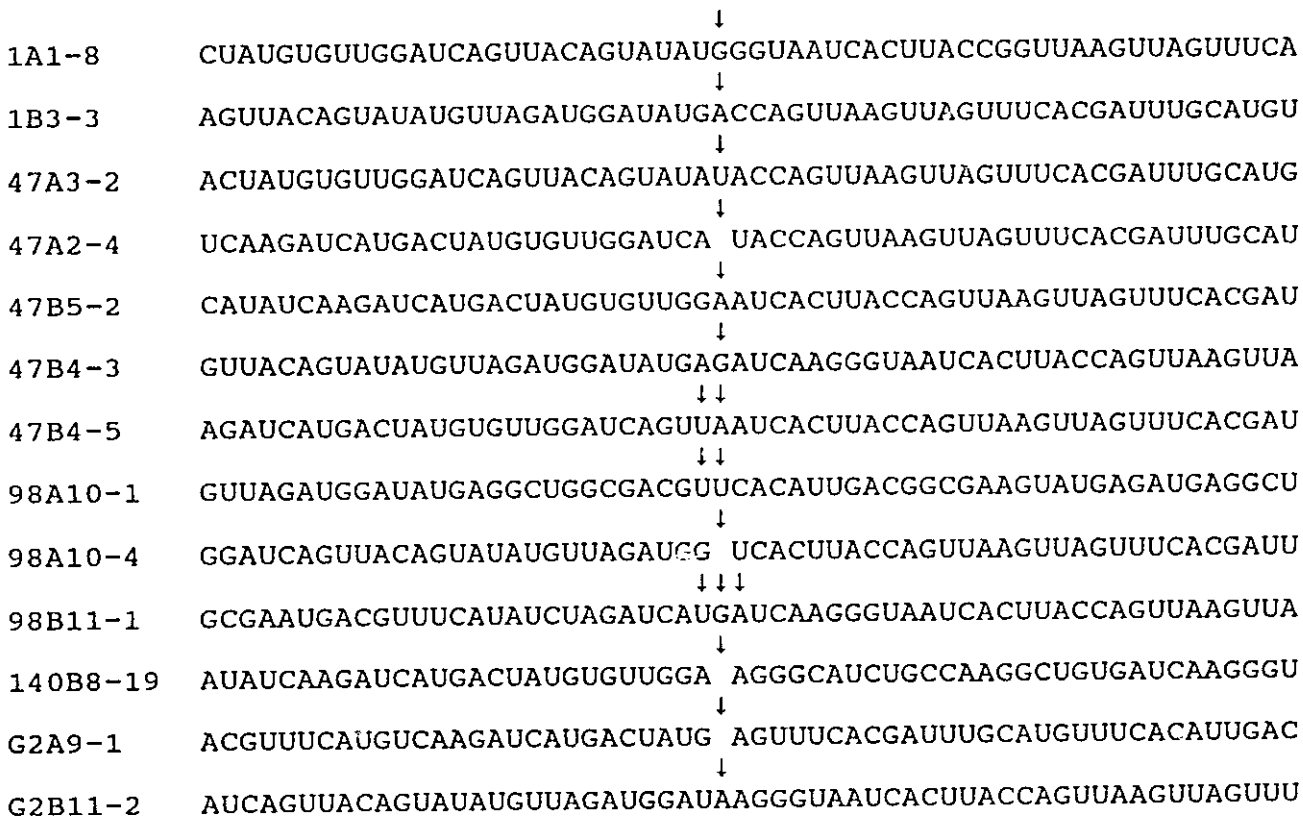

1A1-8 CUAUGUGUUGGAUCAGUUACAGUAUAUGGGUAAUCACUUACCGGUUAAGUUAGUUUCA
1B3-3 AGUUACAGUAUAUGUUAGAUGGAUAUGACCAGUUAAGUUAGUUUCACGAUUUGCAUGU
47A3-2 ACUAUGUGUUGGAUCAGUUACAGUAUAUACCAGUUAAGUUAGUUUCACGAUUUGCAUG
47A2-4 UCAAGAUCAUGACUAUGUGUUGGAUCA UACCAGUUAAGUUAGUUUCACGAUUUGCAU
47B5-2 CAUAUCAAGAUCAUGACUAUGUGUUGGAAUCACUUACCAGUUAAGUUAGUUUCACGAU
47B4-3 GUUACAGUAUAUGUUAGAUGGAUAUGAGAUCAAGGGUAAUCACUUACCAGUUAAGUUA
47B4-5 AGAUCAUGACUAUGUGUUGGAUCAGUUAAUCACUUACCAGUUAAGUUAGUUUCACGAU
98A10-1 GUUAGAUGGAUAUGAGGCUGGCGACGUUCACAUUGACGGCGAAGUAUGAGAUGAGGCU
98A10-4 GGAUCAGUUACAGUAUAUGUUAGAUGG UCACUUACCAGUUAAGUUAGUUUCACGAUU
98B11-1 GCGAAUGACGUUUCAUAUCUAGAUCAUGAUCAAGGGUAAUCACUUACCAGUUAAGUUA
140B8-19 AUAUCAAGAUCAUGACUAUGUGUUGGA AGGGCAUCUGCCAAGGCUGUGAUCAAGGGU
G2A9-1 ACGUUUCAUGUCAAGAUCAUGACUAUG AGUUUCACGAUUUGCAUGUUUCACAUUGAC
G2B11-2 AUCAGUUACAGUAUAUGUUAGAUGGAUAAGGGUAAUCACUUACCAGUUAAGUUAGUUU

Fig. 3.2 Sequences of M1 fragments. Twenty clones of M1 fragments are shown; several clones were obtained from the same high passage stock and thus have the same breakage/joining point. The sequence of the T1 M1 genome segment is indicated at the top of each column; only sequence differences are indicated for fragments; identical nucleotides are indicated with a dash. Where there was uncertainty as to the origin of nucleotides around the break point these nucleotides have been shown in lower case symbols. The M1 fragments are named according to the reassortant of origin followed by a clone number. The deleted portions of fragments are indicated by solid lines.

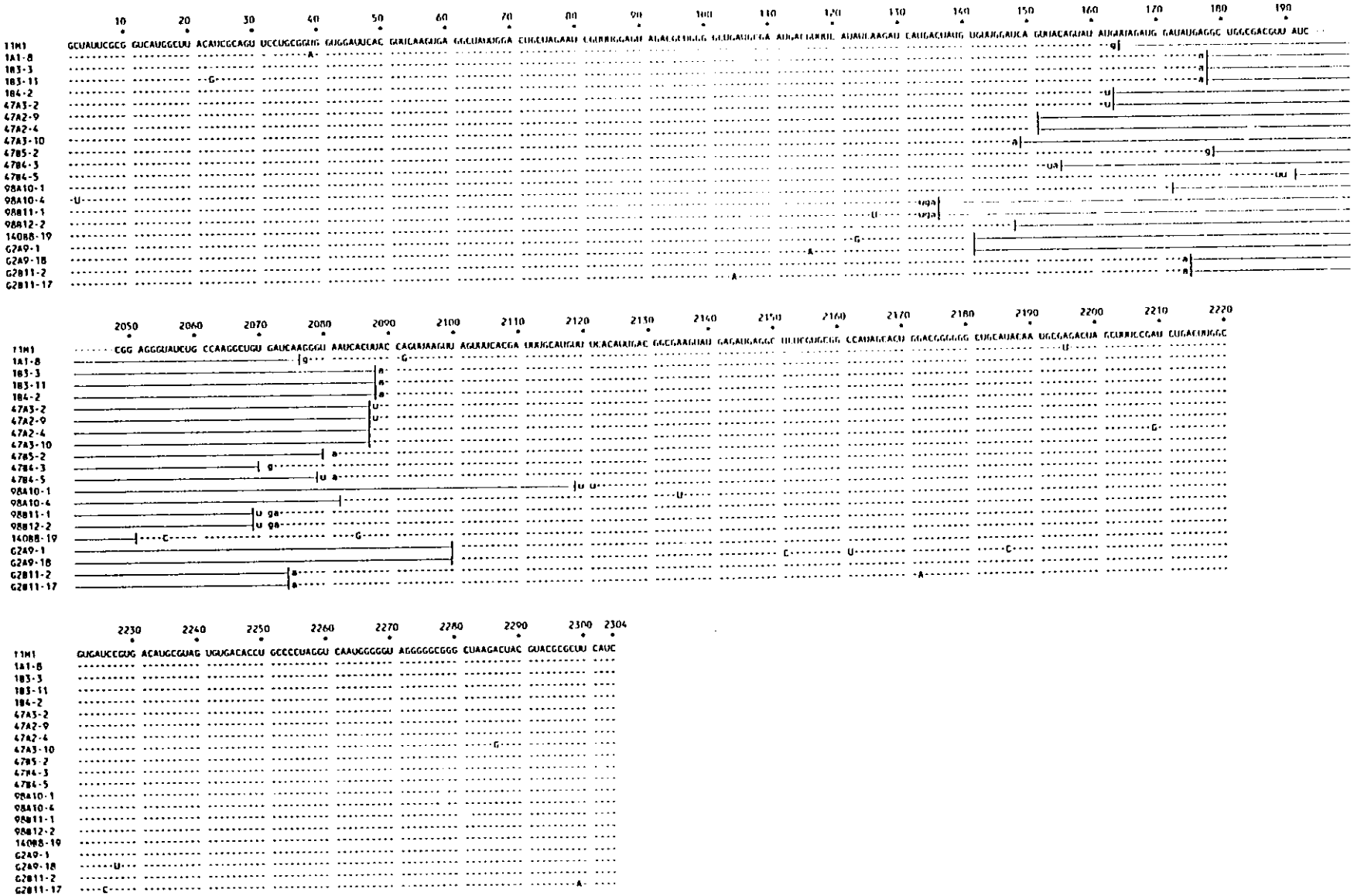
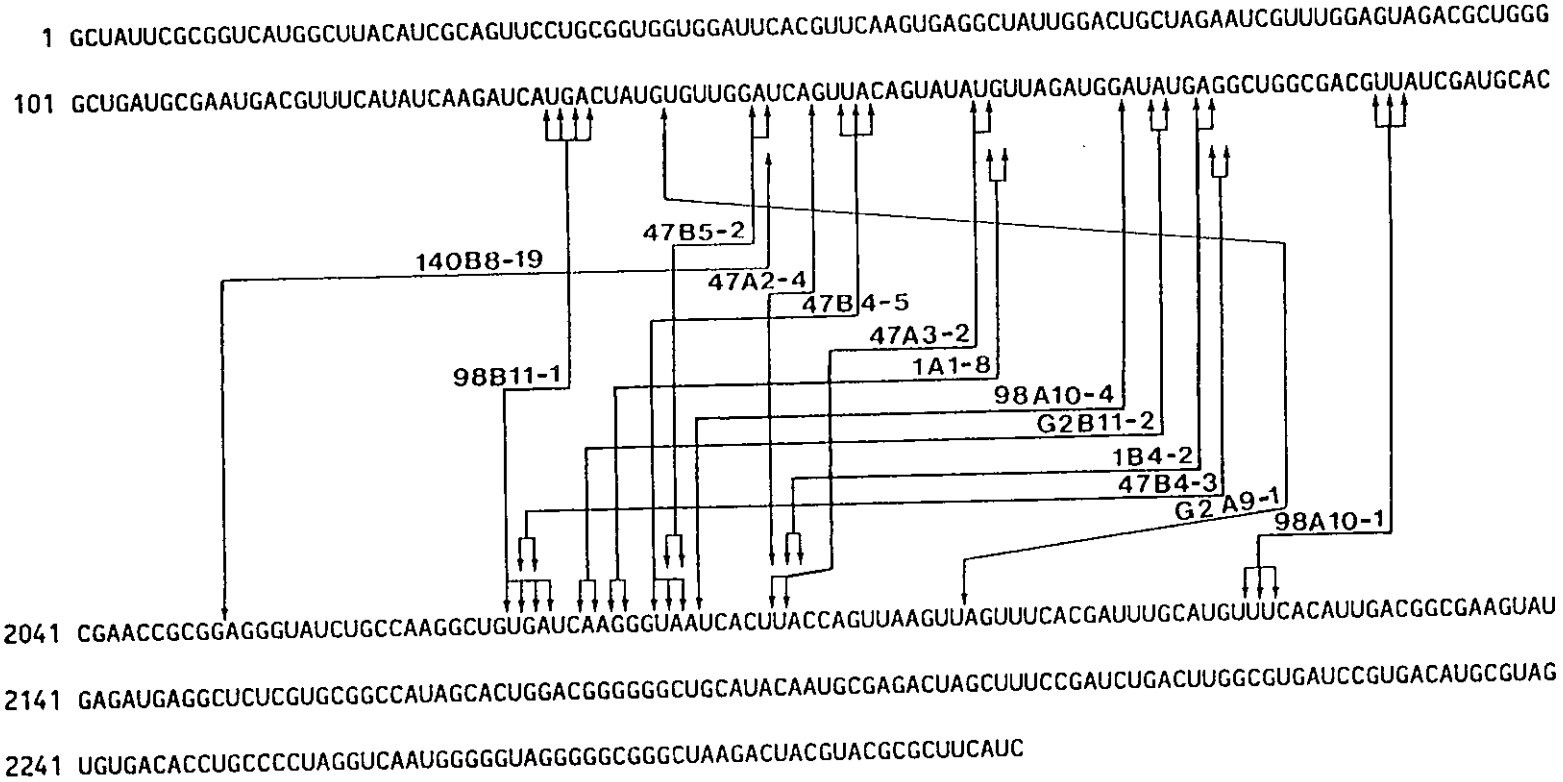


Fig. 3.3 Location of break-points in the M1 segment. The terminal regions of the M1 segment are shown with arrows linking the specific regions that were joined in fragments that had been sequenced. Where the exact location of the break point is uncertain multiple arrows on the same line indicate the possible break points.


1 GCUAUUCGCGGUCAUGGCUUACAUCGCAGUUCCUGCGGUGGUGGAUUCACGUUCAAGUGAGGCUAUUGGACUGCUAGAAUCGUUUGGAGUAGACGCUGGG
101 GCUGAUGCGAAUGACGUUUCAUAUCAAGAUCAUGACUAUGUGUUGGAUCAGUUACAGUAUAUGUUAGAUGGAUAUGAGGCUGGCGACGUUAUCGAUGCAC
47B5-2
140B8-19
47A2-4
47B4-5
47A3-2
1A1-8
98B11-1
98A10-4
G2B11-2
1B4-2
47B4-3
G2A9-1
98A10-1
2041 CGAACCGCGGAGGGUAUCUGCCAAGGCUGUGAUCAAGGGUAAUCACUUACCAGUUAAGUUAGUUUCACGAUUUGCAUGUUUCACAUUGACGGCGAAGUAU
2141 GAGAUGAGGCUCUCGUGCGGCCAUAGCACUGGACGGGGGGCUGCAUACAAUGCGAGACUAGCUUUCCGAUCUGACUUGGCGUGAUCCGUGACAUGCGUAG
2241 UGUGACACCUGCCCCUAGGUCAAUGGGGGUAGGGGGCGGGCUAAGACUACGUACGCGCUUCAUC

of the terminal regions may have been involved in directing the formation of deletion mutants. Scrutiny of the sequences upstream and downstream of the junctions of the terminal regions showed no discernible sequence element of significance. When the ends of the 5' and 3' sequences at the joining sites are mapped to the sequence of the M1 gene it is seen that the break points for the 3' end are more tightly clustered than the break points for the 5' ends (Fig. 3.3).

Only 2 of the deletion fragments (47A3-2 and 47B4-3) maintained the original reading frame at the joining site to produce proteins of 94 and 105 amino acids that consisted of the amino and carboxy terminal portions of the μ2 protein. All of the other deletion fragments would be predicted to terminate translation, at or near the breaking/joining site to produce proteins of 43-70 amino acids. These protein products would not be expected to play a role in replication or assembly since they consist of small fractions of the μ2 protein.

3.4 Discussion

Deletion mutants in reovirus can involve the L1, L2, L3, and M1 genome segments. The genome fragments in deletion mutants can still replicate and be assembled into progeny virus and therefore have maintained the signals essential for their replication and encapsidation, even though replication of the deletion mutant requires helper virus. M1 deletion fragments were identified in high-passage stocks of reovirus reassortants by Northern blot analysis. Since all the fragments of M1 that were detected contained both termini they were amplified by PCR of cDNA using end-specific primers. The smallest

fragments were cloned and sequenced. The results from 13 fragments showed that the minimum length of the M1 deletion fragments was 344 nucleotides, and 132-135 nucleotides from the 5' end of the M1 genome segment and 183-185 nucleotides from the 3' end were conserved in all 13 M1 deletion fragments. Therefore it can be deduced that these two terminal regions contain the minimum of sequence elements essential for replication and encapsidation of this segment. It is not known what part of this sequence harbors specific signals for the recognition events in replication and encapsidation. In addition to specific sequence signals, the deleted fragment might also have a limit in length and 344 nucleotides could be the minimum length essential for replication and/or encapsidation. Some of the sequence may function as spacer required to separate binding sites that form specific secondary or tertiary structures or multicomponent complexes. It is possible that protein-RNA or RNA-RNA intermediates are formed in replication and that spatial and steric parameters must be satisfied.

Each reovirus segment must maintain a dsRNA-dependent transcriptase promoter at the 3' end of the minus strand and a ssRNA-dependent replicase promoter at the 3' end of the plus strand as well as those sequences necessary for assembly. Assembly requires signals for encapsidation and sorting/exclusion since only one of each segment is assembled into each virion. In assembly, an individual segment is recognized as being reoviral as well as being a specific genome segment. The sorting and excluding signals may be identical or separate depending on the mechanism of achieving each function. The 4 terminal nucleotides at the 5' end and the 5 terminal nucleotides at the 3' end common to all 10 segments could be the signals recognized as identifying the viral

genome (Antczak et al., 1982). The segment-specific identifier will be elsewhere since it will differ among segments. Antczak et al. (1982) sequenced the termini of all of the reovirus genome segments and analyzed the sequences for structures that would link or network all of the segments together but such structures could not be found. It is possible that assembly signals involve terminal or interterminal secondary structures formed in conjunction with proteins. Secondary structure predictions of the consensus sequences present in M1 deletion fragments produce an extensively base-paired structure with a free energy of -103.6 kcal/mol; the noncoding regions at the termini produce a panhandle hairpin structure that anneals the first nucleotide of the initiation and termination codons (Zou and Brown, 1992b). M1 RNA is poorly translated in vivo but is efficiently translated in vitro (Roner et al., 1989), indicating that if this is due to secondary structure then host factors are involved. It is possible that host and viral factors operate in replication and assembly of reovirus.

There is no direct evidence that the M1 deletion fragments are defective interfering RNAs, however they are defective and have a replicative advantage relative to wild type since they are amplified on culturing and thus replicate at the expense of wild type virus.

Deletion mutants of other dsRNA viruses have all conserved their terminal regions. The fragments of two deletion mutants of WTV possessed consensus sequences of 319 nucleotides from the 5' end and 205 nucleotides from the 3' end. The smallest dsRNA fragment described to date is a 315-nucleotide fragment consisting of 121 nucleotides from the 5' end and 191 nucleotides from the 3' end of the polyhedrin gene of cytoplasmic polyhedrosis virus (CPV), which is a reovirus that infects insects, (Nuss,

1988). The similarity in size of the regions conserved in CPV and reovirus deletion fragments suggest that the location of replication and encapsidation signals in all members of the Reoviridae may be similar.

It may be a general feature of all viruses with segmented RNA genomes that both terminal sequences play critical roles in genome transcription, replication and packaging (Nuss and Summers, 1984). Conserved common 5' and 3' terminal nucleotides and adjacent segment-specific nucleotide sequences were found in influenza A and B virus (Stoeckle et al., 1987) and in wound tumor virus (Nuss and Summers, 1984; Anzola et al., 1987; Nuss and Dall, 1990). In influenza A, the 22 5' terminal and the 26 3' terminal bases of segment 8 viral RNA were sufficient to provide the signals for RNA transcription, RNA replication, and packaging of RNA into influenza virus particles (Luytjes et al., 1989) whereas the 15 nucleotide 3' terminus contains the promoter for transcription (Parvin et al., 1989). The smallest deletion mutant described for influenza virus contained 83-84 nucleotides from the 5' end and 95-96 nucleotides from the 3' end of the plus strand of segment 8 (Jennings et al., 1983).

With the identification of the sequence elements sufficient for replication and encapsidation of the M1 segment, it becomes possible to design plasmid vectors for the introduction of foreign genes into reovirus via cloned DNA intermediates. This will further the analysis of structure-function relationships in reovirus genes through the application of in vitro mutagenesis for the purposes of reverse genetics. Recently, reovirus has been produced by transfection with ssRNA and/or dsRNA derived from reovirions together with their in vitro-translated products in the presence of helper virus

(Roner et al., 1990). With the development of a packaging and selection system for the introduction of synthetic genome segments into reovirus, the consensus terminal sequences identified in this study can then be altered by in vitro mutagenesis to identify those elements that control specific aspects of replication and assembly.

CHAPTER FOUR

CLONING AND SEQUENCING OF REOVIRUS M1 GENOME SEGMENT

4.1 Introduction

Mammalian reoviruses are represented by 3 serotypes, 1, 2, and 3. Weiner et al. (1989a) determined the sequence of the serotype 3 strain Dearing M1 genome segment which is 2304 nucleotides long with a single large open reading frame from position 14 to 2224 encoding 736 predicted amino acids. There is a downstream in-frame translation initiation codon located at position 161. The sequence of M1 segments of other serotypes have not been determined. As stated before, genetic analyses of reassortant viruses have associated M1 genome segment with viral replication and pathogenesis. Comparison of the M1 nucleotide sequences of different serotypes would identify differences that might be of significance with regard to the M1 gene functions. Furthermore, the production of a packaging and selection system for the introduction of engineered genome segments into reovirus would be based on the cloned M1 segments. Therefore, the M1 genome segments of both type 1 reovirus strain Lang (T1 M1) and type 3 strain Dearing (T3 M1) were cloned and the nucleotide sequence of T1 M1 was compared with the published sequence of T3 M1.

4.2 Methods

The full-length M1 segments of T1 and T3 were cloned into plasmid pGEM 7Zf(+) from purified passage 3 viruses by using a modification of the methods described by

Cashdollar et al. (1982) and Schmid et al. (1987). cDNAs complementary to the + and - strands were primed using the M1 5'+ and M1 5'- oligonucleotides.

For sequencing, overlapping T1 M1 subclones were produced by progressive unidirectional deletion using the Erase-a-Base reaction kit and protocol supplied by Promega. Each M1 subclone was sequenced from both directions and with both dGTP and dITP in the sequencing reactions using SP6 and T7 primers and the Sequenase kit.

The sequence data were analyzed by the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package and the IBI DNA/Protein Sequence Analysis programs.

4.3 Results and Discussion

The T1 M1 genome segment consisted of 2304 nucleotides, the same length as the published T3 M1 sequence and contained the same initiation and termination codons (Wiener et al., 1989a). Comparison of T1 and T3 M1 sequences showed high homology in nucleotide sequences (2252/2304, 97.79%) (Fig. 4.1) and in predicted amino acid sequences (726/736, 98.64%) (Fig. 4.2). There were only 51 nucleotide differences, of which 39 (76.47%) occurred in the third codon position, with 16% and 8% in the 1st and 2nd codon positions respectively. There were no deletions or insertions between the T1 and T3 M1 segments. Out of the 2.2% total nucleotide variability observed, 5.3% of the third base positions were substituted as compared with 1.1% and 0.5% of the 1st and 2nd positions, respectively. The low level of third base substitution indicated that the T1 and T3 M1 segments were closely related and the relatively less variable 1st and 2nd

Fig. 4.1 Nucleotide sequence comparison of the M1 genome segment of reovirus serotype 1 strain Lang with the M1 segment of serotype 3 strain Dearing. Initiation and termination codons are underlined. Only the nucleotide differences are indicated for type 3.

```
T1     1  GCUAUUCGCG GUCAUGGCUU ACAUCGCAGU UCCUGCGGUG GUGGAUUCAC GUUCAAGUGA GGCUAUUGGA CUGCUAGAAU CGUUUGGAGU
T3     1                                                                 G

T1    91  AGACGCUGGG GCUGAUGCGA AUGACGUUUC AUAUCAAGAU CAUGACUAUG UGUUGGAUCA GUUACAGUAU AUGUUAGAUG GAUAUGAGGC
T3    91                C                                                                 C

T1   181  UGGCGACGUU AUCGAUGCAC UCGUCCACAA GAAUUGGUUA CAUCACUCCG UCUAUUGCUU GUUGCCACCC AAAAGUCAAC UACUAGAGUA
T3   181    U                                                   U                                      U

T1   271  UUGGAAAAGU AAUCCUUCAG UGAUACCGGA CAACGUUGAU CGUCGGCUUC GUAAACGACU AAUGCUAAAG AAAGAUCUCA GAAAAGAUGA
T3   271                      C                                                                      G

T1   361  UGAAUACAAU CAACUAGCGC GUGCUUUCAA GAUAUCGGAU GUCUACGCAC CUCUCAUCUC AUCCACGACG UCACCGAUGA CAAUGAUCCA
T3   361               G                                                                              A

T1   451  GAACUUGAAU CAAGGCGAGA UCGUGUACAC CACGACGGAC AGGGUAAUUG GGGCUAGAAU CUUGUUAUAU GCUCCUAGAA AGUACUAUGC
T3   451               G                                   A

T1   541  GUCAACUCUA UCAUUUACUA UGACUAAGUG CAUCAUUCCG UUUGGCAAAG AGGUGGGUCG UGUUCCUCAC UCUAGAUUUA AUGUUGGCAC
T3   541          G                                        U                  C

T1   631  AUUUCCAUCA AUUGCUACCC CGAAAUGUUU UGUCAUGAGU GGGGUUGAUA UUGAGUCCAU CCCAAAUGAA UUCAUCAAGU UGUUUUACCA
T3   631        G                                                                  U

T1   721  GCGCGUCAAG AGUGUUCACG CCAAUAUACU AAAUGACAUA UCACCUCAGA UCGUCUCUGA CAUGAUAAAC AGAAAGCGUU UGCGCGUUCA
T3   721                          U  C                 U                                      C

T1   811  UACUCCAUCA GAUCGUCGAG CCGCGCAGUU GAUGCAUUUG CCCUACCAUG UUAAACGAGG AGCGUCUCAC GUCGACGUUU ACAAGGUGGA
T3   811                                                U

T1   901  UGUUGUAGAC GUGUUGUUAG AGGUAGUGGA UGUGGCCGAU GGGUUGCGCA ACGUAUCUAG GAAACUAACU AUGCAUACCG UUCCGGUAUG
T3   901            A       C                                                                          U

T1   991  UAUUCUUGAA AUGUUGGGUA UUGAGAUUGC GGACUAUUGC AUUCGUCAAG AGGAUGGAAU GUUCACAGAU UGGUUCCUAC UUUUAACCAU
T3   991                                                                  C

T1  1081  GCUAUCUGAU GGCUUAACUG AUAGAAGGAC GCAUUGUCAA UACUUGAUUA AUCCGUCAAG UGUGCCUCCU GAUGUGAUAC UUAACAUCUC
T3  1081                G                                   G

T1  1171  AAUUACUGGA UUUAUAAAUA GGCAUACAAU CGAUGUCAUG CCUGAUAUAU AUGACUUCGU UAAACCCAUU GGCGCUGUGC UGCCUAAGGG
T3  1171                          A                   C

T1  1261  AUCAUUUAAA UCAACAAUUA UGAGAGUUCU UGAUUCAAUA UCAAUAUUAG GAGUCCAGAU CAUGCCGCGC GCGCAUGUAG UUGACUCGGA
T3  1261                                                      A    A                                    A

T1  1351  UGAGGUGGGC GAGCAAAUGG AGCCUACGUU UGAGCAUGCG GUUAUGGAGA UAUACAAAGG GAUUGCUGGC GUUGACUCGC UGGAUGAUCU
T3  1351                                    G

T1  1441  CAUCAAGUGG GUGCUGAACU CGGAUCUCAU UCCGCAUGAU GACAGGCUUG GCCAAUUAUU UCAAGCGUUU CUGCCUCUCG CAAAGGACUU
T3  1441               U                                        U                  U

T1  1531  GUUAGCUCCA AUGGCCAGAA AGUUUUAUGA UAACUCAAUG AGUGAGGGUA GAUUGCUGAC AUUCGCUCAU GCCGACAGUG AGUUGCUGAA
T3  1531  A                                                                 A

T1  1621  CGCAAAUUAC UUUGGUCAUU UAUUGCGACU AAAAAUACCA UAUAUUACAG AGGUUAAUCU GAUGAUUCGC AAGAAUCGUG AGGGUGGAGA
T3  1621           U

T1  1711  GCUAUUUCAG CUUGUGUUAU CGUAUCUAUA UAAAAUGUAU GCUACUAGCG CGCAGCCUAA AUGGUUUGGA UCAUUAUUGC GAUUGUUAAU
T3  1711                     U

T1  1801  AUGUCCCUGG UUACAUAUGG AGAAAUUAAU AGGAGAAGCA GACCCGGCAU CUACGUCGGC UGAAAUUGGA UGGCAUAUCC CUCGUGAACA
T3  1801                                                                          G

T1  1891  CCUGAUGCAA GAUGGAUGGU GUGGAUGUGA AGAUGGAUUC AUUCCCUAUG UUAGCAUACG UGCGCCAAGA CUGGUUAUGG AGGAGUUGAU
T3  1891                                    C                                     A

T1  1981  GGAGAAGAAC UGGGGCCAAU AUCAUGCCCA AGUUAUUGUC ACUGAUCAGC UUGUCGUAGG CGAACCGCGG AGGGUAUCUG CCAAGGCUGU
T3  1981                                                                          U

T1  2071  GAUCAAGGGU AAUCACUUAC CAGUUAAGUU AGUUUCACGA UUUGCAUGUU UCACAUUGAC GGCGAAGUAU GAGAUGAGGC UCUCGUGCGG
T3  2071               C                                                          U

T1  2161  CCAUAGCACU GGACGGGGGG CUGCAUACAA UGCGAGACUA GCUUUCCGAU CUGACUUGGC GUGAUCCGUG ACAUGCGUAG UGUGACACCU
T3  2161                    U  A           G

T1  2251  GCCCCUAGGU CAAUGGGGGU AGGGGGCGGG CUAAGACUAC GUACGCGCUU CAUC
T3  2251   U
```

Fig. 4.2 Comparison of the predicted amino acid sequence of the $\mu 2$ proteins of reovirus serotype 1 strain Lang and serotype 3 strain Dearing. Only the amino acid differences in serotype 3 relative to type 1 are indicated.

```
T1     1  MAYIAVPAVV DSRSSEAIGL LESFGVDAGA DANDVSYQDH DYVLDQLQYM LDGYEAGDVI DALVHKNWLH HSVYCLLPPK SQLLEYWKSN
T3     1

T1    91  PSVIPDNVDR RLRKRLMLKK DLRKDDEYNQ LARAFKISDV YAPLISSTTS PMTMIQNLNQ GEIVYTTTDR VIGARILLYA PRKYYASTLS
T3    91    A                                                                 R

T1   181  FTMTKCIIPF GKEVGRVPHS RFNVGTFPSI ATPKCFVMSG VDIESIPNEF IKLFYQRVKS VHANILNDIS PQIVSDMINR KRLRVHTPSD
T3   181

T1   271  RRAAQLMHLP YHVKRGASHV DVYKVDVVDV LLEVVDVADG LRNVSRKLTM HTVPVCILEM LGIEIADYCI RQEDGMFTDW FLLLTMLSDG
T3   271                                   M  F                                                          L

T1   361  LTDRRTHCQY LINPSSVPPD VILNISITGF INRHTIDVMP DIYDFVKPIG AVLPKGSFKS TIMRVLDSIS ILGVQIMPRA HVVDSDEVGE
T3   361              M                                                                                 I

T1   451  QMEPTFEHAV MEIYKGIAGV DSLDDLIKWV LNSDLIPHDD RLGQLFQAFL PLAKDLLAPM ARKFYDNSMS EGRLLTFAHA DSELLNANYF
T3   451         Q

T1   541  GHLLRLKIPY IIEVNLMIRK NREGGELFQL VLSYLYKMYA TSAQPKWFGS LLRLLICPWL HMEKLIGEAD PASTSAEIGW HIPREQLMQD
T3   541

T1   631  GWCGCEDGFI PYVSIRAPRL VMEELMEKNW GQYHAQVIVT DQLVVGEPRR VSAKAVIKGN HLPVKLVSRF ACFTLTAKYE MRLSCGHSTG
T3   631                          I

T1   721  RGAAYNARLA FRSDLA
T3   721       S
```

base codon positions indicated that the μ2 protein is structurally conserved. This is not surprising since μ2 is an internal core component that must interact with the other core proteins σ2, λ1, λ2, λ3 and possibly with the dsRNA genome.

The differences in phenotype of T1 and T3 that have been associated with the M1 genome segment, such as cytopathic effect, plaque size, the capacity to replicate in different cells and pathogenesis in infected animals (Moody and Joklik, 1989; Matoba et al., 1991, 1993; Sherry and Fields, 1989; Sherry and Blum, 1994; Haller et al., 1995), must be due to the 51 nucleotide substitutions and/or the 10 predicted amino acid substitutions. Although 41 out of the 51 nucleotide substitutions (1 in the 3' noncoding region and 40 others in the coding region) did not alter μ2 sequence, they may affect the secondary structure of the M1 mRNA and thus affect its interactions with cellular factors. Of the 10 amino acid substitutions, 5 were predicted to be non-conservative according to the properties of the corresponding amino acids (T1-T3: Q-R, L-F, F-L, H-Q, and N-S at amino acid positions 150, 302, 347, 458, and 726 respectively) (Zou and Brown, 1992a). One or more of these must be responsible for the phenotypic differences.

CHAPTER FIVE

CONSTITUTIVE EXPRESSION OF REOVIRUS μ2 PROTEIN IN MAMMALIAN CELLS

5.1 Introduction

Characterization of reovirus M1 deletion mutants identified the consensus termini of M1 required for replication and encapsidation, which consists of 132-135 nucleotides (nt) from the 5' end and 183-185 nt from the 3' end of the M1 plus strand. This information is applicable to production of a packaging and selection system for the introduction into reovirus of an M1 analogue containing the identified consensus sequences. However, reoviruses possessing an M1 analogue in place of wild type M1 will be replication defective. One approach to complement the defective function of the M1 gene was to develop stable cell lines expressing the M1 gene product, μ2 protein.

Viral proteins expressed from transfected DNA constructs are capable of complementing mutations in the homologous gene of defective viruses. For example, expression of the fusion protein gene of a virulent Newcastle disease virus strain provides an active fusion protein and complements the spread of infection by avirulent Newcastle disease virus (Morrison et al., 1991). Stable expression of the vaccinia virus K1L gene in rabbit cells complements the host range defect of a vaccinia virus mutant (Sutter et al., 1994).

Expression of a gene stably in mammalian cells involves the generation of DNA constructs for expression, introduction of the DNA constructs into host cells

(transfection), selection and screening of cells expressing the protein of interest.

Generally, an expression vector contains (1) an efficient promoter element for transcription initiation, (2) mRNA processing signals including mRNA cleavage and polyadenylation sequences and frequently intervening sequences, (3) polylinkers containing multiple endonuclease restriction sites for insertion of foreign DNA, and (4) selectable markers that can be used to select cells that have stably integrated the plasmid DNA (reviewed by Kaufman, 1990). Some vectors contain an SV40 origin of replication for amplification to high copy number in cells expressing the SV40 large T antigen such as COS monkey cells. Certain vectors have a segment of the bovine papillomavirus (BPV) DNA, either the entire viral genome or a fragment (the 69% transforming fragment), which confers the plasmid vector the ability of replicating episomally in the nucleus.

The promoters used can be constitutive or inducible. The latter can be used to express a protein which is potentially cytotoxic but it is important to ensure that the inducing stimulus does not interfere with the properties under study. Transcription termination, 3' end cleavage, and polyadenylation of precursor mRNAs are essential steps for the biogenesis of mRNA. Efficient signals for polyadenylation from the SV40 early transcription unit, the hepatitis B surface antigen transcription unit, and the mouse ß-globin gene are commonly used. The selectable markers can also be amplifiable, of which dihydrofolate reductase (DHFR) is the most widely used. Selection for amplification of the marker gene usually results in coamplification of the gene to be expressed and thus high expression of the gene. However, it is important that the gene

or its products not interfere with amplification or be toxic to the cell. If toxic genes are amplified it is possible to select for mutations in the coamplified gene which permit cell viability and thus the protein obtained after selection for expression at high level could be different from that encoded by the original transfected gene.

Polycistronic expression vectors have also been used to express foreign genes in mammalian cells. Polycistronic mRNAs are translated either through internal initiation mediated by the cap-independent translation initiation element (CITE) from picornaviruses (Kaufman et al., 1991; Wood et al., 1991) or potentially through termination-reinitiation (Kaufman et al., 1987). In either case, the polycistronic mRNA expression vectors containing the gene to be expressed in the 5' open reading frame and the selectable marker gene in the 3' position ensure that the desired gene is expressed in the selected cells.

Methods for introducing DNA into mammalian cells can be: (1) direct microinjection; (2) delivery of genetic information by viral vectors; (3) formation of complexes between DNA and chemical agents followed by active cellular uptake or (4) physical methods such as electronic field-mediated DNA transfer (electroporation) and (5) a microprojectile method in which DNA packaged into a "projectile" is "fired" into cells. Chemical-mediated procedures include formation of complexes of DNA with calcium phosphate, DEAE-dextran, Polybrene, or natural or synthetic cationic lipids (reviewed by Keown et al., 1990).

In this study, μ2-specific antibody was first produced through immunization of rabbits with fusion μ2 proteins expressed in E. coli for the detection of μ2 expression.

M1-containing monocistronic or dicistronic DNA constructs were then generated and transfected into cells followed by selection and screening of transfectants using the μ2-specific antibody. Stable μ2-expressing cell lines were established through transfection with dicistronic constructs. Furthermore, the μ2 protein expressed in these cells was shown to complement the growth at nonpermissive temperature of a reovirus temperature-sensitive (ts) mutant that has a defect in the M1 genome segment. This suggests the feasibility of complementing the M1 gene function defect of reoviruses containing a rescued M1 analogue. In addition, the μ2-expressing cell lines may be used to study structure-function relationship as well as interactions between μ2 protein and host factors since μ2 is implicated in pathogenesis and virulence.

5.2 Methods

5.2.1 Cells and viruses

Mouse fibroblast L929 cells, the Swiss mouse embryo cell line, NIH/3T3, and the mouse mammary tumor cell line, C127I, were used in this study.

The reovirus ts mutant (tsH11.2) with the ts phenotype mapped to the M1 segment was generated in K.Coombs' laboratory. The μ2 gene in tsH11.2 has two substitutions at nt 1209 and 1254 resulting in amino acid mutations, from $M^{398} \rightarrow T$ and $P^{414} \rightarrow H$, respectively (K. Coombs, personal communication). The efficiency of plating of tsH11.2 at 39^{0}C and higher is 1000 times lower than that at permissive temperature, 32^{0}C (K. Coombs, personal communication).

5.2.2 Generation of μ2-specific antibody for the detection of μ2 expression

The anti-reovirus serum derived from reovirus-immunized rabbits reacts very poorly with μ2 protein as shown in the precipitation of in vitro translation of transcripts from the cloned M1 genome segment. In order to detect μ2 expression in transfected mammalian cells, anti-μ2 antibody was induced in rabbits immunized with a μ2 fusion protein expressed in E. coli. T1 M1 and T3 M1 DNAs were subcloned into vector pATH so that the M1 gene coding sequence was fused to the E. coli Trp E gene according to the manufacturer's instructions (Oncogene Science, Inc.). Serum from immunized rabbits were then tested by RIPA of reovirus-infected cell lysate.

5.2.3 Generation of M1 gene-containing DNA constructs

Both monocistronic and dicistronic constructs were generated for the expression of μ2 proteins in transfected cells. T1 M1 and T3 M1 gene DNA sequences cloned in pGEM were subcloned into a mammalian expression vector pKJ1 (a gift from Dr. M. McBurney, University of Ottawa) under the control of the mouse phosphoglycerate kinase (pgk) promoter (Boer et al., 1990; McBurney et al., 1991). The neomycin resistant gene from transposon Tn5 (Beck et al., 1982; Thomas and Capecchi, 1987) was chosen as the selectable marker for the selection of transfectants. For generation of monocistronic constructs (pKJ1M1neo), the neo^r marker plus the SV40 promoter and the poly A signal were first cut out of plasmid pMAMneo (CLONTECH laboratories, Inc.) with Bam HI and inserted into pKJ1 linearized by Hind III downstream of the pgk polyadenylation signal (pKJ1neo). The plasmid pKJ1neo was then linearized by Kpn I

located in the polycloning site (PCS) between the pgk promoter and the pgk poly A signal and the M1 DNA was inserted by blunt-end ligation (pKJ1M1neo). The dicistronic constructs (M1CN) were generated by insertion of the neo^r sequence into pKJ1 cut open by Bam HI in the PCS followed by insertion of M1 upstream of the neo^r marker at the Kpn I site. Neo^r sequences were obtained from plasmid pMAMneo by polymerase chain reaction (PCR). The neo gene was translated due to the presence of a cap-independent translation initiation element (CITE) from encephalomyocarditis virus (EMCV). CITE was cut out of plasmid pCITE-1 (Novagen) with Eco RI and Nco I and inserted between M1 and neo^r at the Sma I site such that neo was fused with the two in-frame AUGs in CITE that were 9 nucleotides apart. The AUG of Neo was 9 nucleotides away from the 2nd AUG in CITE (AAUAUGGCCACAACCAUGGGGGAUCAUAUG). The dicistronic construct, M1CN, was transcribed using the pgk promoter. A further modification was the insertion of the bovine papillomavirus (BPV) transforming fragment (69% portion) derived from plasmid p306 (a gift of Dr. J. Campione, Health Canada) following Hind III and Bam HI digestion into the Hind III site following the pgk poly (A) signal of M1CN (M1CN-BPV). The three types of constructs are shown in Fig. 5.1.

5.2.4 Transfection, selection and screening

L929 cells were first transfected with pKJ1M1neo and M1CN using lipofectin and the protocol from GIBCO-BRL, selected with G-418 at 500 μg/ml for 14 days. Resistant colonies of cells were picked and screened by RIPA using μ2-specific antibody. Later, M1CN-BPV was transfected into L929, NIH/3T3 and C127I cells and μ2-expressing cells

were selected.

5.3 Results

5.3.1 The Trp-E-μ2 fusion protein-induced antibody was μ2-specific

In order to detect μ2 expression in transfected mammalian cells, anti-μ2 antibody was induced in rabbits immunized with a Trp-E-μ2 fusion protein expressed in E. coli. The anti-reovirus serum derived from rabbits immunized with purified reovirus reacts poorly with μ2 protein as indicated by poor precipitation of translation products of in vitro transcripts of the cloned M1 genome segment (Fig. 5.2). μ2 protein is barely detectable after immunoprecipitation. Clone 6 of the T1 M1 DNA (T1M1-6) has a deletion mutation at nucleotide 300 resulting in an early termination at base 329. The shorter translation product from this clone must be from other initiation codons. Fig. 5.3 shows the Trp-E-μ2 fusion proteins and the purified products used for immunization. The fusion protein band is not present in lysates of pATH-transformed E. coli or of untransformed bacteria but pATH-transformed E. coli has a band ~33 kDa which is the product of the Trp-E gene in the vector. Before immunization of rabbits, prebleeds were collected and tested by RIPA of reovirus-infected L929 lysate. Weekly serial bleeds from the immunized rabbits were collected for 7 weeks before testing for precipitation of the μ2 protein from reovirus-infected cells. In Fig. 5.4 the RIPA of all bleeds showed that in the T1 μ2-immunized rabbit even the first bleed possessed antibody activity but in T3 μ2-immunized rabbit the antibody activity developed more gradually. There was only one dominant protein band precipitated by each antibody. Antiserum produced

Fig. 5.1 M1 gene-containing DNA constructs used for transfection. The monocistronic pKJ1M1neo contains M1 driven by the pgk promoter and Neo driven by the SV40 promoter. The dicistronic M1CN has M1 and Neo under the same pgk promoter and the EMCV CITE between M1 and Neo. M1CN-BPV is M1CN plus the 69% transforming fragment of BPV.

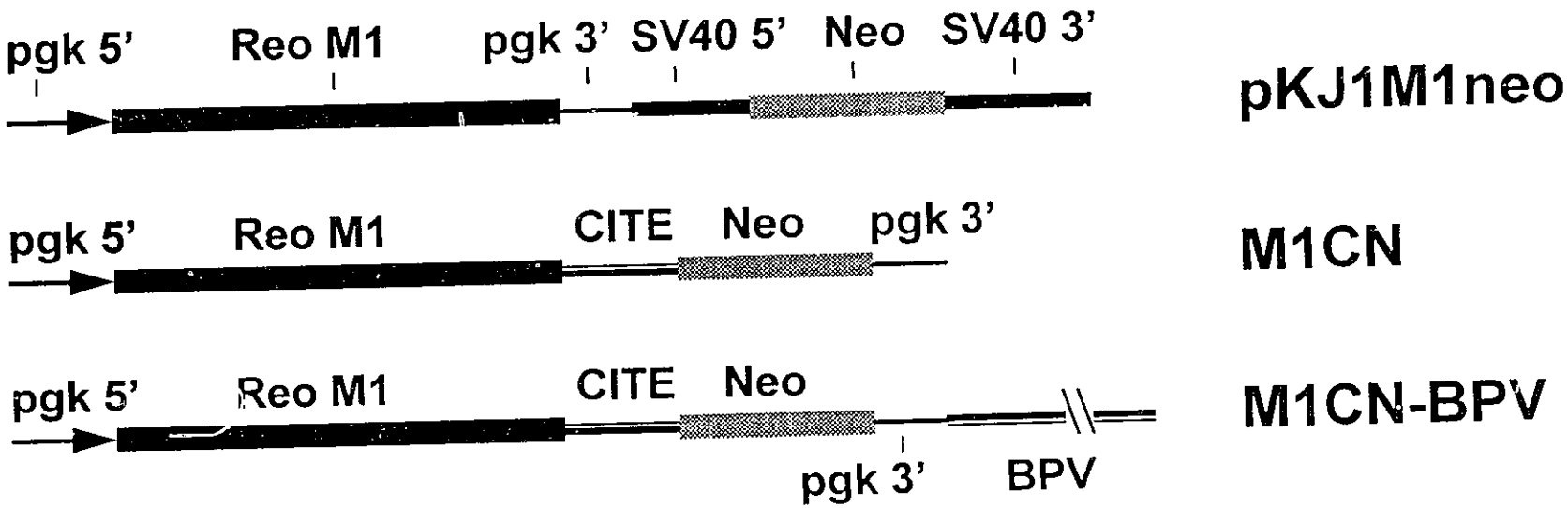

pgk 5'
Reo M1
pgk 3'
SV40 5'
Neo
SV40 3'
pKJ1M1neo
pgk 5'
Reo M1
CITE
Neo
pgk 3'
M1CN
pgk 5'
Reo M1
CITE
Neo
pgk 3'
BPV
M1CN-BPV

Fig. 5.2 RIPA of in vitro translation products of transcripts from the cloned M1 genome segment. T1M1 and T3M1 cloned in pGEM7 were transcribed in vitro with T7 RNA polymerase and the transcripts were translated in rabbit reticulocyte lysate in the presence of ^{35}S-methionine. Translation products were subjected to precipitation by anti-T1 Lang antibody. Both precipitated and unprecipitated samples were separated by 7.5% SDS-PAGE. u, unprecipitated; p, precipitated; cap, addition of cap analogue to the translation reaction; unc, no cap analogue in the translation reaction; T1M1-7, T3M1-18, T1M1-6, individual cDNA clones. T1M1-6 has a deletion at nucleotide 300 resulting in an early termination at base 329. Control has no mRNA in the translation reaction.

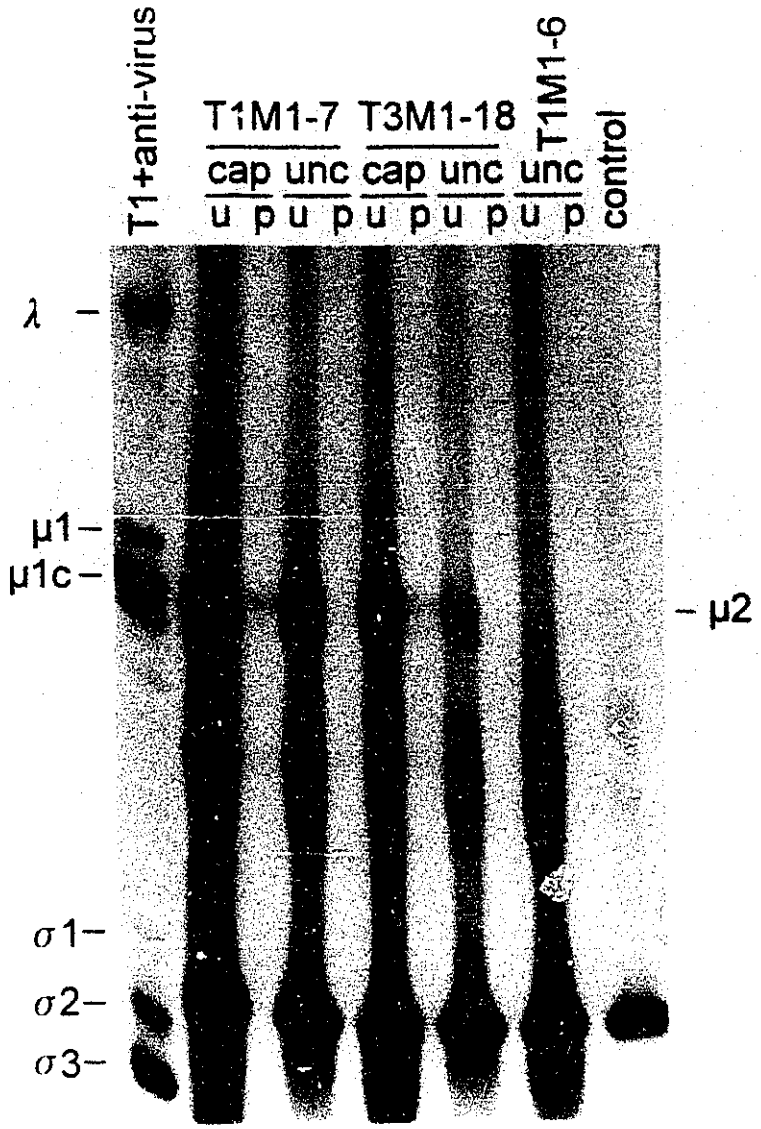

T1+anti-virus
T1M1-7
T3M1-18
T1M1-6
cap
unc
cap
unc
unc
u p u p u p u p u p
control
λ –
μ1 –
μ1c –
– μ2
σ1 –
σ2 –
σ3 –

Fig. 5.3 Purified fusion $\mu 2$ proteins expressed in E. coli. The left side shows screening of Trp-E-T1$\mu 2$ and Trp-E-T3$\mu 2$ fusion proteins. Two samples for different pATH vector clones of T1 and 4 samples for T3 were separated together with the lysate of untransformed E.coli RR1 and that of RR1 transformed by the expression vector pATH which produces a 33-kDa Trp-E protein. The SDS-PAGE-purified fusion proteins are shown.

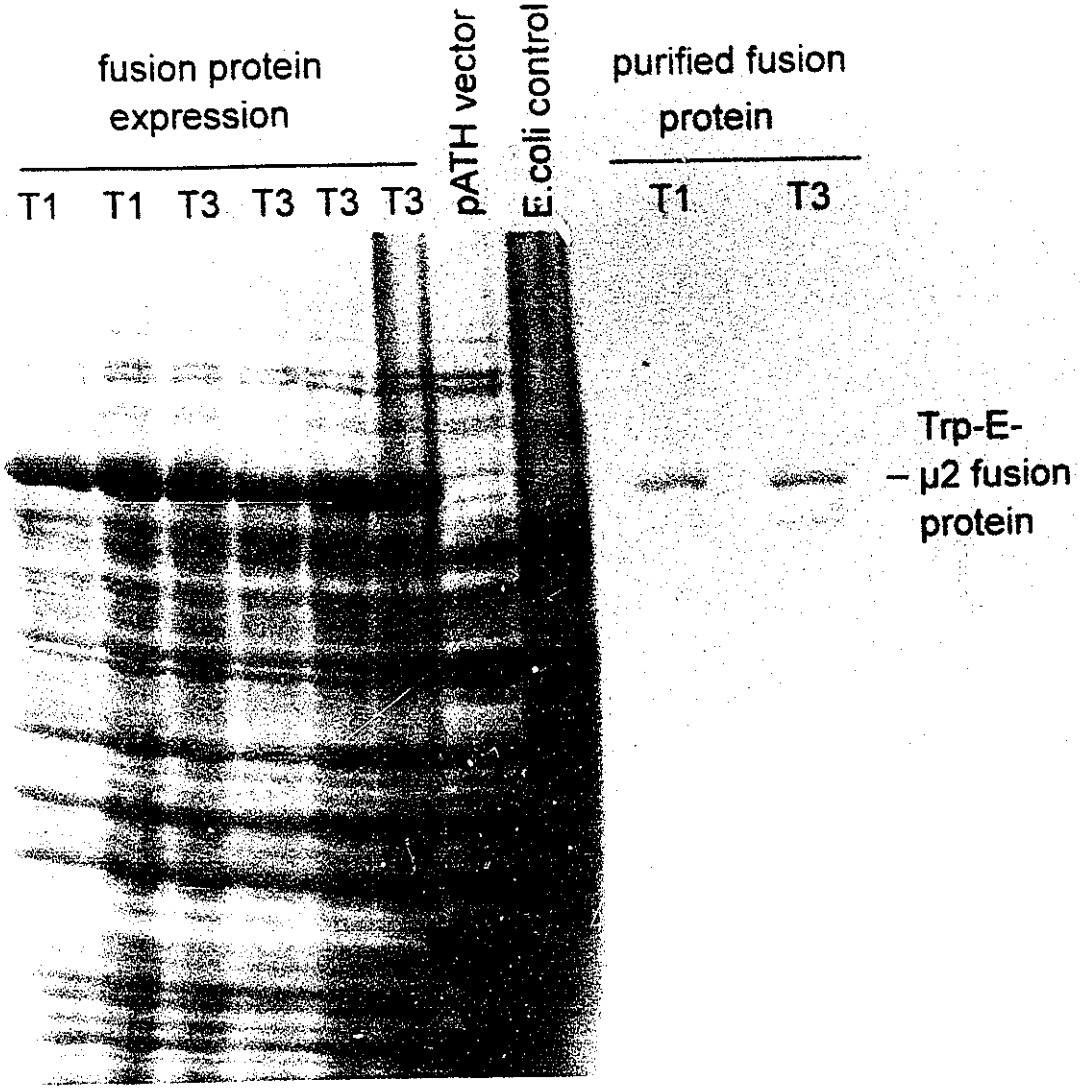

fusion protein expression
T1
T1
T3
T3
T3
T3
pATH vector
E.coli control
purified fusion protein
T1
T3
Trp-E-
– μ2 fusion
protein

Fig. 5.4 RIPA of T1 reovirus-infected L929 lysate by all bleeds from immunized rabbits. Two immunized rabbits (one by the T1 $\mu 2$ fusion protein and one by the T3 $\mu 2$ fusion protein) were bled once a week following boost and the sera were tested by precipitation of radiolabelled $\mu 2$ protein from T1 virus-infected L929 lysates. Precipitated proteins were separated on 7.5% polyacrylamide gels. T1, T3, lysate of infected L929 precipitated by anti-virus antibody.

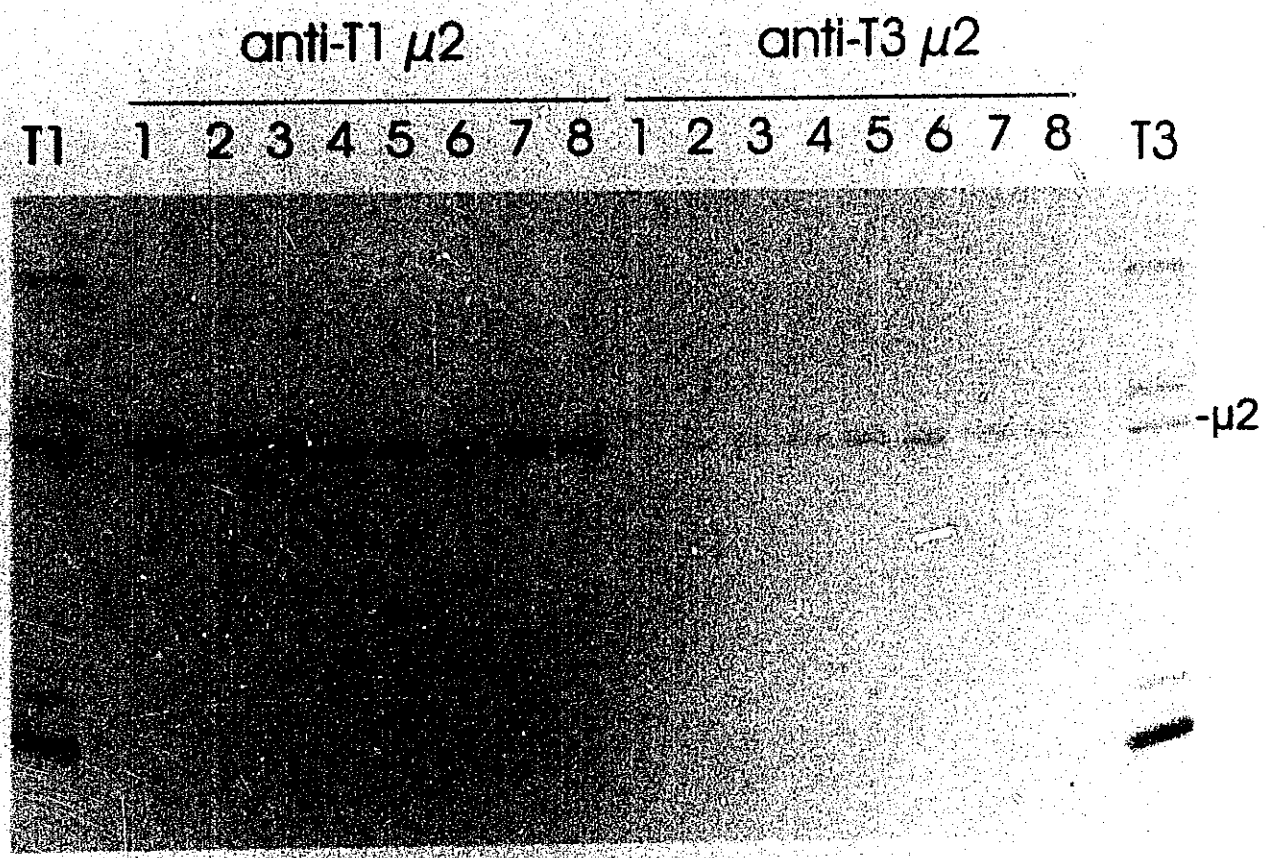

anti-T1 μ2
anti-T3 μ2
T1
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
T3
-μ2

Fig. 5.5 RIPA of μ2 proteins from in vitro translation products of M1 cDNA transcripts and melted reovirion dsRNA. The left side shows differential precipitations of T1-infected L929 lysate first by anti-T1 virus and then by anti-μ2 or vice versa. prebleed, precipitated by prebleed from Trp-E-μ2 immunized rabbit; mock, L929 mock infected. The other lanes show unprecipitated and precipitated in vitro translation products of in vitro T7 transcripts of cloned M1 cDNA or melted viral dsRNA. Two translation reactions of T1M1 ssRNA were included in the gel.

λ
μ1
μ2
μ1c
σ1
σ2
σ3
lysate
anti-virus T1
2nd precip anti-μ2
anti-μ2
2nd precip anti-T1
preblee
mock+anti-μ2
T1 dsRNA translation
T3 +anti-μ2
T1 dsRNA translation
T3
T1 M1 ssRNA
T3 translation
T1
T1 M1 ssRNA
T3 translation
T1 +anti-μ2
T3 infection+anti-μ2
T1 infection+anti-μ2
T1 infection
T1 infected

against T1 and T3 μ2 protein cross-reacted strongly with heterologous μ2 protein (data not shown), which was not surprising since the μ2 proteins of T1 and T3 have 98.6% amino acid homology. The antibodies were then tested further for precipitation of μ2 proteins from in vitro translation products of M1 cDNA transcripts and melted dsRNA of reoviruses (Fig. 5.5). The first five lanes show the proteins in T1-infected L cell lysates that were sequentially immunoprecipitated with anti-T1 reovirus serum followed by anti-μ2 fusion protein or vice versa. The amount of μ2 protein precipitated is similar whether or not immunoprecipitation with the anti-T1 reovirus immune serum preceded immunoprecipitation with the anti-μ2 antibody as predicted by the lack of immune response to μ2 protein. The σ proteins in the lane "2nd precip anti-μ2" were probably due to "carry-over" of protein A beads from the first immunoprecipitation with the anti-virus antibody since precipitation with the anti-μ2 antibody first (lane "anti-μ2") did not yield these protein bands. It can also be seen from this sequential precipitation that the amount of μ2 protein in infected cells was relatively high when compared with the abundant proteins μ1c and σ3, even though quantitation from analysis of the immunoprecipitated proteins is not accurate. Neither antiserum to T1 nor T3 reovirus was able to bind μ2 protein either by western blotting or by RIPA (data not shown). Protein with the mobility of μ2 protein was not precipitated from normal L929 cell lysate by anti-Trp-E-μ2 antibody nor from lysates of reovirus-infected L929 cells by preimmunization serum. The μ2 antibody precipitated a single major protein from in vitro translations of both M1 cDNA transcripts and melted reovirion dsRNA, which aligned with the protein precipitated from reovirus-infected cells when analyzed by SDS-

PAGE. RIPA of in vitro translation products gave other smaller bands that presumably represented short translation products from the same reading frame as the full size μ2 protein or degradation products. The results shown above indicated that the antibody induced in rabbits by μ2 fusion proteins expressed in E. coli reacted specifically with reovirus μ2 proteins.

5.3.2 Constitutive expression of μ2 in L929 with M1CN

Transfection of L929 cells with the T1 and T3 μ2 monocistronic constructs pKJ1M1neo followed by G418 selection gave a low but detectable level of T1μ2 and possibly T3μ2 expression in the uncloned pools of G418 resistant transfectants, which was not present in transfections with constructs containing the reverse orientation of the M1 gene (Fig. 5.6). However, screening of 18 clones of cells transfected by T1 M1 and 15 clones of cells transfected by T3 M1 in a repeat transfection did not yield any clones that expressed a detectable level of μ2 protein indicating that expression was poor from this type of construct. It was reasoned that if both M1 and Neo were put into one construct under one promoter with M1 upstream of Neo, selection with G418 would select for higher levels of μ2 expression. The utilization of polycistronic mRNA expression vectors containing the desired gene in the 5' open reading frame and a selectable gene in the 3' position had been reported to select cells that expressed high levels of the gene product in the 5' position (Kaufman et al., 1987; Levinson, 1990). Transfection of L929 with the dicistronic constructs (T1 and T3) of M1CN did give higher μ2 expression, with T1 μ2 much higher than T3 μ2 (Fig. 5.7). Furthermore, 4

Fig. 5.6 μ2 expression in L929 cells transfected with the monocistronic constructs pKJ1M1neo. After transfection and Geneticin selection resistant colonies of cells were pooled and tested for μ2 expression by RIPA using the anti-μ2 antibody. T1 infection, lysate of T1 reovirus-infected L929 cells; T1+anti-virus, precipitation of T1-infected cell lysate by anti-T1 virus immune serum; T1+prebleed, precipitation of T1-infected cell lysate by prebleed; T1M1/T3M1, precipitation of T1 or T3 M1-transfected cell lysate by anti-μ2 antibody; T1M1R/T3M1R, precipitation of lysate of cells transfected by DNA constructs containing T1 or T3 M1 gene in reverse orientation with regard to the pgk promoter.

T1 infection
T1+anti-virus
T1+prebleed
pKJ1M1neo
+anti-μ2
T1M1R
T1M1
T3M1R
T3M1
μ2

out of 4 clones of cells from transfection by T1 M1 and 2 out of 6 clones of cells from transfection by T3 M1 were positive for μ2 expression. The highest level of μ2 expression was seen for clone T1-11-1 that was ~5% of the level in infected cells as determined by densitometry of the autoradiogram.

5.3.3 The μ2 protein expressed constitutively in L cells is stable

To assess the possibility that the reduced level of μ2 protein was due to a decreased μ2 protein stability in transfected cells the stability of μ2 protein was determined by pulse-chase. Both T1-infected L929 cells and the clone of μ2-expressing L929 cells (T1-11-1) were labelled with ^{35}S-methionine at 50 μCi/ml for 60 min followed by removal of the labelling medium, wash with PBS twice and overlay with complete MEM. At indicated times (30 min, 60 min, 2 hrs, 4 hrs, 8 hrs) following complete MEM overlay, the cells were lysed with lysing buffer for RIPA (see general materials and methods). μ2 proteins were precipitated using anti-μ2 antibody and separated by SDS-PAGE in 7.5% polyacrylamide gels. The quantitation of μ2 protein was done by densitomitry. The pulse-chase experiment showed that the stability of μ2 protein in infected and transfected L929 cells was similar, with a half life of 5.8 hrs and 6.1 hrs, respectively, as determined by quantitation of the μ2 protein band. Amounts of μ2 proteins did not decrease significantly during a 4 hr chase period (Fig. 5.8), indicating that the μ2 proteins were relatively stable when expressed alone or during infection with the full complement of viral proteins. The μ2 protein expression level was stable in the μ2-expressing cell lines since the μ2 expression level remained unchanged after passage of

Fig. 5.7 μ2 expression in L929 cells transfected with the dicistronic constructs M1CN. Following transfection and selection resistant colonies of cells were grown up and tested by RIPA with the μ2 antibody. T1, lysate of T1-infected L929; mock, mock infected L929; M1-Neo fusion, precipitation by μ2 antibody of the pool of resistant colonies from transfection of L929 cells by a construct in which Neo was fused to the T1M1 5'-terminal 135 nucleotides and the M1-neo was driven by the pgk promoter. T1-9/T1-11 were clones of M1CN T1M1 constructs and T3-1/T3-6 were clones of M1CN T3M1 constructs. The pool of transfectants from T3-6 was lost during passage.

T1+anti-virus
T1+anti-μ2
T1+preblеed
mock+anti-μ2
M1-Neo fusion
M1CN+anti-μ2
T1-9
pool
clone 1
clone 2
T1-11
pool
clone 1
clone 2
T3-1
pool
clone 1
clone 2
T3-6
clone 1
clone 2
T1+anti-μ2
-μ2

Fig. 5.8 Pulse-chase of T1 reovirus-infected L929 cells and the μ2-expressing L929. The cells were labelled with ^{35}S-methionine for 60 min followed by chase for 30 min, 60 min, 2 hrs, 4 hrs, or 8 hrs. Cells were lysed and precipitated with anti-T1 μ2 serum and separated by 7.5% polyacrylamide gels.

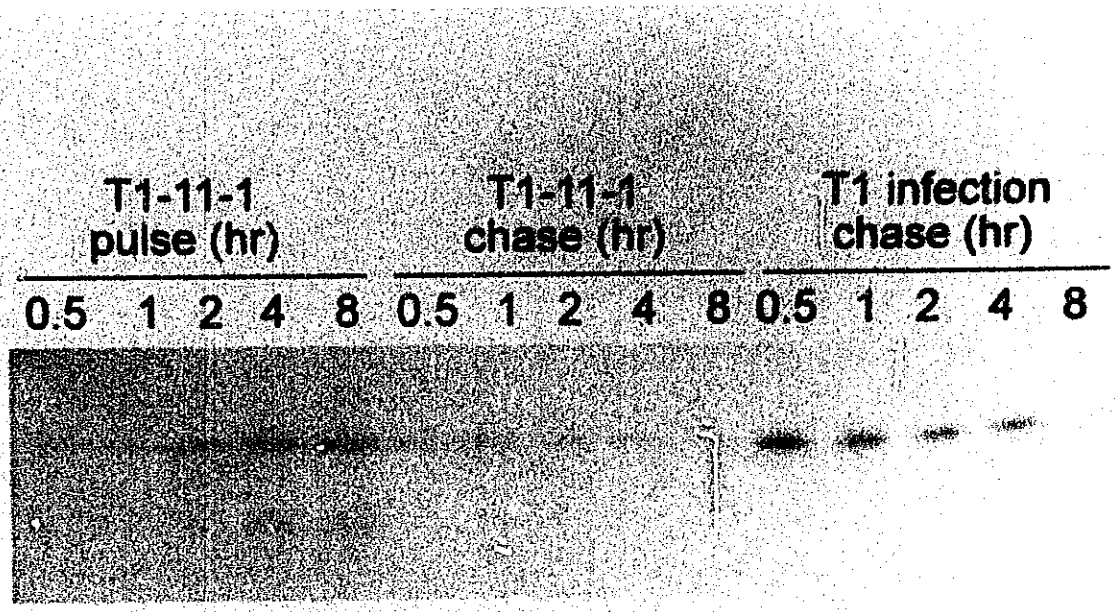
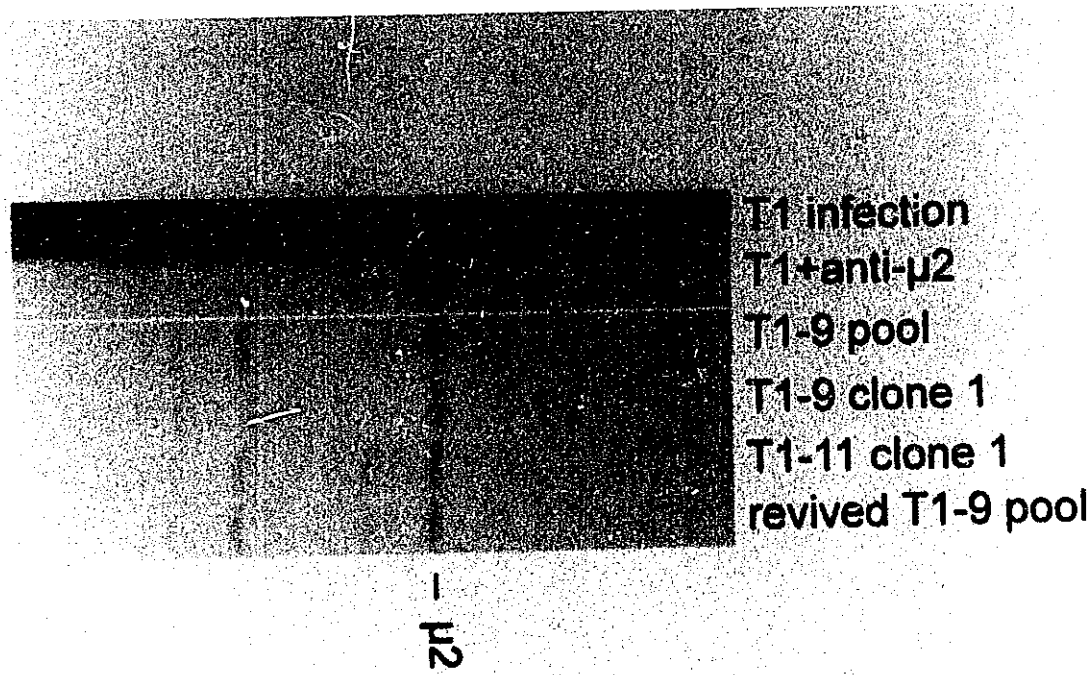

T1-11-1
pulse (hr)
T1-11-1
chase (hr)
T1 infection
chase (hr)
0.5 1 2 4 8 0.5 1 2 4 8 0.5 1 2 4 8

Fig. 5.9 μ2 expression in transfected L929 cells after passage for over 6 months. The μ2-expressing L929 cells were passaged as stationary culture at 37^{0}C without Geneticin. Cells were subcultured every week. After 6-month of passage cells were trypsinized and frozen in MEM with 10% fetal calf serum and 10% DMSO in liquid nitrogen. Cells were labelled with ^{35}S-methionine, and μ2 was immunoprecipitated with anti-μ2 antibody before analysis by SDS-PAGE. T1 infection, lysate of T1 reovirus-infected L929 cells; T1+anti-μ2, precipitation of T1-infected cell lysate by anti-μ2 antibody; T1-9, T1-11, DNA clones of M1CN constructs; T1-9 pool, precipitation of pool of T1-9 transfectants by anti-μ2 antibody; T1-9 clone 1, precipitation of clone 1 of T1-9 transfectants; T1-11 clone 1, precipitation of clone 1 of T1-11 transfectants (T1-11-1); Revived T1-9 pool, precipitation of pool of T1-9 transfectants revived from liquid nitrogen storage.


T1 infection
T1+anti-μ2
T1-9 pool
T1-9 clone 1
T1-11 clone 1
revived T1-9 pool
- μ2

the cells for over 6 months or after liquid nitrogen storage of the cells (Fig. 5.9). Passage of clone T1-11-1 for over two years (in the absence of G418) maintained the same level of expression (data not shown).

5.3.4 The μ2 protein from transfected cells was indistinguishable from viral μ2 protein by peptide mapping

To further test if the μ2 protein expressed in transfected cells was identical to the counterpart produced during infection, μ2 proteins were subjected to peptide mapping using partial proteolysis and SDS-PAGE. Proteins were purified by precipitation with μ2 antibody from T1 Lang infection, from in vitro translation of T1 M1 cDNA transcripts as well as from the T1 μ2-expressing cell line T1-11-1 and then digested with Staphylococcus aureus V8 protease as described by Cleveland et al. (1977). The μ1c protein which runs very close to μ2 on SDS-PAGE was also precipitated with the anti-reovirus antibody from T1 Lang-infected cells and digested with graded doses of V8 protease. The digestion patterns of μ2 proteins from different sources were indistinguishable from each other but were clearly different from that of μ1c, providing further evidence that the μ2 protein stably expressed by transfection was the same as the authentic μ2 protein (Fig. 5.10).

5.3.5 Expression of T3μ2 was improved by including the transforming fragment of bovine papillomavirus in the dicistronic plasmid M1CN to produce M1CN-BPV

Although reovirus M1 was constitutively expressed in L929 cells the level of μ2

Fig. 5.10 μ2 protein constitutively produced in L cells was indistinguishable by peptide mapping from authentic μ2 protein. μ2 protein was precipitated with anti-μ2 antibody from T1 virus-infected L929 and the μ2-expressing L929, T1-11-1. μ1c was precipitated with anti-T1 virus antibody from T1-infected L929. They and the in vitro translation product of the T7 transcript of M1 DNA cloned in pGEM were separated by SDS-PAGE, μ1c and μ2 protein bands were cut out and loaded onto 15% polyacrylamide gels containing SDS. V8 protease as indicated was then added to each sample well. After electrophoresis until the bromophenol blue tracer was 1 cm from the separating gel the power was turned off for 15 min and then resumed till the dye was out of the gel (Cleveland et al., 1977).

undigested
T1 viral µ1c
T1 viral µ2
in vitro T1µ2
T1-11-1 µ2
0.001µg V8
viral µ1c
viral µ2
in vitro µ2
T1-11-1 µ2
0.01µg V8
viral µ1c
viral µ2
in vitro µ2
T1-11-1 µ2
0.1µg V8
viral µ1c
viral µ2
in vitro µ2
T1-11-1 µ2

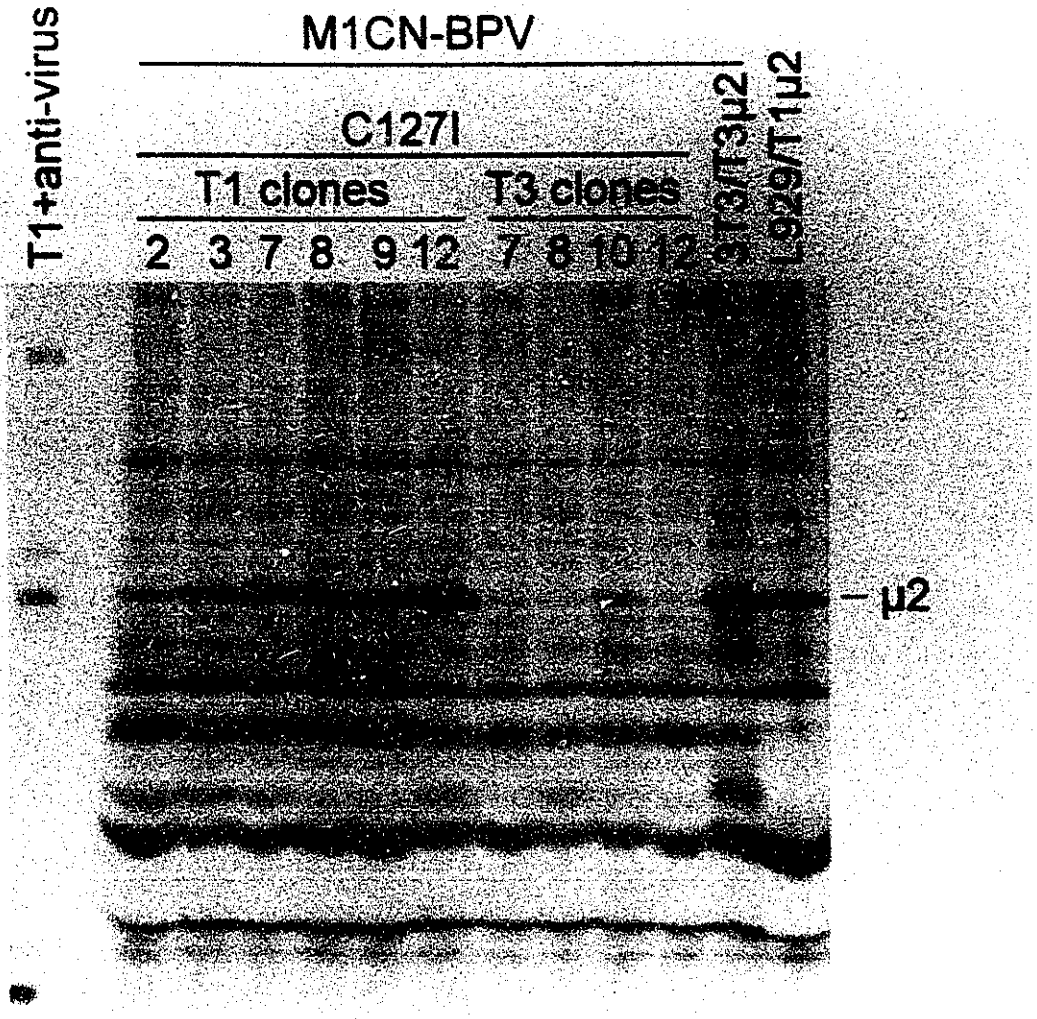
expressed was relatively low especially for T3 μ2 protein. To increase the level of expression, the transforming fragment of bovine papillomavirus was introduced into the M1CN constructs, M1CN-BPV was constructed and transfected into L929, NIH/3T3, and C127I cells. After selection with G418, more resistant colonies were obtained from transfections with M1CN-BPV than from M1CN transfections (Table 5.1). Screening

Table 5.1 Efficiency of G418-resistant colony production following transfection with different constructs

type of constructs	cell type	DNA clone	No. of colonies
pKJ1M1neo	L929	T1-12	18
	L929	T3-4	15
M1CN	L929	T1-9	2
	L929	T1-11	2
	L929	T3-1	2
	L929	T3-6	4
M1CN-BPV	L929	T1-9-1	10
	L929	T3-15-33	33
	3T3	T1-9-1	4
	3T3	T3-15-33	39
	C127I	T1-9-51	26
	C127I	T3-15-33	14

of the pool and 4 to 15 clones of transfectants from each transfection by RIPA showed that the expression in L929 cells was not improved whereas expression of T3μ2 in 3T3 cells was much higher than in L929. Expression of T1 μ2 was much higher in C127I cells than in T1-11-1, the L929 cell line expressing the highest level of T1μ2 via construct M1CN (Fig. 5.11). The level of T3μ2 expression was considerably lower than T1μ2 in C127I cells (Fig. 5.11) whereas the expression of T1μ2 was similar to T3μ2 in pools and most clones of 3T3 cells (data not shown). In one clone of 3T3 transfected

Fig. 5.11 μ2 expression in NIH/3T3 and C127I transfected with M1CN-BPV. 3T3 and C127I cells were transfected with M1CN-BPV constructs of T1M1 and T3M1 using lipofectin. Transfectants were selected with geneticin at 500 μg/ml for 14 days. Cloned cells of transfectants were labelled and lysed and μ2 was precipitated by anti-μ2 antibody and analyzed in 7.5% polyacrylamide gels. Representative positive clones of C127I transfectants, the 3T3 clone expressing the highest level of T3μ2 on transfection with the M1CN-BPV construct (T3-15-33 clone 1) and the L929 clone expressing the highest level of T1μ2 on transfection with the M1CN construct (T1-11-1, lane "L929/T1μ2") are shown in this figure. Precipitation of T1 virus-infected L929 lysate with anti-T1 serum is included as a size reference (lane "T1+anti-virus").


M1CN-BPV
C127I
T1 clones
T3 clones
2 3 7 8 9 12 7 8 10 12
T3/T3μ2
T1/T1μ2
T1+anti-virus
μ2

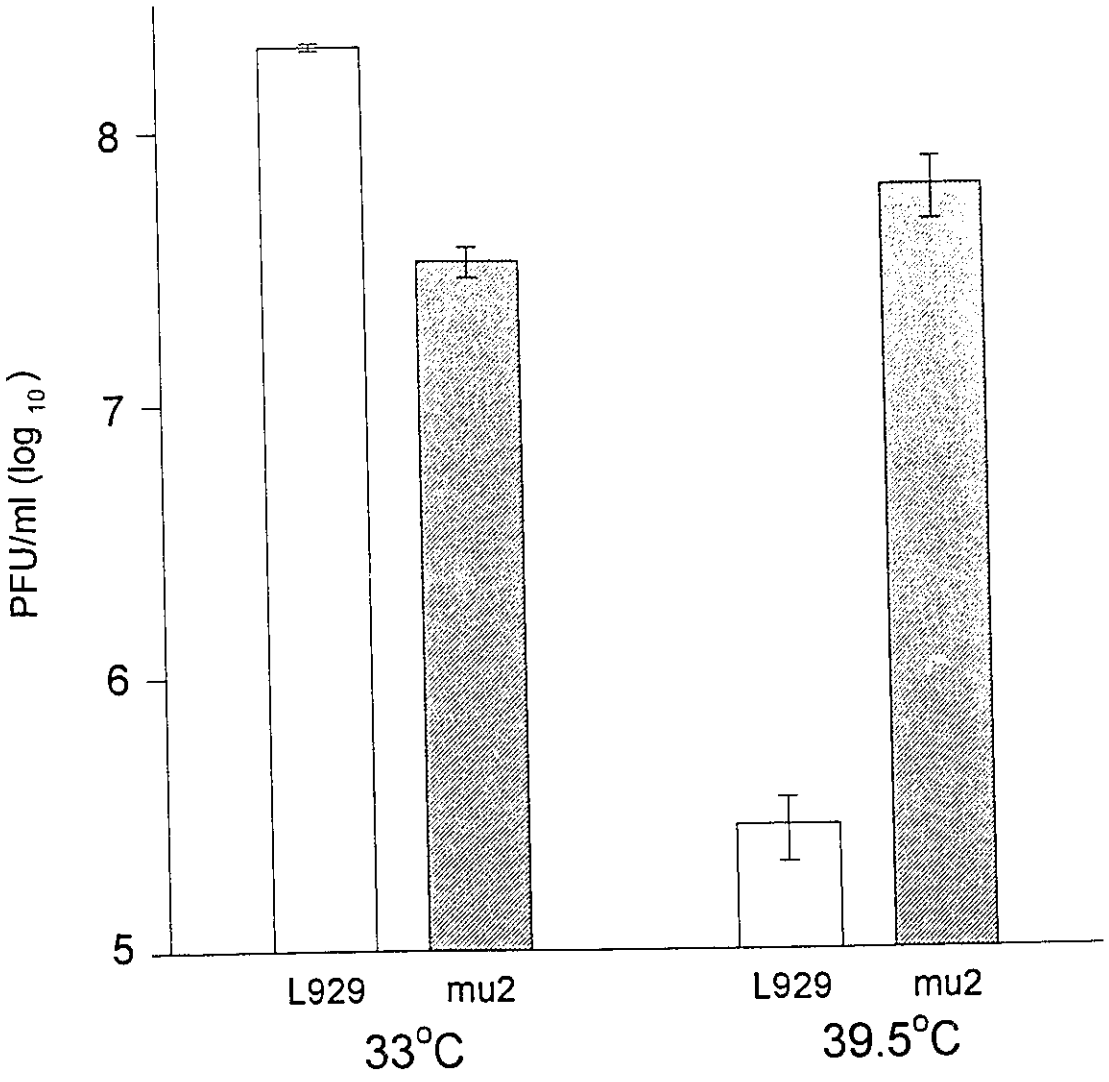
by T3M1-containing M1CN-BPV (T3-15-33 clone 1, lane "3T3/T3μ2" in Fig. 5.11), the expression of T3μ2 was much higher than that of T1μ2 and also higher than that of T3μ2 in C127I cells. However, this level of μ2 expression was less stable and decreased to a lower but still detectable level after continuous passage of the cell lines (data not shown). Nevertheless, this work showed that it is possible to get higher expression of μ2 and that different types of mouse cells have different abilities to express μ2 protein.

5.3.6 The μ2-expressing cells supported the growth of the M1 ts mutant, tsH11.2

One of the goals of expressing μ2 stably in mammalian cells was to complement the defective function of μ2 protein for reoviruses containing a foreign gene in place of authentic M1. The μ2-expressing L929 clone T1-11-1 generated with M1CN was infected with a reovirus M1 ts mutant tsH11.2 to assess the ability of μ2 expression to complement the ts defect. tsH11.2 is restricted in growth at 39.6^{0}C due to mutations in the M1 gene (K. Coombs, personal communication). The plaque-forming efficiency of tsH11.2 on μ2-expressing cells was over 200 times higher than that on normal L929 cells at 39.6^{0}C (6.2×10^7 PFU/ml versus 2.8×10^5 PFU/ml) whereas at 33^{0}C the plaque-forming efficiency on normal L929 cells was slightly higher than that on μ2-expressing L929 cells (2.0×10^8 PFU/ml versus 3.3×10^7 PFU/ml), indicating that the μ2 protein constitutively expressed in L929 cells was able to complement the growth of the M1 ts mutant, tsH11.2 (Fig. 5.12).

To test if the complementation is specific to the M1 gene defect, normal L929 cells, μ2-expressing T1-11-1 cells and L929 cells transfected by pKJ2, a plasmid that contains

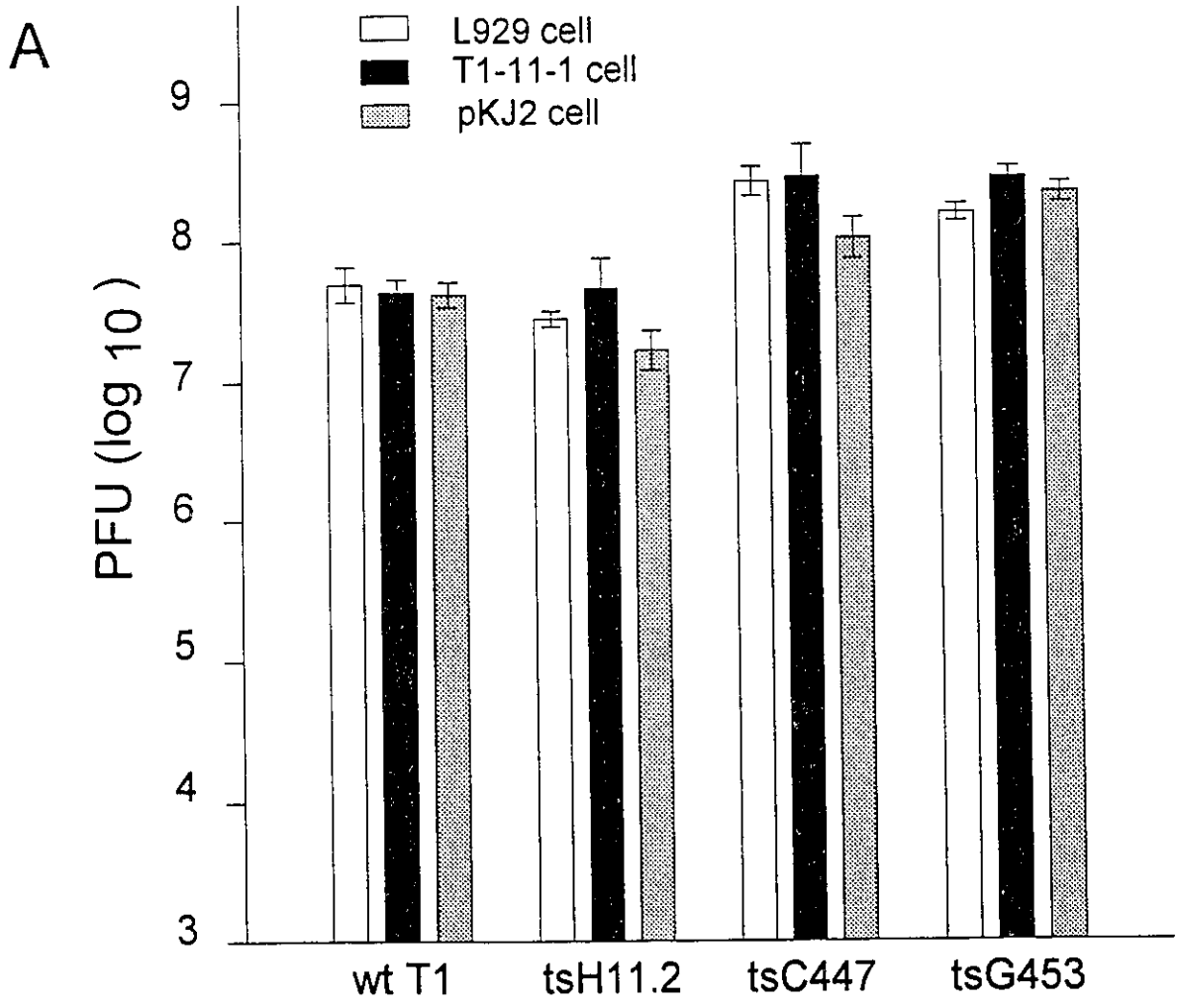
Fig. 5.12 Plaque formation of the M1 ts mutant, tsH11.2, at permissive and nonpermissive temperatures on normal L929 and the μ2-expressing T1-11-1 cells. The mutant reovirus tsH11.2 was passaged in normal L929 twice before assay on normal L929 cells and the μ2-expressing L929 cell line, T1-11-1. The data are the result of triplicates of the assay at 39.5^{0}C and duplicates at 33^{0}C.


PFU/ml (log 10)
8
7
6
5
L929
mu2
L929
mu2
33°C
39.5°C

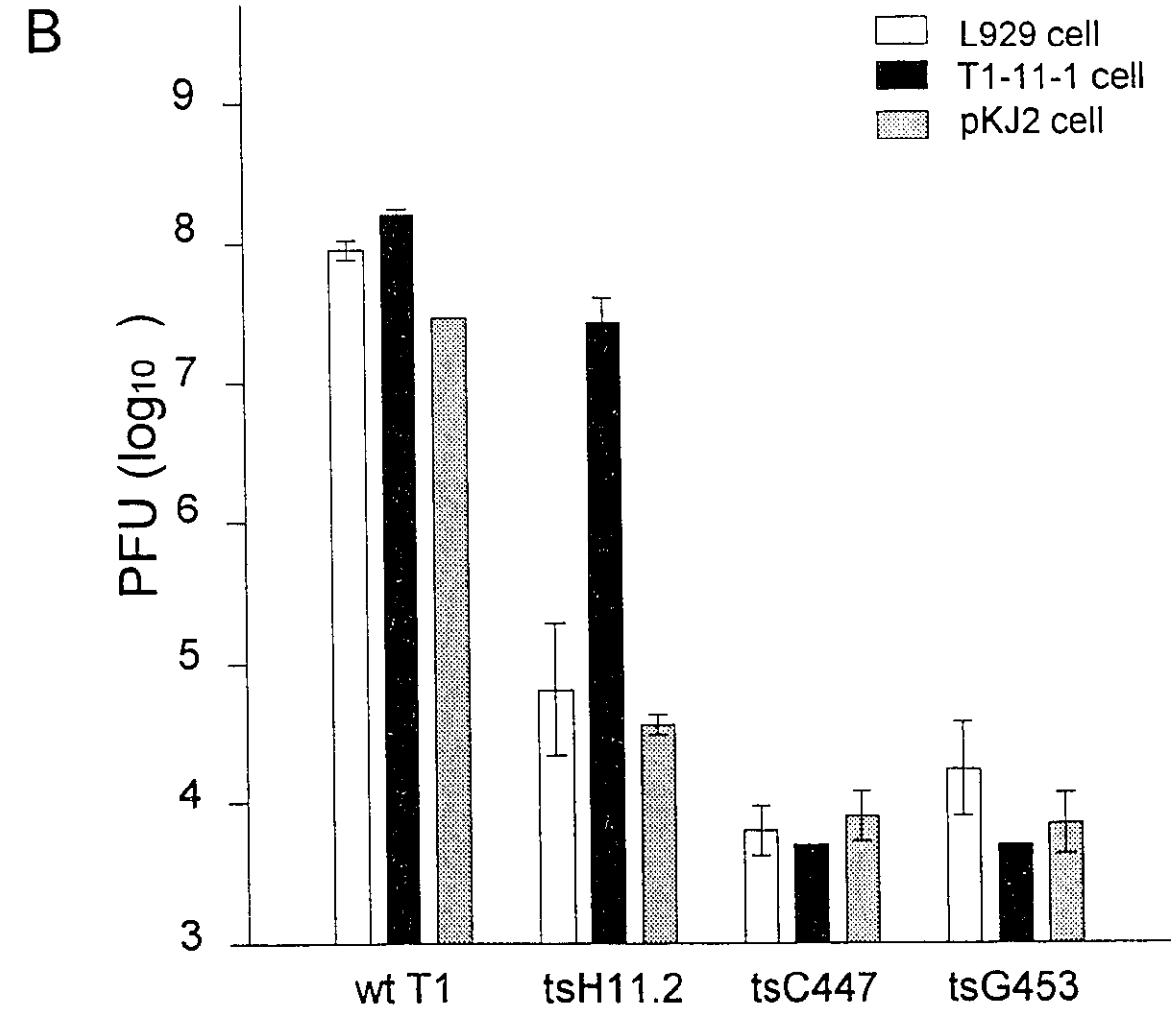
the neo gene driven by pgk promoter (a gift of M. McBurney), were infected at m.o.i. 3 by wild type reovirus type 1 (wt T1), tsH11.2, tsC447 and tsG453 that have ts mutations mapped to segment S2 and S4 respectively (gifts of K. Coombs). Following incubation at 33°C or 39.5°C for 72 hrs viruses were harvested and titrated in normal L929 cells at 33°C. Triplicate infections by tsH11.2 or tsC447 and duplicate infections by wt T1 or tsG453 were performed for each cell type and at each temperature condition. Fig. 5.13 shows the virus yield from different viruses in different cells at 33°C or 39.5°C; the virus yields were assayed at permissive temperature on L929 cells. At 33°C the growth of each virus in the three types of cells showed no significant difference. In all three cell types at 39.5°C the growth of wt T1 was not adversely affected but the growth of tsC447 and tsG453 was all inhibited yielding titre about 10,000 times lower than those at 33°C. However, the yield of tsH11.2 in the μ2-expressing T1-11-1 cells at 39.5°C was complemented, such that the titre was similar to the yield at 33°C ($2.9x10^7$ versus $5x10^7$ PFU/ml), compared to the dramatic decrease in virus yield at 39.5°C relative to 33°C in normal L929 cells ($9x10^4$ versus $2.8x10^7$ PFU/ml) or in the control transfectant pKJ2 cells ($3.7x10^4$ versus $1.7x10^7$ PFU/ml), indicating that the complementation in T1-11-1 cells was not due to properties of the plasmid vector background. The results clearly demonstrated that (1) the μ2-expressing cell line T1-11-1 complemented the growth of the M1 ts mutant tsH11.2; (2) the complementation was specific for the M1 gene defect as neither of the other two reovirus ts mutants could be complemented by the μ2-expressing cell line.

To confirm that the complementation was phenotypic and not the result of genetic

Fig. 5.13 Complementation of the M1 ts mutant, tsH11.2, by μ2 protein expressed in T1-11-1 L929. Panel A, yield of virus at 33^{0}C. Panel B, yield of virus at 39.5^{0}C. Normal L929, the μ2-expressing L929 cell line (T1-11-1), and L929 cells transfected by the vector control (pKJ2) were infected separately with wild type reovirus T1 (wt T1), tsH11.2, tsC447, and tsG453 at an m.o.i. of 3. Duplicate infections were performed for wt T1 and tsG453 and triplicate infections for tsH11.2 and tsC447. Following incubation at 33^{0}C or 39.5^{0}C for 72 hrs viruses were harvested by freeze-thaw three times and titrated at 33^{0}C in normal L929 cells. In panel B, the titres of virus yield from infection of T1-11-1 by tsG453 and tsC447 were less than 10^4 PFU/ml.



growth of different viruses in different cells at 33^{0}C



growth of different viruses in different cells at 39.5^{0}C

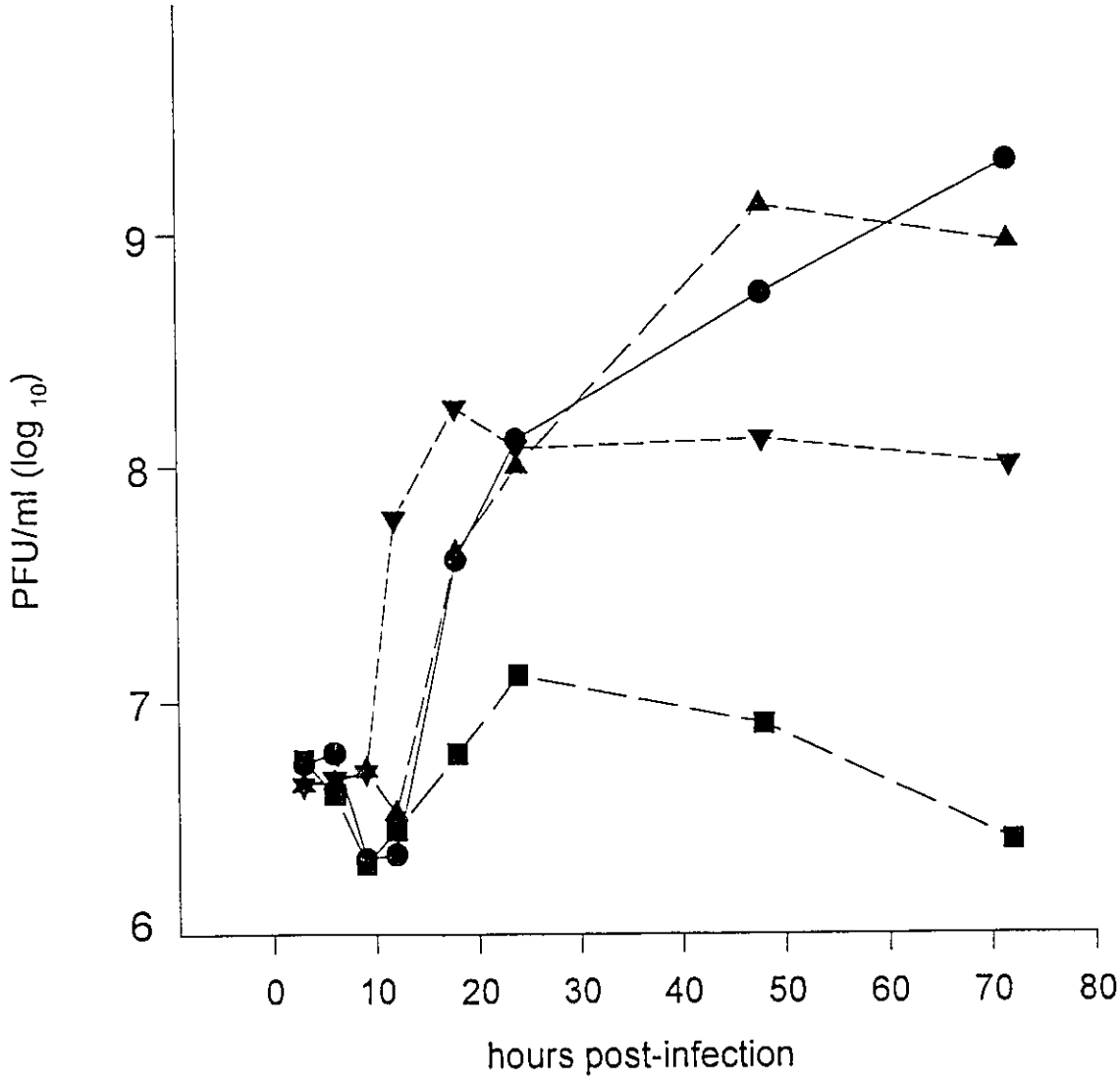
reversion, the virus harvests of tsH11.2 and those of tsC447 from T1-11-1 cells at 39.5^{0}C were also titrated at 39.5^{0}C. The titres for tsH11.2 at 39.5^{0}C versus 33^{0}C were $4.5x10^4$ and $2.9x10^7$ PFU/ml, respectively and those for tsC447 were both below 10^4 PFU/ml, respectively, indicating that the complementation was indeed phenotypic and was not due to genetic reversion since the efficiency of plating of tsH11.2 was similar whether grown on wild type L929 cells ($1.4x10^{-3}$) or μ2-expressing, T1-11-1, cells ($1.5x10^{-3}$).

Further time course studies showed that in μ2-expressing L929 cells the ts mutant grew faster and reached peak titre 6-12 hrs earlier than in normal L929 cells at 39.6^{0}C. Furthermore, in normal L929 cells at the nonpermissive temperature, the virus titre decreased after reaching its peak at 24 h postinfection, suggesting that virus produced in wild type cells was not as stable as those produced in μ2-expressing L929 cells (Fig. 5.14).

5.4 Discussion

The level of protein expression from heterologous genes introduced into mammalian cells depends on multiple factors, including DNA copy number, transcription efficiency, mRNA processing, mRNA transport, mRNA stability, and translational or post-translational events. The pgk promoter used in this study drives the normal house-keeping gene, phosphoglycerate kinase and thus was a promising promoter for constitutive expression. The pgk driven Neo constructs were shown to give rise to more than 10 times as many drug resistant colonies in P19 embryonal carcinoma cells as

Fig. 5.14 Growth kinetics of tsH11.2 in normal L929 and the μ2-expressing L929. 35mm dishes of L929 and of T1-11-1 were infected by tsH11.2 at an moi of 15 and incubated at 33^{0}C or 39.6^{0}C. Dishes were frozen at various times before harvesting by freeze-thaw (3x) and titration by plaque assay. The following symbols are used: ■, growth of tsH11.2 on normal L929 at 39.5^{0}C; ▼, on T1-11-1 at 39.5^{0}C; ●, on normal L929 at 33^{0}C; ▲, on T1-11-1 at 33^{0}C.


PFU/ml (log 10)
9
8
7
6
0
10
20
30
40
50
60
70
80
hours post-infection

pSV2neo that has a SV40 promoter (McBurney et al., 1991), indicating that it is a strong promoter in mouse cells. Transfection of L929 cells with constructs containing the neo gene under pgk promoter showed stable expression of moderate levels of the neo gene product asdetected by specific antibody (data not shown).

Although monocistronic DNA constructs have been successfully used for stable expression of many proteins, it was necessary to generate dicistronic constructs that possessed two advantages: high expression frequency and higher expression level. For one transfection with the monocistronic constructs, screening of 15 clones of transfectants failed to yield one positive clone even though the uncloned pool of G418-resistant transfectants expressed a detectable amount of μ2. However, when dicistronic constructs (M1CN) were used, 4 out of 4 clones of T1-transfected cells and 2 out of 6 clones of T3 M1-transfected cells were positive for μ2 expression.

Inclusion of the transforming fragment of BPV into the expression vector has been reported to increase expression efficiency as the BPV fragment allows episomal replication of the transfecting plasmid and thus provides amplification of the gene to be expressed by a gene dosage effect (reviewed by DiMaio, 1987). It has been shown that in 3T3 or C127 cells, BPV-containing constructs tend to exist as plasmids with multiple copies whereas in some other cells they tend to become integrated and consequently give low expression (DiMaio et al., 1982; Sambrook et al., 1985). Higher expression was indeed achieved in 3T3 and C127I cells with BPV-containing constructs in this study in a type-specific manner such that T1μ2 was expressed better in C127I cells and T3μ2 was expressed to similar level of T1μ2 in 3T3 cells. T3-15-33 clone 1 was an exception that

produced a higher level of T3μ2 protein than that from transfection by T1M1-containing M1CN-BPV. However, the expression of T3 μ2 by this clone decreased after passage of the cell line which contrasts with the cell lines generated with M1CN. Presumably the copy number of BPV-containing plasmid decreased, or the plasmid was rearranged during serial passage as has been previously documented for this type of vector (DiMaio, 1987; Kitamura et al., 1991).

Cell lines generated with dicistronic constructs M1CN are highly stable. Cells maintained in liquid nitrogen or passaged for over two years (e.g., the highest level μ2-expressing L929 clone T1-11-1) without G418 pressure showed the same level of expression. The level of μ2 expression reached in the stable cell lines was only about 5% of that in infected cells. However, transient expression levels of μ2 in COS-1 cells (data not shown) as well as in L929 cells driven by the T7 RNA polymerase from recombinant vaccinia virus were much higher than in infected cells (see chapter six). It is conceivable that stable expression of the μ2 gene requires a balanced situation and that only moderate levels of expression can be maintained stably without being deleterious to the cell. This notion follows from the possibility that the μ2 gene product is toxic as suggested by the control of cytopathic effect by the M1 genome segment (Moody and Joklik, 1989).

There have been reports on the complementary effect of viral gene products expressed in transfected cells on the growth of viruses possessing defects in the corresponding gene (Morrison et al., 1991; Sutter et al., 1994). A poliovirus defective genome was complemented by a recombinant vaccinia virus which provided poliovirus P1 capsid

precursor in trans (Ansardi et al., 1993). The final goal of this research is the development of a packaging and selection system for the introduction of a engineered gene into reovirus and consequently the generation of reovirus as an expression vector. The complementation of tsH11.2 indicates that the μ2 protein expressed from transfected M1 DNA constructs is functional and that the expression level reached in T1-11-1 is sufficient to support reovirus replication. Therefore, these L929 cell lines that constitutively and stably express μ2 protein may provide a way to cultivate engineered reoviruses containing foreign genes.

As for the mechanism of complementation, presumably the wild type μ2 protein expressed in the transfectants supplied the missing function of the mutant tsH11.2. Given that about 12 copies of μ2 protein are present in the interior of the virion core it is assumed that this virion-associated protein supplies at least one critical, probably enzymatic function. If this function preceded transcription then complementation of that defect would not be possible unless the virion was functionally wild type but genetically mutant as is tsH11.2 grown at permissive temperature. The μ2 protein is present at relatively high concentration in infected cells, relative to other abundant proteins μ1c and σ3, and may serve other functions before or concomitant with virion assembly where the μ2 protein is sequestered in the core of the virion. Without knowing the functional and structural roles of μ2 protein I cannot predict the mechanism for complementation. As the μ2 protein from tsH11.2 could not be differentiated from the wild type μ2 either by electrophoresis or by antibody detection (data not shown), I could not show if the wild type μ2 was incorporated into the virion or just functioned in trans. The reduced

stability of tsH11.2 produced at the nonpermissive temperature in wild type L929 cells relative to μ2-expressing T1-11-1 cells suggested that viruses incorporating the wild type μ2 protein from the μ2-expressing host cells were more stable. Further work on the nature of complementation by μ2 expression may shed light on the specific stages(s) in reovirus infection at which μ2 protein plays a role.

The μ2-expressing cell lines can also be used to study the function of μ2 and the interactions of μ2 with host cells. T1 and T3 μ2 expression levels differed significantly in the same type of cells and varied in different type of cells. In L929 cells transfected with the monocistronic constructs, pKJ1M1neo, or the dicistronic constructs, M1CN, expression of T1μ2 protein was always significantly higher than that of T3μ2 protein, which was true for both transient and stable expression. With constructs M1CN-BPV, expression of T1μ2 was much higher than T3μ2 in C127I cells but it was not so in 3T3 cells. These data suggest interactions either between host factors and the M1 mRNA or between host factors and the μ2 protein. In infected L929 cells, the yield of T1 virus is always higher than that of T3 virus. Possibly the interactions of M1 mRNA or μ2 protein with the host contribute to this difference. Previous data on the biological and genetic effects of μ2 protein on plaque size, CPE, growth of reovirus in cardiac cells and bovine aortic endothelial cells, pathogenesis and virulence in liver cells of infected animals (Moody & Joklik, 1989; Matoba et al., 1991; Matoba et al., 1993; Sherry & Fields, 1989; Sherry and Blum, 1994; Haller et al., 1995) are also consistent with interaction of μ2 with host factors. Apparently, either the 51 nucleotide substitutions and/or the 10 amino acid differences between T1 and T3 (Zou and Brown, 1992a) are

responsible for the difference in μ2 expression. It can be proposed that μ2 protein or M1 mRNA interacts with host factors in such a way that T3 μ2 or T3 M1 mRNA has a stronger inhibitory effect than T1 μ2 or T1 M1 mRNA on the host and consequently a unbalanced situation, less optimal for virus growth is reached between the host and the virus in T3 infection, and therefore, virus yield is reduced. Alternatively it is possible that the μ2 protein level directly controls virus yield and that specific host interactions lead to decreased levels of T3μ2 protein and decreased yield of virus.

In summary (1) the antibody generated from μ2 fusion protein-immunized rabbits was specific to reovirus μ2 protein; (2) reovirus μ2 proteins were constitutively expressed in L929 cells using dicistronic constructs and the μ2-expressing cell lines were stable; (3) the expression levels of T1 and T3 μ2 proteins were different and varied for different cell types; (4) expression of μ2 protein complemented the growth of reovirus defective in the M1 gene.

CHAPTER SIX

THE TRANSLATION OF REOVIRUS M1 GENE INITIATES FROM THE FIRST AUG CODON IN BOTH INFECTED AND TRANSFECTED CELLS

6.1 Introduction

Mammalian reovirus has a genome of 10 segments of double-stranded RNA (dsRNA) and the M1 segment encodes a minor core protein $\mu 2$. The sequences of M1 genome segments for both serotype 3 Dearing (T3) and serotype 1 Lang (T1) are 2304 nucleotides in length with a large open reading frame extending from nucleotide (nt) 14 to 2224. There are two in-frame translation initiation codons starting at nt 14 (AUG_{14}) and nt 161 (AUG_{161}) respectively (Wiener et al., 1989; Zou and Brown, 1992a). It was reported that in vitro translation of reovirus M1 can initiate at either of the two in-frame initiation codons but in reovirus-infected cells only AUG_{161} is used (Roner et al., 1993).

Following the identification of consensus M1 termini required for M1 replication and encapsidation by analyses of M1 gene deletion mutant reoviruses, we set out to develop a system for the introduction into reovirus of a foreign gene flanked by the consensus M1 termini. The breakthrough that in vitro transcripts of reovirus can be packaged to produce infectious virus (Roner et al., 1990) indicates that incorporation of transfected RNA into progeny viruses might be possible. Introduction of a synthetic RNA derived from the M1 DNA analogue into reovirus would provide a system for the characterization of specific genetic signals in the M1 gene and also the development of reovirus as an expression vector.

However, reoviruses containing a introduced M1 analogue in place of the wild type M1 would be defective due to a lack of the M1 function. One of the ways proposed to complement such an M1 gene defect was to develop cell lines that stably express reovirus μ2 protein. By transfection of L929 cells with M1-containing dicistronic DNA constructs driven by the mouse phosphoglycerate kinase gene (pgk) promoter, we obtained cell lines which express μ2 protein stably at a level of about 5% of that in reovirus-infected cells. Although this level of expression was later shown to be sufficient to complement the growth of a reovirus ts strain with an M1 gene defect (described in chapter five), efforts were made to try to increase the expression level.

The M1-containing dicistronic DNA constructs were first modified by deletion of the 5' terminal 144 nucleotides or the 5' terminal 160 nucleotides with or without changes in the AUG_{161} sequence context, followed by removal of the 3' untranslated region (UTR). It was found that deletion of the 3' UTR did not improve μ2 expression and that truncation of the 5' terminal sequence abolished the expression. The T7 promoter was then inserted upstream of this series of modified M1 DNA constructs. The expression driven by recombinant vaccinia virus showed that the protein products from constructs with the 5' terminal 144 or 160 nucleotides deleted were smaller than the μ2 protein expressed from the full size M1 constructs whereas the latter was the same size as μ2 protein precipitated from virus-infected cells by anti-μ2 antibody. These data demonstrate that the translation of reovirus M1 gene initiates from the first AUG in both infected and transfected cells.

6.2 Methods

The ligation independent cloning method developed by Jones and Howard (1991) was used for deletion, mutation or insertion modifications of DNA constructs in this study. DNA segments were modified by using amplifying primers that add homologous ends to the PCR products. These homologous ends underwent recombination in vivo following transformation of recA- E.coli strains. For deletion of a region, e.g. M1 5' terminal 144 nucleotides, primer A (5' CTAGTCTCGAGGATCAGTTACAGTA 3') had a 5' end overlapping with the plasmid sequence and 3' end overlapping with nucleotide 145 to 159 of M1 and primer B (5' CATTGTCATCGGTGACG 3') was complementary to nucleotides 445 to 429 of M1. Standard PCR was carried out using M1CN or pGEM-M1 DNA as the template. The construct to be modified was digested with BglII (cuts M1 at nucleotide 343) and Xho I (cuts in the polycloning site) to remove the region of M1 from nucleotide 1 to 343. The digested plasmid was then mixed with the PCR product and transformed into E.coli strain DH5α made competent by the $CaCl_2$ method (Sambrook et al., 1989, pp.1.76-1.84). After screening by restriction digestion of plasmid minipreparations, the modifications were confirmed by sequencing of the M1 region upstream of nucleotide 429 using primer B and the Sequenase kit and protocol (United States Biochemical Corp.). For generation of a point mutation, the mutation was included in one of the two amplifying primers. For insertion of a segment from another plasmid, the recipient plasmid was cut open and the segment to be inserted was amplified using two primers that carried ends overlapping with the ends of the linearized recipient plasmid.

The M1-containing dicistronic constructs (M1CN) generated for stable μ2 expression were the basis for modifications. M1CN contained the pgk promoter, T1 M1 or T3 M1, a downstream neomycin resistant gene (Neo) and a cap-independent translation initiation element (CITE) from encephalomyocarditis virus (EMCV) between M1 and Neo to initiate the translation of Neo. For both T1 M1 and T3 M1, the basic constructs M1CN (a)(see Fig. 6.1 for diagram of constructs described in this section) were first modified by deletion of the 5' terminal 144 nucleotides of M1 using PCR and in vivo recombination (b). The modified regions produced by PCR and the cross over regions were sequenced to confirm that clones were of the predicted modified sequence and were free of adventitious mutations. The -3 or the -3 together with +4 positions of AUG_{161} were then mutated to replace U with A for both positions (c, d). The 3' UTR (80 nucleotides) was deleted from both the basic constructs M1CN (e) and also from the above modified constructs b, c, d (f, g, h). This was followed by deletion of the 5' terminal 160 nucleotides of M1 from the basic constructs M1CN and change of the +4 position of AUG_{161} from U to A (i). Deletion of the 3' UTR from constructs i yielded constructs j and insertion of another CITE upstream of the M1 gene in constructs i yielded constructs k in which the M1 AUG_{161} overlapped with the viral AUG of EMCV located at the end of CITE. In construct l-f the Neo gene replaced the region of T1 M1 from nucleotide 136-2072 and Neo was in frame with the AUG_{14} of M1. In construct l-m the four AUGs within the M1 5' end at nucleotide 14, 105, 111, 132 were mutated to GUGs.

Constructs a, b, d, i, k were further manipulated by insertion of the T7 promoter

immediately after the pgk promoter between the Sst I site and Xho I site by using an oligonucleotide primer that was terminally complementary with both the 3' overhang of Sst I and the 5' overhang of Xho I. The constructs a, b, d, i, and k were cut open with Sst I and Xho I and the oligonucleotide containing the T7 promoter was allowed to anneal to the overhangs generated by the restriction digestion, followed by Klenow treatment, phosphorylation and ligation. These constructs were called pT7M1CN, pT7M1CN2, pT7M1CN3, pT7M1CN5, and pT7M1CN5+CITE, respectively (see Fig. 6.2). Other constructs included T1 M1 and T3 M1 cloned at the Sma I site in plasmid pGEM7Zf(+) (Promega) under control of the T7 promoter (pGEM-M1), T3 M1 in pGEM with the M1 5' terminal 144 nucleotides deleted, and pGEM-S7-M1 in which the cDNA copy of the genome segment 7 of influenza virus A/FM/1/47 (S7, 1027 nucleotides encoding the matrix protein M1) was inserted between the T7 promoter and the M1 gene in pGEM-M1 following linearization with Apa I and Kpn I. Segment 7 of influenza was also inserted between the pgk promoter and the M1 DNA in M1CN at the Xho I site (pS7M1CN) and between the T7 promoter and the M1 sequence in pT7M1CN (pT7S7M1CN).

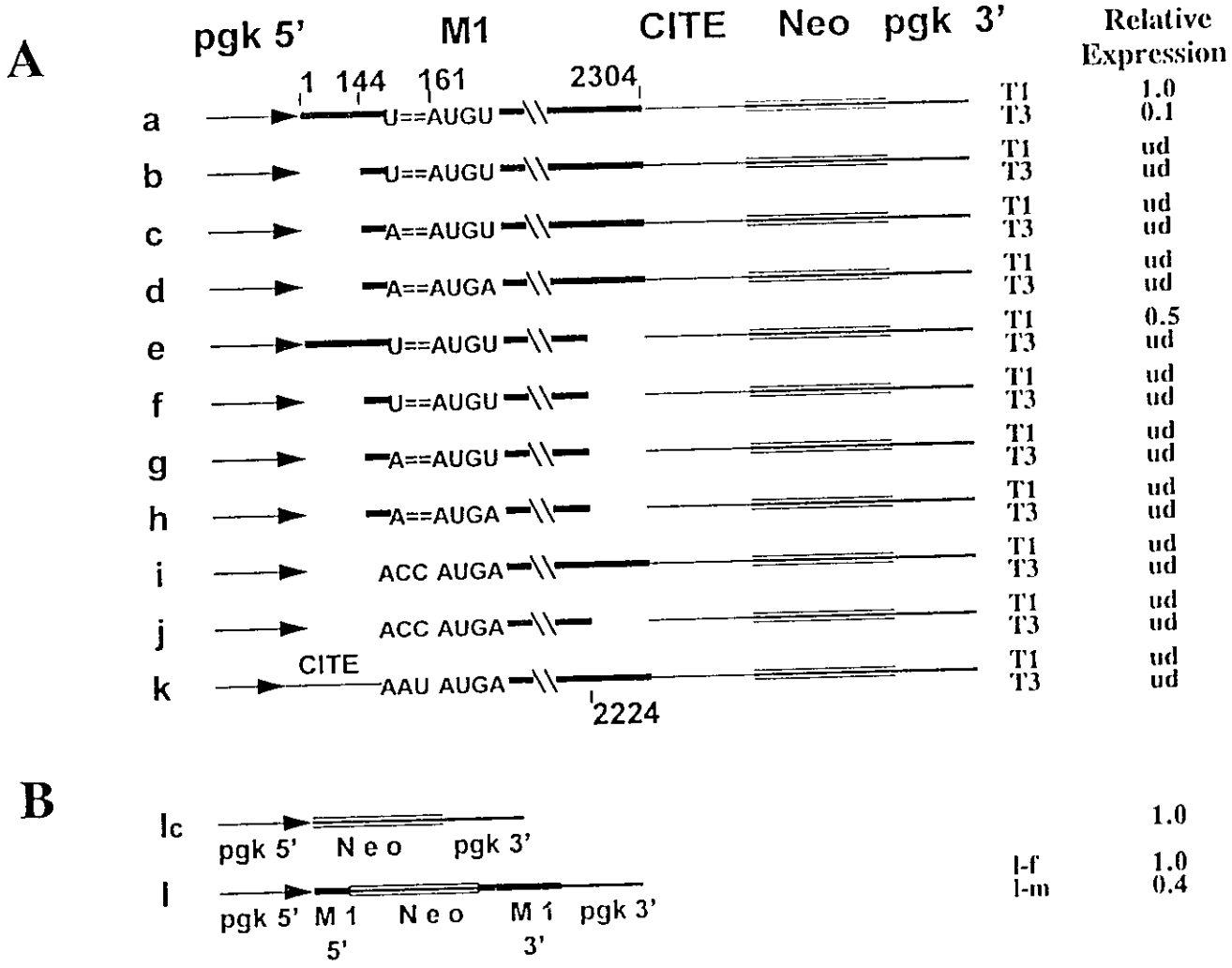
6.3 Results

6.3.1 Deletion of the M1 5' terminal 144 or 160 nucleotides abolished μ2 protein expression

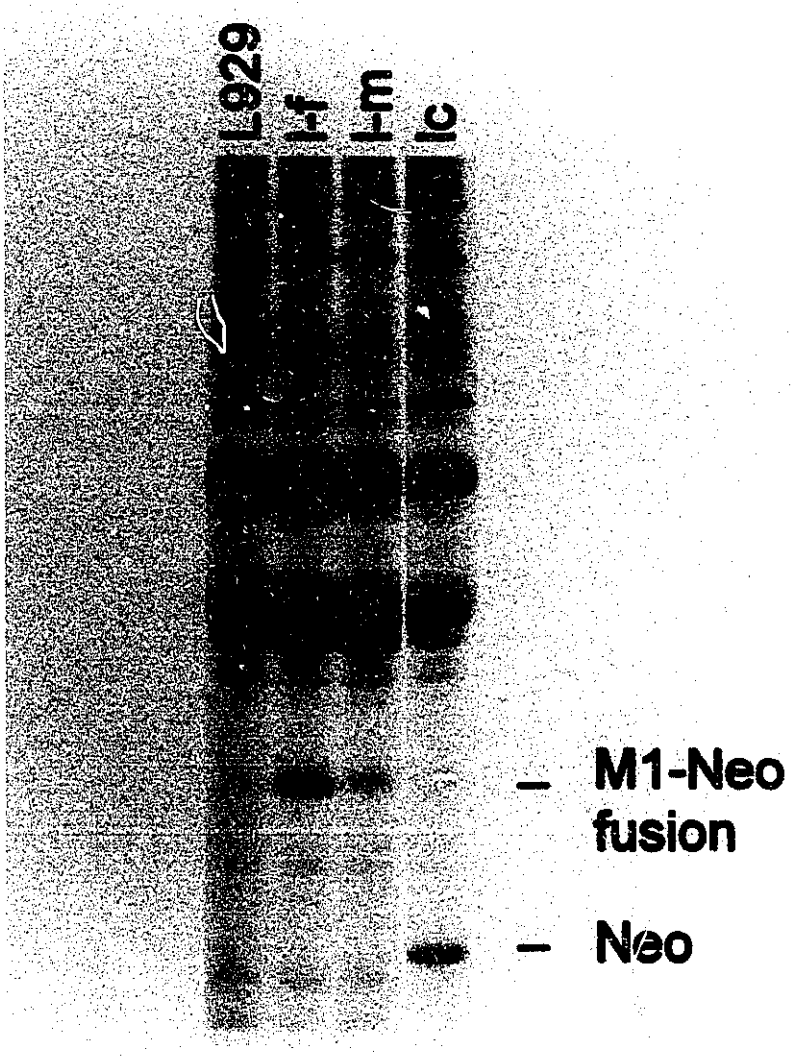
Constructs a through k were transfected into L929 cells and assayed for μ2 expression. After selection with Geneticin, the pools and 4 to 8 individual drug-resistant clones from

transfection by each construct were tested by RIPA using anti-μ2 antibody. Unexpectedly, none of the 5' end-truncated constructs gave detectable μ2 expression and none of the constructs with the 3' UTR deleted but without the 5' terminal truncation gave expression at levels comparable to that obtained with the basic constructs containing the intact M1 gene (Fig. 6.1A). However, the M1-Neo fusion construct (l-f) that possessed 135 nucleotides from the 5' end of the μ2 gene and the Neo gene fused in frame with AUG_{14} of the μ2 gene gave expression of an M1-Neo fusion protein that was precipitated with anti-Neo antibody and present at levels similar to that obtained with the Neo DNA construct driven by the same type of promoter (pgk) but without extraneous reovirus sequences (lc in Fig. 6.1B and 6.1C). The M1-Neo fusion protein product would contain 41 amino acids encoded by the M1 5'-terminal sequence if translation was initiated from AUG_{14} of the M1 gene. Indeed, the size difference of proteins produced by plasmid lc and by construct l-f indicated that AUG_{14} of M1 was used (Fig. 6.1C). The construct l-m in which all AUGs were mutated to GUGs in the M1 5' terminal 135 nucleotides even gave detectable expression of a slightly larger M1-Neo fusion protein than l-f due to additional sequences upstream of the Neo AUG initiation codon, which further supported the hypothesis that AUG_{14} (now mutated to GUG) was used for translation initiation. Replacement of the 5' 160 nucleotides with CITE reduced μ2 expression to below detectable levels, which implied not only that AUG_{14} was the initiation codon but also suggested that either the 5' end sequence was required for translation or stability of M1 mRNA or the amino terminus of μ2 protein was required for μ2 protein stability. In either case, the above results showed that the 5'-end-truncated

Fig. 6.1 Effect of M1 gene modification on μ2 expression. For each type of construct (a through k) in panel A, both T1 M1 and T3 M1 genes were modified, transfected into L929 cells and tested for μ2 expression by RIPA of stably transfected clones of cells using the anti-μ2 antibody. Both pools and 4 to 8 clones of cells from each transfection were screened and the results for the clone that showed the highest level of expression was shown for each construct. ud, undetectable. In panel B, the expression from M1-Neo fusion constructs without or with mutation of M1 AUGs to GUGs (l-f or l-m, accordingly) was compared to that from Neo itself (lc) by RIPA using antibody specific to the Neo gene product. The immunoprecipitated protein samples were separated by SDS-PAGE in 7.5% polyacrylamide gels and the relative expression was determined by densitometry of the protein bands. In panel A, μ2 expression from M1CN aT1 was set as the reference and in panel B, Neo expression from lc was the basis for comparison. Panel C shows the size difference of protein products from plasmid lc that had only the Neo gene and from constructs l-f and l-m that contained 135 extra nucleotides of the M1 gene 5' terminus.


A
pgk 5'
M1
CITE
Neo
pgk 3'
Relative Expression
1
144
161
2304
a
U==AUGU
T1 1.0
T3 0.1
b
U==AUGU
T1 ud
T3 ud
c
A==AUGU
T1 ud
T3 ud
d
A==AUGA
T1 ud
T3 ud
e
U==AUGU
T1 0.5
T3 ud
f
U==AUGU
T1 ud
T3 ud
g
A==AUGU
T1 ud
T3 ud
h
A==AUGA
T1 ud
T3 ud
i
ACC AUGA
T1 ud
T3 ud
j
ACC AUGA
T1 ud
T3 ud
CITE
k
AAU AUGA
T1 ud
T3 ud
2224
B
Ic
pgk 5'
Neo
pgk 3'
1.0
I
pgk 5'
M1
5'
Neo
M1
3'
pgk 3'
I-f 1.0
I-m 0.4

C



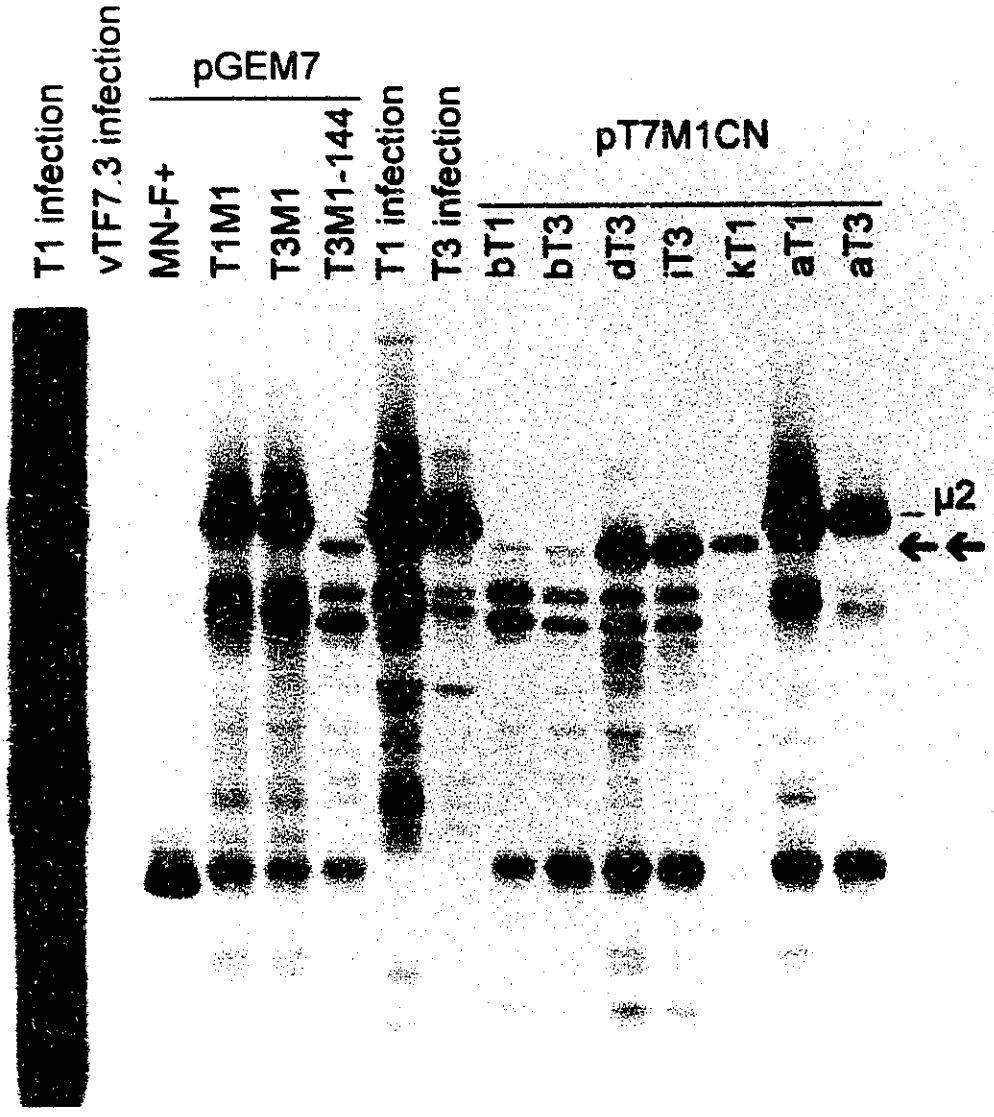
M1 constructs did not express detectable amount of full size μ2 protein. Demonstration of a smaller size product from the 5' deletion mutants would be direct evidence that the AUG_{14} codon was used for translation initiation of the authentic μ2 protein. However, as the expression level was low with the pgk promoter the smaller size product, if any, would have been masked by the background bands.

6.3.2 The 5' end-truncated M1 gene produced a smaller protein product than μ2 protein

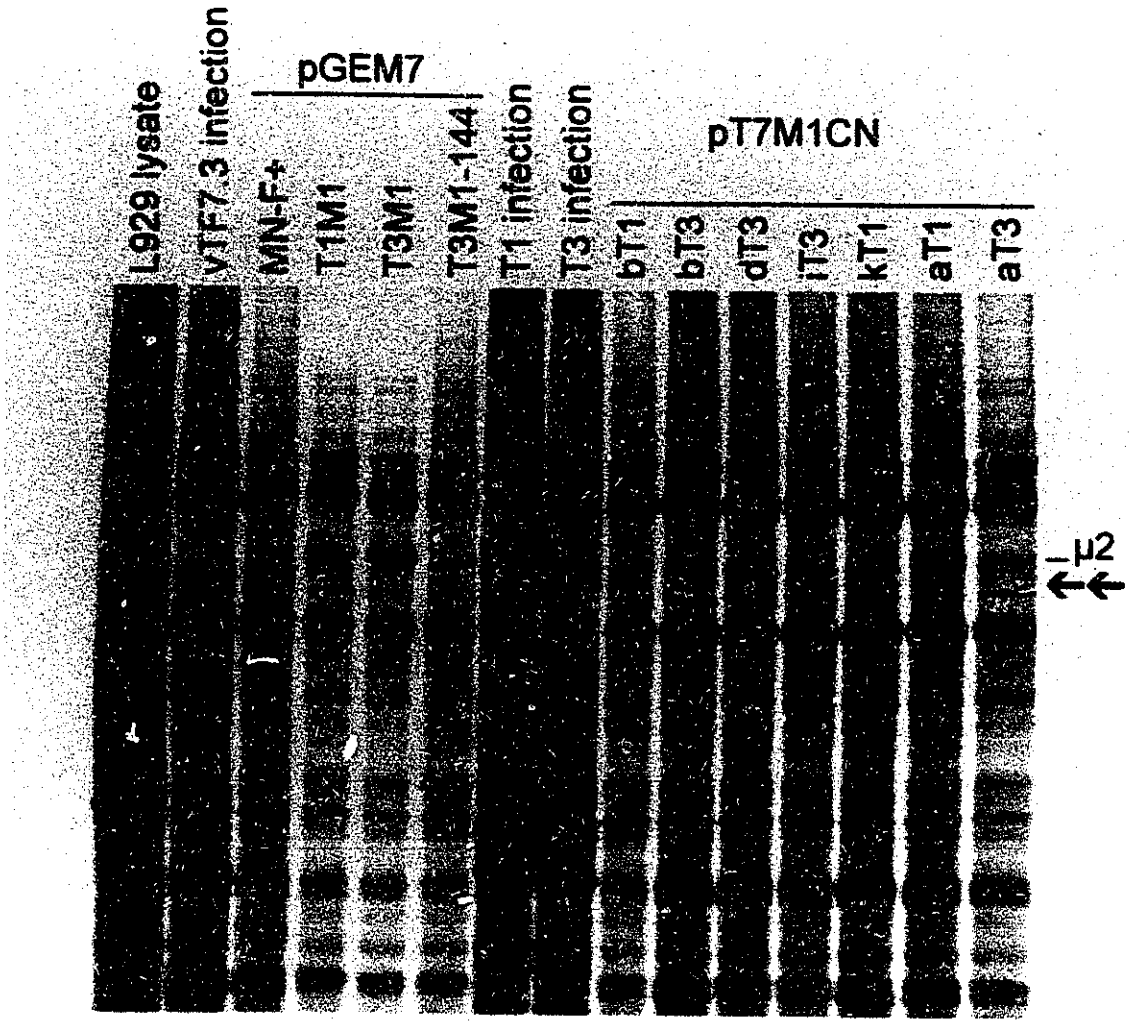
To further investigate which AUG was used to initiate translation of the M1 gene the T7 promoter was inserted immediately upstream of M1 in the following constructs as described above: full size M1 (a → pT7M1CN), deletion of the 5' terminal 144 nucleotides (b → pT7M1CN2), deletion of the 5' terminal 144 nucleotides plus modification of the -3 and +4 positions of AUG_{161} (d → pT7M1CN3), deletion of the 5' terminal 160 nucleotides plus change at the +4 position of AUG_{161} (i → pT7M1CN5), replacement of the 5' terminal 160 nucleotides with CITE (k → pT7M1CN5+CITE). pT7M1CN3 and pT7M1CN5 constructs with the T7 promoter were only generated with T3 M1 whereas for pT7M1CN5+CITE the T7 promoter was only inserted into the T1 M1-containing construct k. As either a T1 M1 or T3 M1 construct of each type should be able to address the same issue no further efforts were made to generate the corresponding T3 M1 or T1 M1 constructs. The above constructs were transfected into L929 cells followed by recombinant vaccinia virus infection to provide T7 RNA polymerase. Cells were labelled and cell lysates were tested by RIPA or directly

Fig. 6.2 Expression of two different size proteins from the intact or 5'-end-truncated M1 constructs. L929 cells were transfected by lipofection (5-hr incubation) followed by infection with the recombinant vaccinia virus vTF7.3. Cells were labelled at 16 hrs p.i. with ^{35}S-methionine for 4 hrs. Cell lysates were extracted and tested by RIPA using the anti-μ2 antibody (panel A) or directly separated by SDS-PAGE on 7.5% polyacrylamide gels (panel B). MN-F+, Neo gene fused to the M1 5'-terminal 135 nucleotides; T3M1-144, the T3M1 5'-terminal 144 nucleotides deleted; b, the M1 5'-terminal 144 nucleotides deleted; d, deletion of the 144 nucleotides plus mutations of the -3 and +4 positions; i, deletion of 160 nucleotides with a +4 mutation; k, CITE in place of the 5' 160 nucleotides; a, full length M1. The double arrow indicates the smaller protein from the truncated M1 gene.

A


T1 infection
vTF7.3 infection
pGEM7
MN-F+
T1M1
T3M1
T3M1-144
T1 infection
T3 infection
pT7M1CN
bT1
bT3
dT3
iT3
kT1
aT1
aT3
μ2

B


L929 lysate
VTF7.3 infection
pGEM7
MN-F+
T1M1
T3M1
T3M1-144
T1 infection
T3 infection
pT7M1CN
bT1
bT3
dT3
lT3
kT1
aT1
aT3
μ2

separated by SDS-PAGE. Panel A in Fig. 6.2 shows that the immunoprecipitated products expressed from those constructs were of two different sizes, with the proteins expressed from the 5'-end-truncated M1constructs being smaller than those from the full size M1 constructs. The full length M1 construct produced μ2 protein that aligned with μ2 proteins from T1 Lang and T3 Dearing infections. The two constructs with the 5' terminal 144 nucleotides deleted but without any change in the context of AUG_{161} gave the lowest level of expression, which is not surprising since the AUG is in a very poor context for initiation with U at -3 and +4 positions. Mutations that produced a more favourable Kozak consensus sequence at -3 and +4 positions gave much higher levels of the smaller protein product. Furthermore, the protein band from the construct pT7M1CN5+CITE was also smaller than the authentic μ2 proteins and was the same apparent size as those from other 5'-end-truncated constructs. Because translation initiation with pT7M1CN5+CITE was from the EMC virus AUG which overlapped with the M1 AUG_{161} the smaller product ($\sim$68 kDa, relative to $\sim$73 kDa for full length μ2 protein) was not the result of translation initiation from an AUG codon further downstream at position 332 (weak Kozak sequence) or 443 (good Kozak sequence ACAAUGA). Therefore the smaller protein expressed from all the 5'-end-truncated M1 constructs was indeed translated as a result of initiation at AUG_{161} of the M1 gene. The size difference between the full-size μ2 protein and the deletion mutant initiating at AUG_{161} is consistent with the loss of 49 amino acids ($\sim$ 5 kDa) from the amino terminal end. SDS-PAGE analysis of unprecipitated lysates (panel B) clearly showed the absence of the full size μ2 protein band in cells transfected by the 5' end-truncated M1 constructs

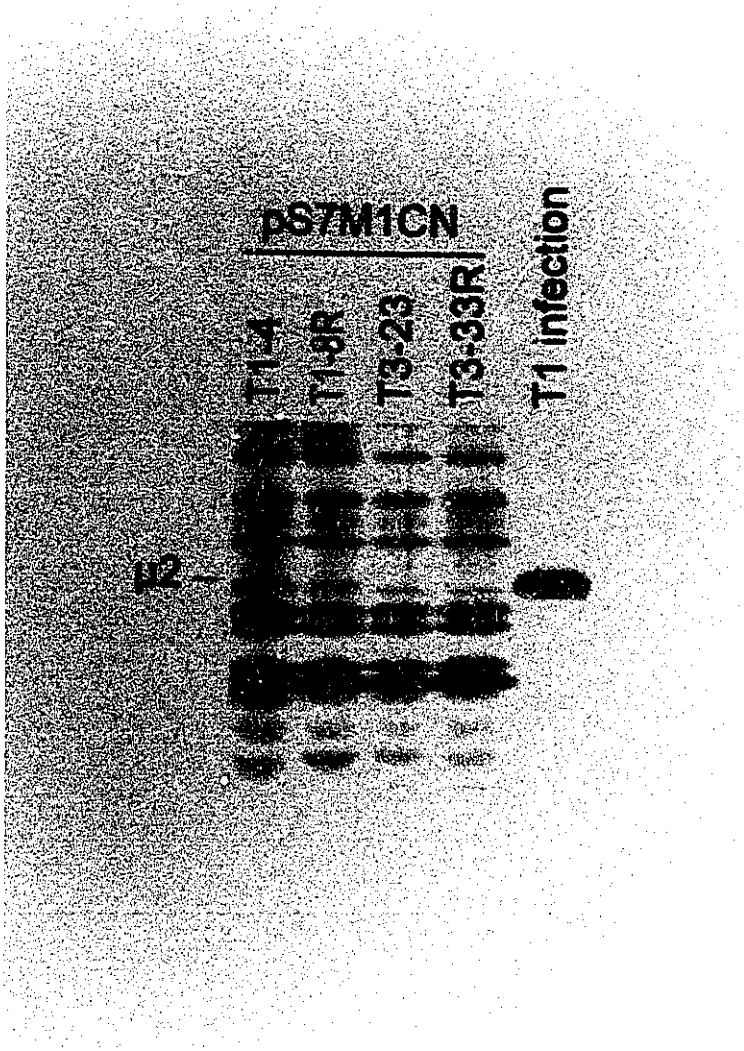
and thus ruled out the possibility that the observed size difference was an artifact due to immunoprecipitation or that other large translation products in other reading frames were produced but not detected with a μ2 specific antibody. It can be concluded that translation of the reovirus M1 gene initiates from the first initiation codon, AUG_{14}, in both infected and transfected cells.

6.3.3 AUG_{14} was used in different backgrounds of plasmid sequences

To address the effect of upstream and downstream vector sequences on the usage or choice of alternative translation initiation codons, μ2 protein expression from the M1 gene in different backgrounds was analyzed. Panel A of Fig. 6.3 shows that T1 μ2 protein was expressed following transfection of 3T3 cells by the construct, pS7M1CN, in which the cDNA copy of segment 7 of influenza virus A was inserted between the pgk promoter and the M1 gene in M1CN. It was surprising that μ2 protein was detectable from this construct that produced an mRNA containing influenza segment 7 (1027 nucleotides long) upstream of the reovirus M1 gene. The level of μ2 protein expression for the T1 construct was similar to the level of M1CN but the T3 construct was lower when segment 7 was in the forward orientation. Reversing the orientation of segment 7 decreased expression of the downstream M1 gene. Panel B shows the expression in L929 and 3T3 cells transfected with constructs pGEM-S7-M1, which contained M1 in pGEM7Zf(+) plus segment 7 between the T7 promoter and the M1 gene. Expression of μ2 was very low for these constructs in L929 cells but was much higher in 3T3 cells. In 3T3 cells the translation of T1μ2 protein was better than T3μ2 protein whereas flu

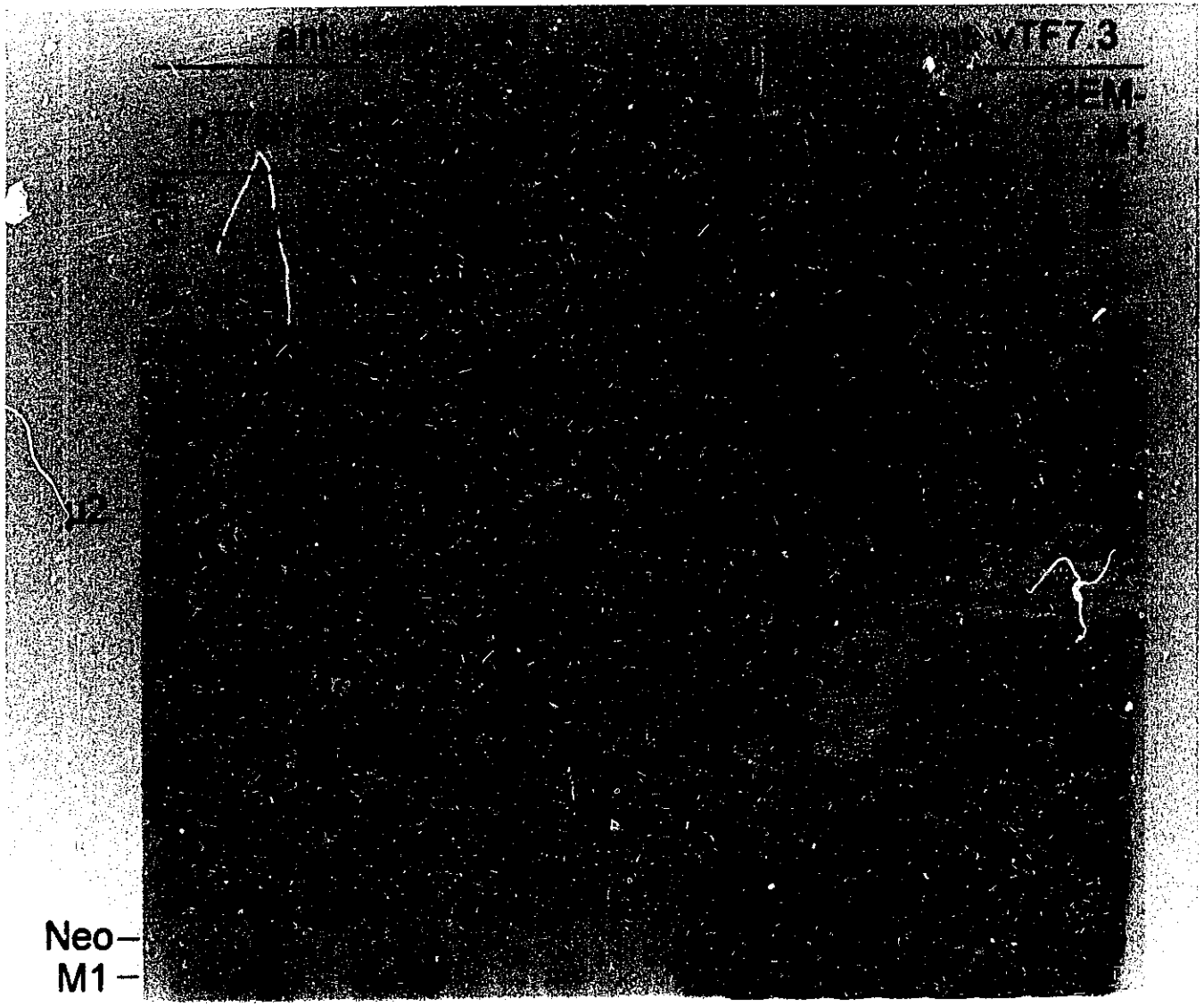
Fig. 6.3 Translation of M1 gene preceded by the influenza segment 7 gene and in different background sequences. Panel A shows transient expression in 3T3 cells transfected by pS7M1CN and detected by RIPA using the anti-μ2 antibody. T1-8R/T3-33R, reverse orientation of segment 7 of influenza virus A/FM/1/47. The position of the μ2 protein band is indicated. Panel B shows the transient expression of μ2 in L929 and 3T3 cells transfected by pGEM-S7-M1 followed by vTF7.3 infection and detected by anti-μ2. R, reverse orientation of segment 7. Less DNA was used in transfection for T1-1R and T3-19R. Shown in panel C is the expression of μ2 protein from L929 cells transfected by pT7S7M1CN and infected with vTF7.3. The same sample from L929 cells transfected with pGEM-S7-M1 was included in panel C as a reference standard and size control. Cell lysates from transfected L929 cells were precipitated using the anti-μ2 antibody and the antibody to influenza virus M1 (segement 7 product) or the anti-Neo and anti-vTF7.3 antibody and separated by 7.5% SDS-PAGE. T1-12R/T3-21R had the reverse orientation of the T7 promoter and all pGEM-S7-M1 constructs had segment 7 in reverse orientation. T1, infection by T1 reovirus; Flu, infection by influenza A/FM/1/47.

A



B

C



segment 7 translation was similar, indicating that transcription was equivalent for both plasmids. In these constructs initiation is due to leaky scanningor reinitiation suggesting that the T1 M1 gene possesses a superior translation initiation mechanism than the T3 M1 gene. Similar transfections that included the 2A gene of poliovirus, which inhibits cap dependent translation initiation (Hellen et al., 1991; Sun and Baltimore, 1989) inhibited expression of both the upstream and downstream M1 genes, influenza matrix and reovirus μ2 proteins, suggesting that both proteins are initiated by cap dependent scanning of ribosomes (data not shown). The expression of μ2 protein from L929 cells transfected with pT7S7M1CN is shown in panel C. Comparing panel B to C shows that the translatability of μ2 was dependent at least in part on the 3' region of the message since the inclusion of the neo gene translated with the CITE element resulted in decreased expression of influenza M1 protein and reovirus μ2 protein from the upstream genes. In the tricistronic construct pT7S7M1CN, the first and last genes were translated by cap-dependent and cap-independent mechanism respectively. μ2 was poorly translated, presumably due to its requirement for cap-dependent initiation of translation. The μ2 proteins expressed in all three experiments aligned with the authentic μ2 protein from infection.

μ2 protein was also transiently expressed in COS-1 cells transfected by a construct in which the M1-CITE-Neo cassette was driven by the CMV promoter of pcDNA3 (Invitrogen) and again the protein, which was produced in large amounts, aligned with the μ2 protein from infection (data not shown). From Fig. 6.2 the proteins produced from the M1 gene in the pGEM7 background (pGEM-M1) or the M1CN construct in the

pKJ background (pT7M1CN) were the same size. Taken together, extraneous sequences upstream or downstream of M1 in these constructs did not affect the usage of translation initiation codons.

6.4 Discussion

Translation initiation in eukaryotic cells generally follows the first AUG rule. According to the scanning model the 40S ribosomal subunit (carrying Met-tRNA$_i^{met}$ and initiation factors eIF-3, eIF-4C, and eIF-2) binds initially at the 5' end of mRNA (in the presence of additional initiation factors) and then migrates, stopping at the first AUG codon in a favourable context for initiating translation (Kozak, 1978). Both the position (first AUG) and the context are important as a survey of 699 vertebrate mRNAs showed that the "first-AUG-rule" held for some 90-95% of the mRNA sequences analyzed and that GCCGCC(A/G)CCAUGG was the consensus sequence for initiation (Kozak, 1987). Later the scanning model was updated to account for exceptions to the rule (Kozak, 1989). Initiation at downstream AUG codons occurs under three specific conditions: when fewer than 10 nucleotides occur between the cap and the first AUG codon; when the first AUG codon is in an unfavourable context (i.e., C or U is at position -3; or position +4 is not G); or when an in-frame terminator codon closely follows the first AUG but is upstream from the second. Under the first two conditions leaky scanning may occur and the downstream AUG codon can be used. In the third case the ribosome reinitiates translation at the downstream AUG after the translation of the upstream "minicistron". As long as the position and context are optimal, the first-AUG rule holds

even when a second AUG codon follows closely upon the first (Kozak, 1995).

For picornaviruses, translation initiation can occur internally (Duke et al., 1992; Borman and Jackson, 1992). The cap-independent translation initiation element (CITE) from encephalomyocarditis virus (EMCV) was used in dicistronic vectors to initiate protein synthesis from the downstream cistron (Wood et al., 1991; Kaufman et al., 1991). The EMCV CITE is about 600 nucleotides long and translation of the gene fused to CITE initiates from the viral AUG located near the end of CITE.

Reovirus M1 segment mRNA is translated inefficiently in infected cells (Gaillard and Joklik, 1985). The highest level of stable expression of μ2 protein obtained in transfected cells was about 5% of that in infected cells. Efforts were made to characterize those elements that control expression, which would help to elucidate ways to increase the expression in transfected cells. The M1 gene has a single large open reading frame starting from a translation initiation codon at nucleotide 14 (Wiener et al., 1989a; Zou and Brown, 1992a) although there is a recent report that AUG_{161} is the normal initiation site (Roner et al, 1993). The sequence context of these two AUGs and several inframe AUGs further downstream (up to nucleotide 560) are shown in table 6.1. The fact that the first AUG has a short (13 nucleotides) noncoding region suggested the possible involvement of leaky scanning in translation of the M1 gene, which is consistent with the report that AUG_{161} was used in reovirus-infected cells (Roner et al., 1993). It was reasoned that if leaky scanning did occur then the unused first AUG and the unfavourable context of the AUG_{161} would presumably be responsible for the low level of μ2 expression. Therefore, the 5' terminal sequence upstream of AUG_{161} was deleted

and/or the context of AUG_{161} was modified to test the effect on μ2 protein expression. The 3' UTR was also deleted in some constructs to see if there was any effect on expression. Unexpectedly, it turned out that not only was the 5' terminal sequence required for expression but also the first AUG was used in both infected and transfected cells.

Table 6.1 Sequence context of AUG codons in frame with AUG_{14}

starting position of AUG (+1)	sequence context	
Kozak consensus sequence	GCCGCC(A/G)CCAUGG	
14	UUCGCGGUCAUGGCUUAC	
161	UUACAGUAUAUGUUAGAU	(T1)
	UUACAGUACAUGUUAGAU	(T3)
332	AAACGACUAAUGCUAAAG	
437	ACGUCACCGAUGACAAUG	
443	CCGAUGACAAUGAUCCAG	(T1)
	CCGAUGACAAUGAUACAG	(T3)
560	UCAUUUACUAUGACUAAG	

When the context of AUG_{14} (GCGGUCAUGG) and that of AUG_{161} (CAGUAUAUGU for T1 M1 and CAGUACAUGU for T3 M1) were checked carefully using the first-AUG-rule, it is not surprising that AUG_{14} was used in spite of the short 5' UTR. AUG_{14} has an almost optimal context, with only two differences from the consensus core sequence GCC(AG)CCAUGG, with the most critical nucleotide at +4 and the second most critical nucleotide at -3 position being correct. However, for AUG_{161}, neither the

+4 nor the -3 position is correct and there are seven (for T1) or six (for T3) mismatches compared to the consensus sequence. The 13-nucleotide UTR is more than long enough to allow initiation from AUG_{14}. Actually, another genome segment of reovirus, S1, encodes protein σ1 from AUG_{14} and the translation efficiency is higher than that of M1 gene (Gaillard and Joklik, 1985; Roner et al., 1989) although the AUG context in S1 (GCGCCUAUGG)(Gaillard et al., 1982) is less favourable than that in M1, according to the Kozak consensus sequence. Taken together, translation initiation of the reovirus M1 gene is consistent with the first-AUG-rule.

Certainly, further upstream and downstream sequences and secondary structures formed by mRNA may have an effect on the efficiency of translation initiation or even the choice of initiation codons (Roner et al., 1989; Grünert and Jackson, 1994; Kozak, 1995). It was also proposed that attached plasmid sequences might affect the availability of initiation codons (Roner et al., 1993). However only one protein, equivalent in size to the μ2 seen in infected cells was expressed in cells transfected by M1 in a pGEM or pKJ1 background, which clearly have different sequences upstream of the M1 gene insert. This was true whether M1 was followed by CITE-Neo or M1 was preceded by segment 7 of influenza virus A. The contradictory results of this study to the results reported previously (Roner et al., 1993) cannot be explained unless there were additional 5' sequences in the constructs of Roner et al that may have provided a new, in frame, initiation site, resulting in an unnaturally long μ2 protein.

Another point worth further mention here is the possibility of internal translation initiation of the M1 gene. We were surprised to detect a full size μ2 protein in 3T3 cells

transfected with the tricistronic construct pS7M1CN, in which transcription was driven by the pgk promoter with the M1 gene as the second gene. If expression of $\mu 2$ from this construct indeed resulted from internal initiation then it would provide an explanation for the translation of uncapped messengers late in reovirus infection (reviewed by Schiff and Fields, 1990). However, when the poliovirus 2A protein gene was cotransfected into cells the expression of the first gene as well as $\mu 2$ was reduced. This result did not support a strong internal initiation process in M1 translation even though this possibility cannot be completely ruled out. It is possible that the surrounding sequences of AUG_{14} or interaction of this region with other regions of the M1 mRNA could form a structure which helped the landing of ribosomes ahead of AUG_{14}. The most likely explanation is that utilization of the M1 AUG_{14}, following the segment 7 gene, was through a reinitiation mechanism. It was reported that when a cistron is preceded by one or even two short cistron(s), reinitiation does occur at the AUG of the downstream cistron, though at much reduced efficiency (Kaufman et al., 1987). This suggests that when AUG_{14} is near the 5' end of the message it initiates inefficiently since a similar amount of protein results from reinitiation after translation of an upstream gene, which normally decreases translatability by $>95\%$. AUG_{14} may be unavailable for initiation due to secondary structure or host factor interaction. Nevertheless, these results indicated that AUG_{14} could still be used even when preceded by a 1 kb gene and thus is indeed a strong initiation codon.

Attempts to sequence $\mu 2$ proteins initiated at AUG_{14} and AUG_{161} were unsuccessful because the $\mu 2$ protein immunoprecipitated from infected cells or from transfected cells

and purified by SDS-PAGE were blocked at the amino terminus.

The question still to be answered is why the μ2 protein was expressed at low efficiency in vivo but not in vitro (Gaillard et al., 1985; Roner et al., 1989). One possibility is that translation efficiency is controlled by interaction between μ2 protein or M1 mRNA and a component of the host cells. The results described above and from previous studies of μ2 protein expression carried out in this laboratory showed that expression of T1μ2 and T3μ2 driven by T7 polymerase from recombinant vaccinia virus both T1 μ2 and T3 μ2 was much higher than in reovirus infected cells. This is in contrast to stable μ2 expression achieved with the dicistronic constructs where the highest level of expression of T1 μ2 protein was only about 5% of that in infected cells and the level of T3 μ2 was even lower. Genetic studies using reassortants have associated the M1 gene or μ2 protein with the extent of cytopathic effect and plaque size in L929 cells (Moody and Joklik, 1989), replication in vitro in heart cells and bovine aortic endothelial cells (Matoba et al., 1991; Matoba et al., 1993), murine myocarditis (Sherry and Fields, 1989; Sherry and Blum, 1994), and virulence in the liver of infected animals (Haller et al., 1995). Our data show differences in translation of T1 and T3 μ2. This evidence supports the notion that interactions occur between host factors and the M1 mRNA or its protein product and that it is these interactions that limit the level of stable μ2 protein expression in transfected cells. Another possible limiting factor could be that the M1 mRNA or μ2 protein might have inhibitory or toxic effects on cells and thus growth of cells in which the level of expressed μ2 protein was limited may have been favoured. As for the difference in expression between T1 μ2 and T3 μ2 proteins it would be of

interest to identify the specific differences in the gene or protein that are responsible for the host dependent expression profile observed.

Why CITE in place of the M1 5' terminal 160 nucleotides (in construct k) did not improve expression of the M1 gene product could be due to the reduced stability of the truncated M1 mRNA or the reduced stability of the protein product missing the amino terminus. Alternatively, the DNA construct bearing two identical CITE sequences might have undergone recombination in transfected cells resulting in the deletion of the M1 gene. Cells harboring the M1-deleted construct might have outgrown M1-containing cells during the selection process. However, the low level of expression of μ2 from this construct was even seen for transient expression driven by the T7 promoter, which is probably not explained by rearrangement of the plasmid in the cytoplasm.

The evidence derived from this study that translation of the reovirus M1 genome segment initiates from the first AUG codon and that the M1 5'-terminal region is required for the gene expression not only clarified the mechanism of translation initiation for the M1 gene but also suggested that efficiency of translation initiation per se can not be the reason for poor μ2 expression in either infected or transfected cells unless this modulation is mediated by other host factors. Further study on the stability of the M1 mRNA and the interactions between host factors and the M1 gene or its gene product are needed to understand the control of μ2 protein expression.

CHAPTER SEVEN

PRELIMINARY WORK ON INTRODUCTION OF A FOREIGN GENE INTO REOVIRUS

7.1 Introduction

As stated before, characterization of M1 deletion mutants identified the consensus termini that possess the signals for M1 replication and encapsidation. Cloning, sequencing and expression of full size M1 in L929 cells generated cell lines supporting the growth of reovirus that has a defective M1 gene. Production of μ2-specific antibody provided a sensitive detection assay for reovirus μ2 protein. All of these paved the way for developing methodology for the introduction of a cloned or engineered gene into reovirus. Once developed, this methodology could be used to characterize the specific signals within the M1 termini that are required for replication and encapsidation and subsequently to develop reovirus as an expression vector.

For plus sense RNA viruses and DNA viruses, introduction of a synthetic/foreign gene into the viral genome has been very successful. However, this is not the case for minus sense RNA viruses or dsRNA viruses since the genomes themselves are not infectious. Because the plus strand of the dsRNA genome does not serve as template for replication dsRNA viruses behave like minus sense RNA viruses in this regard. Recently, extensive effort has been made to manipulate the genome of minus sense RNA viruses and several examples of success have been reported (reviewed by Wertz and Melero, 1993; Garcia-Sastre and Palese, 1993). For paramyxoviruses, transfection of

viral RNA analogues synthesized in vitro from DNA constructs into helper virus-infected host cells led to rescue of the RNA analogues into viruses (Park et al., 1991; Collins et al., 1991; Dimock et al., 1993; De and Banerjee, 1993). For influenza viruses, RNA was synthesized, mixed in vitro with purified viral nucleocapsid proteins and the encapsidated RNA was introduced into helper virus-infected host cells (Luytjes et al., 1989). This system has been successfully used to characterize the influenza virus genome (Parvin et al., 1989; Huang et al., 1990; Luo et al., 1991; Subbarao et al., 1993; Horimoto and Kawaoka, 1994). Another group used purified viral proteins from infected cells instead of proteins synthesized in vitro for the reconstitution of the ribonucleoprotein complexes (Martin et al., 1992). A different approach was tried for VSV (vesicular stomatitis virus), in which RNA was synthesized within host cells expressing T7 RNA polymerase in the cytoplasm. The HDV ribozyme generated the correct 3' end of the synthesized RNA and a complete set of VSV proteins expressed from introduced plasmids provided the encapsidation proteins and other necessary functions. The cloned genome of a defective interfering VSV was rescued by this method (Pattnaik and Wertz, 1990; 1991; Pattnaik et al., 1992). A similar approach has been used for rescue of synthetic genomic RNA analogs of rabies virus (Conzelmann and Schnell, 1994).

The work on dsRNA viruses has met less success. The paramyxovirus approach was tried for rotavirus but only amplification of the introduced RNA was observed and there has been no report of rescue (Gorziglia and Collins, 1992). An in vitro replication assay was also tried for rotavirus in which rotavirus plus sense RNA was replicated to form

dsRNA in a transcription/translation cocktail supplemented with rotavirus-primed cell lysate. This method was able to generate dsRNA but there was no further report of replication of synthetic RNA derived from cDNA (Patton, 1986). A different in vitro replication system was recently reported in which open core particles of rotavirus SA11-4F were prepared by dialysis against low-ionic-strength buffer and both viral and cDNA-derived plus sense RNA templates were replicated using the open core particles (Chen et al., 1994). For reovirus, the only success was with the approach used by Roner et al (1990) which modified the paramyxovirus method by adding all reoviral RNAs and their in vitro translation products in the transfection, resulting in the production of virus from viral RNA transcribed in vitro from activated reovirus cores. This work demonstrated that RNA transcripts produced in vitro can interact with viral proteins in an appropriate way and subsequently be encapsidated (Roner et al., 1990).

From the observations stated above, it seems that helper virus infection provides essential functions and that synthetic reovirus RNA needs to be associated with viral proteins.

The foreign gene used for introduction into viruses can be one of the following: (1) a reporter gene such as CAT; (2) a mutated version of a viral gene which can be detected/selected by differences between the two versions of the gene; (3) a wild type viral gene introduced into a conditionally defective mutant such as a ts mutant so that the virus is selected by variation of cultivation conditions; (4) a genome segment from one serotype of a segmented virus introduced into another serotype so that the virus of interest can be detected by differences in the segment.

7.2 Methods

The general procedure for the work on introduction of a foreign gene into reovirus included (1) preparation of DNA constructs designed to insert a selectable gene between the identified M1 consensus termini for recognition by reoviruses; (2) generation of RNA transcripts from the DNA constructs and introduction into reovirus; (3) selection and enrichment of reoviruses containing the introduced gene.

The neomycin resistant gene (Neo) was chosen as the foreign gene, which would allow the selection of reoviruses containing the introduced gene using G418 treatment.

One of the cloned and sequenced M1 deletion fragments (98B11-1) has a restriction site (Bcl I) at the junction point. The Neo gene was inserted into the Bcl I site in frame with the first AUG (AUG_{14}) of the M1 5' terminus (98B11-1-Neo F). In a second construct all four translation initiation AUGs starting at nucleotides 14, 105, 111, and 132 in the M1 5' terminus were mutated to GUGs (98B11-1-Neo M). The DNA constructs were subcloned into the mammalian expression vector pKJ1 under the pgk promoter (pKJ1-98B11-1-Neo F/M, also called 1-f and 1-m in chapter six) to check if the Neo gene was functional in the constructs.

A T7 promoter element and a Bsm I site were then used to flank the 98B11-1-Neo F/M constructs in both orientations such that the derived dsRNA would have authentic M1 termini (MN-F+/-, MN-M+/-). MN-F+/- would give +ssRNA or -ssRNA transcripts with Neo fused to M1 whereas MN-M+/- would give +ssRNA or -ssRNA transcripts with AUGs in the M1 5' terminus mutated to GUGs.

It was proposed that reoviruses containing the foreign gene would be complemented

by cell lines constitutively expressing μ2 protein, by cell lines persistently infected with reovirus, or simply by wild type helper virus which would presumably be interfered with by the foreign-gene-containing reoviruses and outgrown in serial passages.

Screening for rescue of the Neo-containing M1 analogue was performed by extraction of dsRNA from harvested virus followed by northern blot hybridization or reverse transcription and PCR amplification (RT-PCR).

7.3 Results

7.3.1 The Neo gene flanked by the M1 termini was functionally expressed

Transfection of L929 cells with either pKJ1-98B11-1-Neo F or pKJ1-98B11-1-Neo M and selection by G418 gave over 100 G418 resistant colonies from $2x10^6$ transfected L929 cells. This indicated that not only was the Neo gene expressed in the transfected cells but also that the expressed M1-Neo gene fusion product functioned in conferring cellular resistance to Geneticin.

7.3.2 The initial attempts to introduce the M1 analogue into reovirus

Fig. 7.1 shows the constructs used in attempts to introduce a segment containing a foreign gene into reovirus. Procedures tried initially for the introduction of the M1 analogue into reovirus included transfection of in vitro transcribed RNA into host cells acutely infected with helper reovirus, into cells persistently infected with reovirus, or transfection of synthetic RNA together with reoviral +ssRNAs and their in vitro translation products into host cells acutely infected with helper reovirus. No rescue of

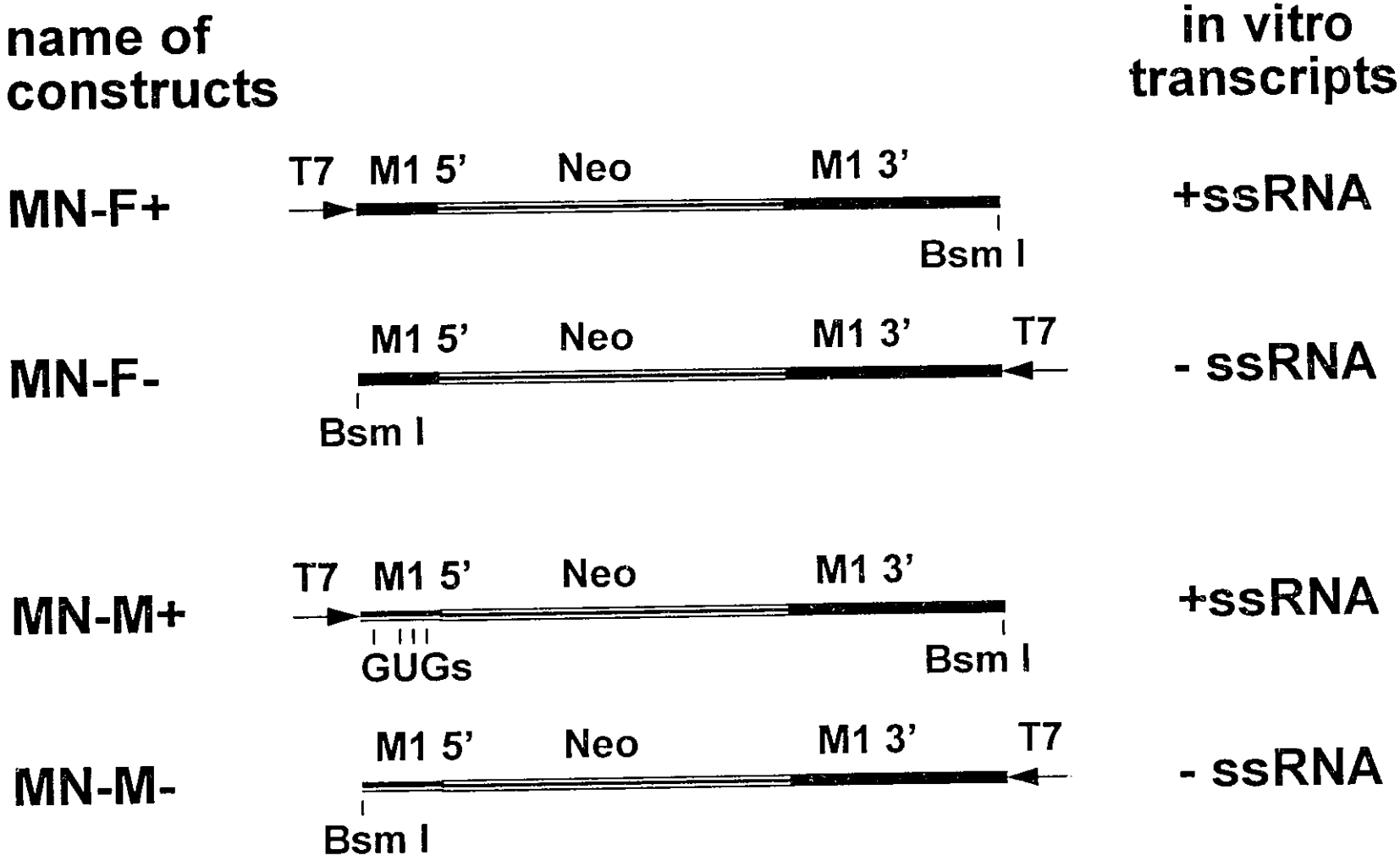
M1-Neo analogue was observed as determined by northern blot hybridization and RT-PCR.

7.3.3 The nuclear T7 RNA polymerase approach

In reovirus replication, +ssRNAs transcribed from the incoming viral cores are assembled by viral proteins into premature progeny in which the 10 segments of +ssRNA are converted to dsRNAs (Schiff and Fields, 1990). The reason for the failure of early experiments could be that adequate amounts of the transfected RNA did not reach the correct location at the correct time to be incorporated into the progeny viruses. It was proposed that continuous RNA transcription of the M1 analogues inside cells might be more likely to allow enough RNA molecules to be present at the appropriate time and in the appropriate compartment to interact with viral proteins and +ssRNAs produced by the helper virus, and thus to be incorporated.

Reovirus +ssRNAs are capped but have no poly(A) tail. Generation of RNA transcripts in the nucleus would presumably solve the capping problem and ribozymes could be used to cleave poly(A) tails and leave a 3' end of the transcripts with the correct nucleotide sequence. Consequently, a nuclear T7 RNA polymerase system was adapted for this study. Constitutive RNA transcription of genome analogues (DNA constructs of Neo flanked by M1 termini) was generated by cotransfection of cells with the DNA constructs and a plasmid pMTT7N expressing T7 RNA polymerase carrying a nuclear localization signal (Lieber et al., 1989). The correct 5' end of transcripts was achieved by the location of the T7 promoter and the correct 3' end was produced by the attached

Fig. 7.1 DNA constructs of the M1 analogue to be introduced into reovirus. The T7 promoter is the same as in pGEM7Zf(+) and the Neo gene was amplified from the plasmid pMAMneo (CLONTECH Laboratories, Inc.). The M1 5' end and the M1 3' end were from the M1 deletion fragment 98B11-1 cloned in pGEM. In MN-F+/-, the Neo gene was fused to the AUG_{14} of the M1 5'-terminus. In MN-M+/-, the four AUGs in the M1 5'-terminus were mutated to GUGs but the Neo gene was still fused to GUG_{14}.


name of constructs
in vitro transcripts
MN-F+
T7
M1 5'
Neo
M1 3'
Bsm I
+ssRNA
MN-F-
M1 5'
Neo
M1 3'
T7
Bsm I
- ssRNA
MN-M+
T7
M1 5'
Neo
M1 3'
GUGs
Bsm I
+ssRNA
MN-M-
M1 5'
Neo
M1 3'
T7
Bsm I
- ssRNA

hepatitis delta (Δ) antigenome ribozyme (a gift from Dr. K. Dimock). The ribozyme sequence in the DNA constructs was followed by a polyadenylation signal from mouse phosphoglycerate kinase gene (derived from the plasmid pKJ1, a gift from Dr. M. McBurney) and a T7 terminator. To prevent the degradation of RNA transcripts and ensure the export of transcripts, both of which have been associated with the poly A tail (Watson et al., 1987; Eckner et al., 1991), the ribozyme was mutated to slow down the cleavage process so that the RNA would have time to get into the cytoplasm before it would be cleaved. It was reported that mutations at nucleotide 17 and 18 reduced the cleavage rate significantly but did not affect the cleavage efficiency (Perrotta and Been, 1991; Been et al., 1992). It was believed that the strength of the design was that the survival of cells after G418 treatment would mean that expression of both the Neo-containing constructs and functional T7 RNA polymerase had occurred.

DNA constructs for transcription of RNA inside cells were generated by addition of the mutated Δ ribozyme, pgk polyadenylation signal, and the T7 RNA polymerase terminator (in this order) to the constructs MN-F+ and MN-M+ such that cleavage of the Δ ribozyme would leave a exact sequence of the M1 3' end. These constructs, called pT7MNd, were confirmed to be correct by sequencing. pT7MNdf stands for M1-Neo fusion, pT7MNdf' for M1-Neo fusion with reversed polyadenylation signal, and pT7MNdm for mutation of viral AUGs to GUGs.

Cell lines resistant to G418 were obtained after cotransfection of L929 with pT7MNd and pMTT7N. The cell lines were then infected with helper reovirus T1 and the harvested virus was treated with DNase I and passaged in normal L929 cells or L929

cells persistently infected with T3 reoviruses. In the latter experiments, G418 selection was used but no resistant colonies were obtained. Following passage with T1 as helper virus, ssRNA and dsRNA were extracted and analyzed by RT-PCR and northern blot. Only the intact M1 gene was detected, indicating failure to rescue the foreign gene.

It was reasoned that the constructs to be rescued must have been transcribed and translated because the cells survived G418 selection but that the level of transcription was insufficient for rescue into reovirus. In addition, the polyadenylation process might have slowed RNA export such that most of the RNA was cleaved and degraded in the nucleus. Therefore, the polyadenylation signal was removed and the new constructs (pT7MNd') were confirmed by sequencing and transfected into L929 cells. No cells survived G418 selection, indicating a failure to produce transcripts that were translated.

To increase the level of transcription, cell lines were screened for high levels of T7 RNA polymerase using a reporter plasmid, pT7ßGal, which expresses ß-galactosidase and was made by replacing the SV40 promoter in plasmid pSV-ß-Gal (Promega, a gift from Dr. K. Dimock) with a T7 promoter. The ß-galactosidase activity in transfected cells was very low .

A second selection process had been reported to improve expression driven by T7 RNA polymerase in the nucleus (Lieber et al., 1989). A construct, pT7puro, was generated by ligating a modified T7 promoter (5'-TTAATTCGACTCACTATACGGAGATA-3')(Lieber et al., 1993) and the puromycin resistant gene from pPGKpuro (a gift of P.W. Laird) into pUC19. The construct was confirmed by sequencing the promoter and the junction region. The modified T7

promoter was used because it had been reported that the standard T7 promoter can be used by the polymerase II in some plasmid sequence backgrounds, although not with Neo cloned in pUC19 (Lieber et al., 1993), whereas the modified T7 promoter was specifically recognized by T7 RNA polymerase (Lieber et al., 1993). Transfection of cell lines, which were produced following pT7MNd transfection and G418 selection, with pT7puro, selection with puromycin (5 μg/ml, medium changed every three days), and screening of resistant cell lines with the reporter plasmid pT7ßGal showed only slightly increased ß-gal activity (data not shown).

To assay directly the level and the accuracy of RNA transcripts generated inside cells, a DNA construct pGEM-MD was made to produce RNA fragments for mapping the 3' end of the intracellularly cleaved RNA transcripts. After digestion of pGEM-MD with Bbv-1 and in vitro transcription, the run-off transcript was complementary to the 137 nucleotides of the M1 mRNA 3' end and the Δ ribozyme sequence from nucleotide 1 to 78. RNase protection assays showed that very low amounts of the RNA transcripts with the correct M1 3' end were present in the established cell lines (Fig. 7.2). To further check for the 3' end of the transcripts, primer extension was also performed with a primer complementary to the 3' end of the Δ ribozyme. No primer extension products could be identified presumably because insufficient RNA was present in cells (Fig. 7.3). The in vitro transcripts of DNA constructs pT7MNd were mapped by RNase protection and primer extension. All of transcripts gave the correct product by primer extension but the RNase protection assay showed that the RNA with a cleaved 3' end accounted for only a small proportion of the total transcripts (Fig. 7.2 and 7.3).

The work described above indicated that (1) the polyadenylation was important probably because of its role in mRNA export from the nucleus to cytoplasm or in mRNA stability; (2) the mutated Δ ribozyme did not work as well inside cells as was reported for an in vitro study which showed slow but efficient (high ratio of cleaved RNA compared to total RNA) and accurate cleavage (Been et al., 1992). Besides, the transfection efficiency (positive transfectants relative to total cells transfected) was very low with this series of constructs. It was thought that the transcripts synthesized in the nucleus by the T7 RNA polymerase might not be capped, resulting in poor translation of the already rare RNA transcripts that may have reached the cytoplasm.

7.3.4 The cytoplasmic T7 RNA polymerase approach

Following the failure with the nuclear transcription approach, the cytoplasmic T7 RNA polymerase approach, using recombinant vaccinia virus vTF7.3 (a gift of Dr. K. Dimock) to drive transcription, was tried. It was not considered in the first place because of the transient nature of transcription and the presence of vaccinia virus. As it was reported that the vaccinia virus capping enzymes can cap other non-vaccinia messengers (Ensinger et al., 1975), vTF7.3 would not only provide the T7 RNA polymerase but also cap the transcripts. The mutated Δ ribozyme in constructs pT7MNd was replaced by the wild type Δ ribozyme (pT7MNdw). Primer extension and RNase protection assay of pT7MNdw in vitro transcripts showed RNA transcripts with the correct 3' termini (Fig. 7.4 and 7.5). Cells were transfected with the constructs and infected with vTF7.3. RNase protection assays with ssRNA extracted from transfected

Fig. 7.2 RNase protection assay of in vitro and in vivo transcripts from pT7MNd constructs. The RNA probe was complementary to the M1 3' end (137 nucleotides) and the immediately downstream delta ribozyme sequence (78 nucleotides). MN-F+, run-off in vitro transcript; L929, RNA extracted from normal L929 cells; T1 inf, RNA from T1-infected L929 cells. In vitro transcripts of pT7MNdf3, 6, pT7MNdm47 were from run-on transcription. The arrow indicates the position the correctly cleaved RNA.

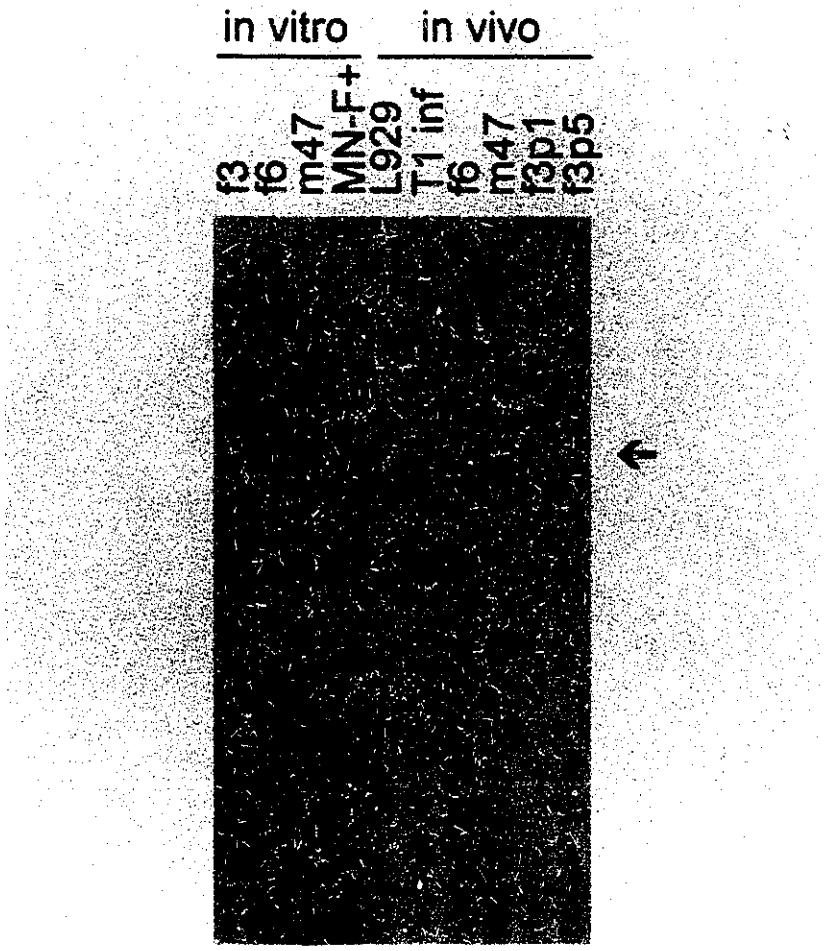
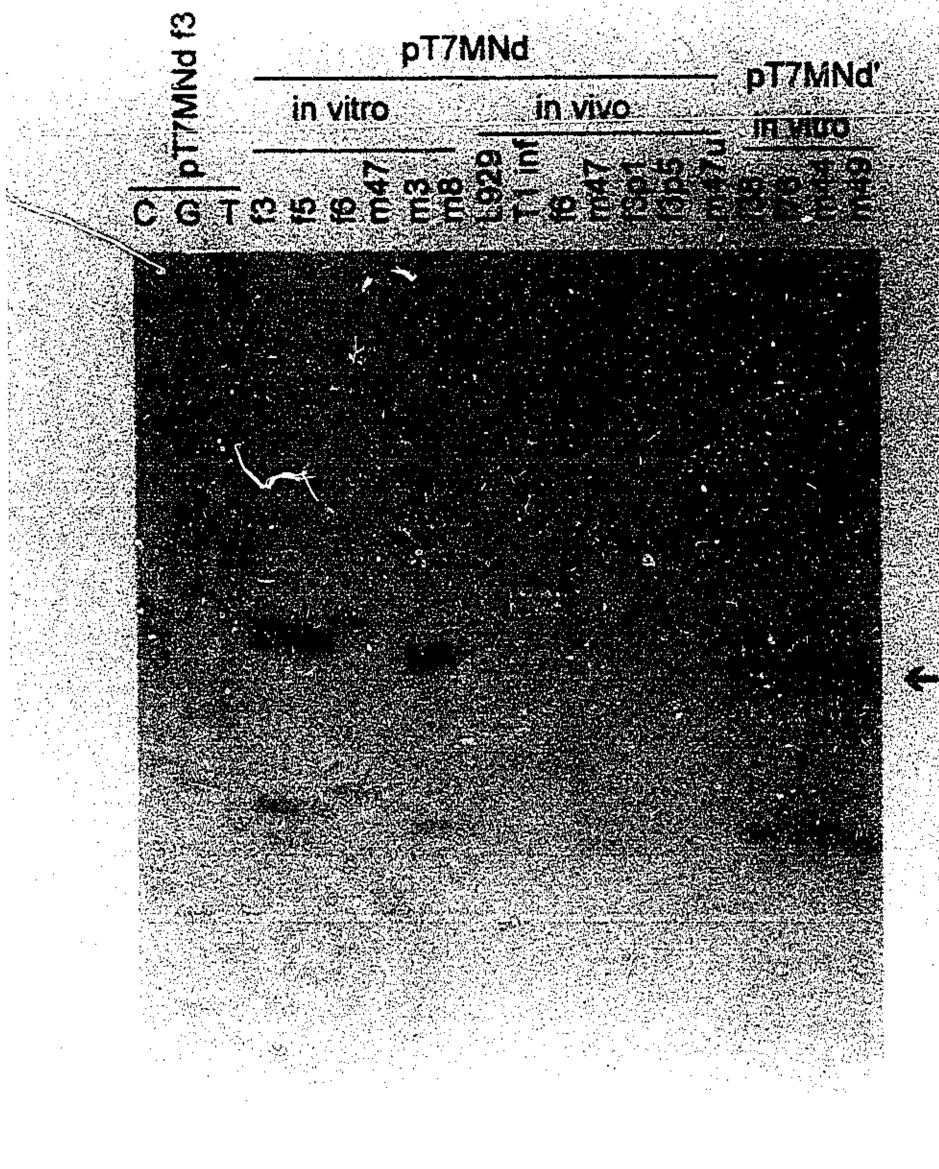

in vitro
in vivo
f3
f6
m47
MN-F+
L929
T1 inf
f6
m47
f3p1
f3p5

Fig. 7.3 Primer extension of in vitro and in vivo transcripts from pT7MNd constructs. The primer was complementary to the 3' end of the Δ ribozyme sequence. The A reaction of the marker sequencing was lost in electrophoresis. f6, m47, f3p1, f3p5, m47u represent RNA samples from different transfectants. The shift in f6 and m47 resulted from a one-nucleotide addition and a three-nucleotide deletion, respectively.


pT7MNd f3
pT7MNd
in vitro
in vivo
pT7MNd'
in vivo
C
G
T
f3
f5
f6
m47
m3
m8
L929
T1 inf
f6
m47
m47u

cells showed that transcripts possessing the correct 3' end were present (Fig. 7.6).

The rescue experiment was subsequently performed in which cells were first infected with helper reovirus T1 at an moi of 20, and then transfected with pT7MNdw followed by infection with vTF7.3 at an moi of 4. Virus was harvested, treated with antibody against vaccinia, RNase A and DNase I. The treated virus was passaged in normal cells together with helper T1 virus at an moi of 5 and dsRNA was extracted and tested by nested RT-PCR. Again, only the intact M1 was detected even though the cytoplasmic system was more efficient than the nuclear system in generating transcripts that possess correct 3' end.

7.4 Discussion

There are several explanations for the inability to rescue a foreign gene into reovirus. With regard to the nuclear T7 RNA polymerase approach, it was recently reported that the bacteriophage T7 promoter can be used by eukaryotic polymerase II quite efficiently. In the absence of T7 RNA polymerase, the expression of reporter genes under control of the T7 promoter in a pGEM or pUC background can be 30-90% of the level achieved with the RSV promoter (Sandig et al., 1993). Although the authors maintained that with the Neo gene the T7 RNA polymerase is required for expression, the flanking M1 termini in our constructs might have enabled the T7 promoter to be recognized by the polymerase II. It was observed in this study that transfection with pT7MNdf alone without pMTT7N generated G418 resistant colonies of cells (data not shown), indicating that polymerase II may have used the T7 promoter. Alternatively it is possible that the

Fig. 7.4 Primer extension of in vitro run-on transcripts from pT7MNdw constructs. The primer was complementary to the 3' end of the Δ ribozyme sequence. The arrow indicates the correct extension product.

pT7MNdw
pT7MNdw 'f37
A C G T
pT7MNdw
f1
f6
f7
f9
f14
f18
f23
f26
f28
m53
m78
m80
m85
m96
pT7MNdw 'f33
pT7MNdw 'f37

Fig. 7.5 RNase protection assay of in vitro transcripts from pT7MNdw constructs. The RNA probe was complementary to the M1 3' end (137 nucleotides) and the immediately downstream Δ ribozyme sequence (78 nucleotides). MN-F+ and MN-M+ were run-off transcripts and all others were run-on transcripts. The arrow indicates the correctly cleaved RNA.

pT7MNdw
pT7MNdw
tRNA
f1
f6
f7
f9
f14
f18
f23
f26
f28
MN-F+
MN-M+
m53
m78
m80
m85
m96

Fig. 7.6 RNase protection assay of in vivo transcripts from pT7MNdw constructs. The RNA probe was complementary to the M1 3' end and the Δ ribozyme sequence. positive control, run-off in vitro transcript; T1 infection, RNA from T1-infected L929 cells. Two DNA clones of each type of construct were used for transfection. The arrow indicates the correctly cleaved RNA.

tRNA control
positive control
T1 infection
no RNA
pT7MNdw

M1-Neo cassette integrated downstream of a cellular promoter. Another report (Perrotta and Been, 1993) indicated that with the mutated ribozyme the changes at positions 17 and 18 caused a 2500-fold decrease in the cleavage rate rather than the 50-fold decrease they reported previously (Been et al., 1992). In view of these reports it is not surprising that the attempts with T7 RNA polymerase in the nucleus and using a mutated hepatitis delta antigenome ribozyme approach were unsuccessful.

For the cytoplasmic transcription approach, infection with vTF7.3 might affect the formation of special structures that are required for reovirus assembly and maturation.

Generally, the differences between RNA derived from cDNA and that derived by viral transcription could be responsible for the failure to encapsidate the M1 analogue into reovirus because it has been shown that viral RNA synthesized in vitro from reovirus cores generated with proteolytic digestion has been assembled into infectious virus (Roner et al., 1990). Modifications such as methylation of the viral RNA which were not present in the DNA-derived RNA could be recognized by the viral assembly mechanism and thus exclude the DNA-derived RNA lacking these modifications. The 3' end generated by the Δ ribozyme was a 2'-3' cyclic phosphate versus a normal hydroxyl group in the viral RNA, which might impair its assembly into the reovirion. Localization of the DNA-derived RNA in cellular compartments may also be a contributing factor as the amount of RNA reaching the correct compartment may be too low for rescue.

The demonstration that μ2 expressed from cDNA complemented the M1 ts mutant shed some light on future rescue work. For example, the ts mutant could be used as the

recipient virus and wild type M1 RNA derived from cDNA for rescue. The virus harvest from the rescue experiment would be passaged in normal cells and selected at an elevated temperature. Reoviruses containing wild type M1 derived from cDNA would be favoured over the parental viruses containing a defective M1 during the passage and thus viruses with the rescued wild type M1 would be enriched. More information about the mechanism of assortment and assembly in reoviruses could then be obtained which should eventually lead to successful rescue of reovirus gene analogues.

CHAPTER EIGHT

SUMMARY

The work carried out in this study answered some of important questions regarding reovirus genome replication, packaging and gene expression and also raised new questions for which more work is needed to find answers.

1. Sequence elements of the reovirus M1 genome segment required for its replication and encapsidation were identified by cloning and sequencing the M1 remnants of reovirus M1 deletion mutants. The termini of the M1 genome segment, 132-135 nucleotides of the M1 5' end and 183-185 nucleotides of the M1 3' end contain all the genetic signals sufficient for replication and encapsidation of the M1 gene segment. These consensus terminal regions can be used for rescue of synthetic viral genome analogues into reovirus and for the development of reovirus as an expression vector.

2. Various DNA constructs containing the neomycin resistant gene (Neo) flanked by the M1 termini required for replication and encapsidation were generated as a first step in developing methodology to introduce a foreign gene into reovirus. Run-off transcription in vitro from some of these constructs generated both ssRNA and dsRNA carrying authentic viral termini. Constructs containing the delta ribozyme produced ssRNAs in transfected cells, which had terminal sequences identical to viral M1 ssRNA. Although several different protocols were attempted to introduce the Neo-containing M1 analogue into reovirus, the M1 analogue could not be rescued. However the results did provide information which will be useful for future efforts. Differences in post-transcriptional modification between the viral RNA and the synthetic RNA analogues,

such as methylation or the nature of the 3' end are probably important for recognition by reovirus components involved in encapsidation.

3. The full length M1 gene of reovirus type 1 Lang was cloned, sequenced and compared with the type 3 Dearing M1 gene. 51 nucleotide substitutions and 10 amino acid substitutions were identified. Some or all of these are responsible for the functional differences between T1μ2 and T3μ2 (e.g. in pathogenesis). Further mutagenic analysis of the T1 and T3 M1 gene segments should identify specific nucleotides or amino acids that lead to the phenotypic differences.

4. The M1 genes of both type 1 and type 3 reoviruses were expressed as Trp-E-μ2 fusion proteins in E. coli and used to produce μ2-specific antibody in rabbits. The antibody provides a specific and sensitive means for the detection of μ2 protein and has been in demand by several collaborators.

5. The reovirus M1 gene was stably expressed in mammalian cells transfected by M1 and Neo gene-containing dicistronic constructs and the μ2-expressing cell line, T1-11-1, was shown to complement the growth of the reovirus tsH11.2 M1 mutant. This work not only demonstrated the ability of μ2 protein expressed from transfected DNA to interact with the viral replication process but also provided cell line for growth of reovirus with defective M1 genes. These cells can presumably be used to cultivate reoviruses containing an M1 gene analogue.

6. The nature of the translation initiation site and the product of the reovirus M1 gene was clarified by in vitro mutagenesis of the M1 gene. Translation of the M1 gene was shown to initiate at the first AUG codon and the resulting protein is about 73 kDa and

predicted to consist of 736 amino acids. The 5'-terminal sequence and the 3' UTR of the M1 gene are not responsible for the low level expression of μ2 protein in transfected cells.

7. Translation of μ2 protein must be affected by host factors since different levels of μ2 proteins were produced in different mouse cell lines. The identity of these host factors and the nature of the interaction will shed light on the control of μ2 translation.

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APPENDIX ONE

Solutions and Buffers Used for Cell Culture

1. Freezing medium for liquid nitrogen storage of cells

MEM supplemented with fetal bovine serum (20%) and DMSO (10%).

2. Geneticin (G418)

From GIBCO BRL, dissolved in deionized distilled water at 50 mg active geneticin per ml (100x stock), filtered with 0.22 μm minipore syringe filter and kept at -20^{0}C.

3. L-Glutamine (3%, 100x)

L-glutamine (Flow) was dissolved in deionized distilled water at 3 g/100 ml, filter sterilized and kept at -20^{0}C.

4. MEM (Minimum Essential Medium) with Eagle's salts

Flow MEM 95.19 g x2 plus $NaHCO_3$ 40 g dissolved in deionized distilled water 20 l, pH adjusted to 7.2-7.4 with HCl or NaOH, filter-sterilized with positive pressure and kept at 4^{0}C.

Supplemented with L-glutamine and penicillin/streptomycin when use.

5. $NaHCO_3$ (Sodium Bicarbonate)

$NaHCO_3$ 25 g dissolved in 500 ml of deionized distilled water, filter sterilized and kept

at 4^0C.

6. PBS (phosphate buffered saline)

Na_2HPO_4	54.5 g
NaH_2PO_4	15.75 g
NaCl	425.0 g
deionized distilled H_2O to	50 l

filter sterilized or autoclaved and kept at 4^0C.

7. PBS + 0.2% Gelatin

Na_2HPO_4	10.9 g
NaH_2PO_4	3.15 g
NaCl	85.0 g
deionized distilled water	10.0 l
Gelatin	20.0 g

Dissolve Gelatin in 1 l of water by stirring and heating and then add it to the PBS solution.

Sterilized by autoclaving for 30 min with liquid cycle.

8. Pen-Strep (penicillin/streptomycin, 100x stock)

Penicillin G Sodium Salt (ICN)	$5x10^6$ International Unit
Streptomycin Sulfate (ICN, 760 mg/g)	6.58 g

Dissolved in 500 ml of deionized distilled water, 0.2 μm filter sterilized and kept at -20^{0}C.

The concentration of Penicillin is 10000 Unit/ml and that of Streptomycin 10000 μg/ml.

9. Trypsin (0.5%, 2x)

NaH_2PO_4	0.315 g
Na_2HPO_4	1.09 g
NaCl	8.5 g
Trypsin (ICN 1-250)	5.0 g
Phenol red	0.02 g
deionized distilled water	1 l

Dissolved with stirring at RT for several hours, filtered through 0.2 μm filter and kept at -20^{0}C.

Dilute 1:2 with PBS when use.

APPENDIX TWO

Oligonucleotide Primers

1. M1 5'- (5' end of the reovirus M1 gene minus strand)

   5' TAACTGCAGGATGAAGCGCGTACGTAG 3'

2. M1 5'+ (5' end of the reovirus M1 gene plus strand)

   5' TAACTGCAGGCTATTCGCGGTCATGGC 3'

3. M1 3-202 (complementary to the M1 plus strand from base 202 to base 185)

   5' GAGTGCATCGATAACGTC 3'

4. SZ1+ (Bcl I Neo 5'+, Bcl I site plus the Neo gene 5' end)

   5' CCCGGGTTTGATCATATGATTGAACAAGATGGA 3'

5. SZ2- (Bam HI Neo 5'-, Bam HI site plus the 5' end of the Neo gene minus strand)

   5' CCCGGGTTGGATCCTCAGAAGAACTCGTCAAG 3'

6. SZ3- (Neo3'- M13'-M, the 3' end of the Neo gene minus strand followed by a synthetic initiation sequence and then by the minus strand of the M1 gene 5'-terminus, with ATGs mutated to GTGs)

5' GCAATCCATCTTGTTCAATGGCCGATCCCATATTGGCTGATCA

CGATCTTGATATGAAACGTCACTCGCACCAGCCCCAGCGTCT 3'

7. SZ4+ (M15'+ MXO, M1 5' end, with ATG mutated to GTG, plus sequence overlapping with the polycloning site of pGEM7Zf(+))

5' CTAGACTCGAGGAATTCGGTACCCCGCT

ATTCGCGGTCGTGGCTTACATCGCAGTTC 3'

8. SZ5+ (T7M15'+, T7 promoter and the M1 gene 5' end)

5' GTAATACGACTCACTATAGCTATTCGCGGTCATGGC 3'

9. SZ6+ (T7M15'+M, T7 promoter and the M1 gene 5' end but the ATG became GTG)

5' GTAATACGACTCACTATAGCTATTCGCGGTCGTGGC 3'

10. SZ7- (Bsm I M15'-, Bsm I site plus the 5' end of the M1 gene minus strand)

5' TATGAATGCAGATGAAGCGCGTACGTAG 3'

11. SZ8- (T7-M15'-, T7 promoter plus the 5' end of the M1 gene minus strand)

5' GTAATACGACTCACTATAGATGAAGCGCGTACGTAG 3'

12. SZ9+ (Bsm I M15'+, Bsm I site plus the M1 5' end)

5' TATGAATGCAGCTATTCGCGGTCATGGC 3'

13. SZ10+ (Bsm I M15'+M, Bsm I site plus the M1 5' end but mutation of ATG to GTG)

5' TATGAATGCAGCTATTCGCGGTCGTGGC 3'

14. SZ11+ (this is a region of the Neo gene about 250 bp from the 5'+ end, for sequencing of Neo)

5' ACTGGCTGCTATTGGGCG 3'

15. SZ13+

5' GTACGCGCTTCATCGGGTCGGCAT

GGCATCAGCACCTCCTCGCGGTCC 3'

16. SZ14-

5' CCCTATGAATGCACGAGCTCGGTACAGC 3'

17. SZ15+ (Sac I site plus nt 145-158 of the M1 gene)

5' CTAGTCTCGAGGATCAGTTACAGTA 3'

18. SZ16- (reverse complementary to the M1 plus strand 429-445)

5' CATTGTCATCGGTGACG 3'

19. SZ17+ (M1 3' end followed by the delta ribozyme sequence)

5' GTACGCGCTTCATCGGGTCGGCATGGCATC 3'

20. SZ18- (reverse complementary to the 3' end of the delta ribozyme sequence)

5' CCCTTAGCCATCCGAGTG 3'

21. SZ19+ (Sac I/Xho I sites plus M1 sequence 145-172, with the -3 position of AUG_{161} mutated from T to A)

5' CAGAGCTCCTCGAGGATCAGTTACAGAACATGTTAGATGGA 3'

22. SZ20+ (Sac I/Xho I sites plus M1 sequence 145-179, with both -3 and +4 positions mutated to A from T)

5' CAGAGCTCCTCGAGGATCAGTTAC

AGAACATGATAGATGGATATGAGG 3'

23. T7P5- (reverse complementary to a region close to the 5' end of the T7 RNA polymerase gene, but about 350 nt downstream of the nuclear localization signal (NLS), common to pMTT7 and pMTT7N)

5' GGCCCGACCGATTGCGCTTGC 3'

24. T7P5+ (a region after the transcription start site but before the NLS region)

5' CTCCGTAGCTCCAGCTTCACC 3'

25. SZ21- (reverse complementary to M1 2206-2224)

5' TCACGCCAAGTCAGATCGG 3'

26. SZ22+ (pCITE-1 2332-2352)

5' CCCGGGAATTCCGCCCCTCTCCCTC 3'

27. SZ23+ (CITE 5' end)

5' GATCAGAGCTCCTCGAGGAATTCCGCCCCTCTCCC 3'

28. SZ24- (5' end of the CITE minus strand)

5' GCCTCATATCCATCTATCATATTATCATCGTGTTTTTCAAAGG 3'

29. SZ25+ (Sac I/Xho I sites plus M1 161-183)

5' GATCAGAGCTCCTCGAGGATGATAGATGGATATGAGGCTGG 3'

30. SZ26- (reverse complementary to M1 115-183 with upstream ATGs mutated to GTGs and -3/+4 positions of ATG_{161} mutated to ACC/A)

5' CCAGCCTCATATCCATCTATCATGGTCTGTAACTG

ATCCAACACACAGTCACGATCTTGATATGAAACG 3'

31. SZ27+ (Sac I/Xho I sites plus M1 sequence 1-18)

5' GATCAGAGCTCCTCGAGGGCTATTCGCGGTCGTGGC 3'

32. SZ28- (Xho I/Not I sites followed by T7 promoter plus Sac I site, for insertion of T7 promoter into M1CN(s) constructs)

5' TCGAGGCGGCCGCCTATAGTGAGTCGTATTACGAGCT 3'

33. T7 M (mutated T7 promoter)

5' TATCTCCGTATAGTGAGTCGAATTA 3'

34. UT plus (Stu I site plus 13 nt of the 5' end of influenza viral RNA, for PCR of T7 promoter out of Clone B6 in pGEM7Zf(+))

5' CACACCAACAACCAAGGCCTTGTTTCTACTTAT 3'

APPENDIX THREE

Plasmid DNA Constructs Used in This Study

1. pGEM-M1

Blunt-end ligation of reovirus M1 cDNA of type 1 and type 3 into plasmid pGEM7Zf(+) (Promega) linearized with Sma I.

T1M1 clones 6, 15 and T3M1 clone 18 are in the orientation of T7 promoter.

T1M1 clone 7 and T3M1 clone 6 are in the orientation of SP6 promoter.

(clone T1-6 has an A deletion at base 300 from the 5'+ end, resulting in an early termination at base 329 because of the reading frame shift)

2. pMAMneo-M1

Blunt-end ligation of T1M1 and T3M1 (from T1-6 and T3-6 in pGEM by Pst I digestion) into pMAMneo cut open by Xho I.

T1M1 clone 9, 12, 18 and T3M1 clone 18 are in correct orientation.

3. pKJ2-M1

Sticky-end ligation of T1M1 and T3M1 (from pGEM-M1 T1-6 and T3-18 by Pst I) into pKJ2 linearized by Pst I.

T1M1 clone 10 and T3M1 clone 72 are in correct orientation.

T1M1 clone 19 and T3M1 clone 92 are in opposite orientation.

(T1M1 clones have the A deletion from pGEM-M1 clone 6)

4. Repairing of pKJ2-M1 clone T1-10

Removal of fragment 192-1200 from T1-10 by Cla I and sticky-end ligation of fragment 192-1200 from pGEM-M1 clone T1-7 to the linearized T1-10.

Clones T1-38 and T1-39 are in correct orientation.

5. pKJ1ΔF-neo, and pKJ1ΔR-neo

Blunt-end ligation of the Neo gene (from pMAMneo by Bam HI) into Hind III-cut pKJ1ΔF/R (pKJ1ΔF/R-neo).

pKJ1ΔF-neo clone 48 has the Neo gene in the same orientation of the pgk promoter.

pKJ1ΔF-neo clone 17 and pKJ1ΔR-neo clone 22 have the Neo gene in opposite orientation of pgk.

6. pKJ1M1neo

Blunt-end ligation of T1M1 (from pKJ2-M1 clone 39 by Pst I) and T3M1 (from pGEM-M1 clone 18 by Pst I) into pKJ1ΔF-neo clone 48 linearized by Kpn I.

T1M1 clones 4, 12 and T3M1 clones 4, 12 are in correct orientation.

T1M1 clone 2 and T3M1 clone 2 are in wrong orientation.

7. pATH-M1

Sticky-end ligation of T1M1 and T3M1 (from pGEM-M1 T1-7 and T3-18 by Pst I) into pATH2 partially digested by Pst I and agarose gel-purified.

T1M1 clones 1, 4, 6, 10, 11, 12, 14 and T3M1 clones 11, 16, 18 are in correct

orientation and correct reading frame.

8. MN-F and MN-M (98B11-1-neo and 98B11-1-neo M)

Sticky-end ligation of the Neo gene (amplified from pMAMneo with primers SZ1+ and SZ2- by PCR and cut with Bcl I and Bam HI) into pUC19 cut open by Sma I.

98B11-1-neo clones 2, 19 have the Neo gene in correct orientation (MN-F)

Digestion of MN-F clone 2 with Nde I and Kpn I, PCR with primers SZ3+ and SZ4- and cotransformation of DH5α (MN-M or 98B11-1-neo M).

MN-M clones 6, 12 are correct.

9. pKJ1-MN-F/M

Sticky-end ligation of the M1-Neo-Mi fragment (cut out from MN-F clone 2 and from MN-M clones 6, 12 by Kpn I and Sac I) into pKJ1ΔF linearized by Kpn I and Sac I.

pKJ1-MN-F clones 2-3, 2-4 and pKJ1-MN-M clones 6-3, 12-3 are correct.

10. MN-F+/- and MN-M+/-

Insertion of the T7 promoter upstream of MN-F and MN-M and Bsm I site immediately downstream of MN-F and MN-M by PCR using primers SZ5+/SZ7- (for generation of +ssRNA from MN-F). SZ8-/SZ9+ (for -ssRNA from MN-F), SZ6+/SZ7- (for generation of +ssRNA from MN-M), and SZ8-/SZ10+ (for -ssRNA from MN-M).

Clones chosen for further work:

Source Clone of MN-F/M	Name of new constructs	Clone Number	ssRNA generated
2	MN-F+	1-3	+ssRNA
6	MN-M+	2-3	+ssRNA
12	MN-M2+	3-3	+ssRNA
2	MN-F-	4-3	-ssRNA
6	MN-M-	5-3	-ssRNA
12	MN-M2-	6-2	-ssRNA

(later as the transcription from clone 2-3 was not very good clone 3-3 was used for further work and sometimes was called MN-M+ instead of MN-M2+)

11. pKJ1ΔF-M1-Neo

Digestion of pKJ1M1neo clones T1-2, 4, 12, T3-2, 4, 12 with Hind III and Xho I to remove the pgk polyadenylation signal and the SV40 promoter.

The following clones are correct:

source clone of pKJ1M1neo	new clones of pKJ1ΔF-M1-Neo
T1-2	1, 2, 3, 4, 5
T1-4	1, 2, 3, 5
T1-12	2, 4, 5
T3-2	1, 2, 3, 4, 5
T3-4	1, 2, 3, 4, 5
T3-12	1, 2, 3, 5

12. M1CN

a. sticky-end ligation of the Neo gene (amplified from pMAMneo with primers SZ1+/SZ2- and cut by Bam HI and Bcl I) into Bam HI-cut pKJ1ΔR.

Clones 1, 2, 4, 7, 8, 10, 11 are in correct orientation.

Clone 3 is in opposite orientation.

Clone 8 was used for further work.

b. Blunt-end ligation of M1 (from pGEM-M1 T1-7 and T3-18 by Pst I digestion) into Kpn I-cut clone 8.

T1M1 clones 2, 3, 6, 7, 10, and T3M1 clone 10 are correct.

T1-2 and T3-10 clones were chosen for further work.

c. Blunt-end ligation of CITE (from pCITE-1 by Eco RI and Nco I digestion and agarose gel purification) into T1-2 and T3-10 linearized by Sma I located between the M1 gene and the downstream Neo gene.

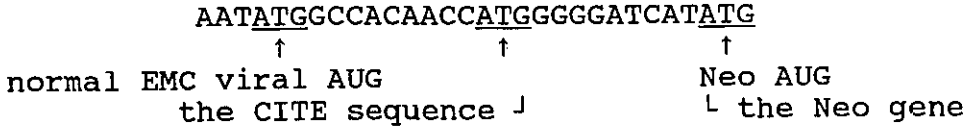
Clones T1-9, 11, 14, and T3-1, 6, 12, 15, 18 are in correct orientation (M1CN).

T1M1 clones 10, 16 and T3M1 clones 3, 4, 5 are in wrong orientation.

d. Reversion of the M1 gene in M1CN clone T1-9 by digestion with Sna BI/Xho I and blunt-end ligation.

Clones 4, 7 were used for further work (T1-9R4 and T1-9R7).

The junction region between CITE and Neo:



13. M1CN-BPV

Blunt-end ligation of the BPV-1 transforming fragment (cut out of p306 by Hind III and Bam HI) into M1CN (clones T1-9, T1-9R4, T3-15) partially digested by Hind III and agarose gel-purified.

The following clones are correct:

T1-9: 1, 9, 14, 15, 51

T1-9R: 24

T3-15: 33, 35, 44

14. pT7MNd(f/f'/m)

a. Blunt-end ligation of the pgk polyadenylation signal (from pKJ1ΔR by Hind III and Bam HI digestion) into the Pst I site between the Δ ribozyme and the T7 terminator of plasmid pIV-catδter partially digested by Pst I and purified.

Clones 9, 10, 28, 58 have pgk poly A signal in correct orientation and clone 40 has it in reverse orientation.

Clones 10 and 40 were chosen for further work.

b. PCR of clones 10 and 40 with primers SZ13+/SZ14- to amplify the Δ-pgk poly A signal-ter fragment and cotransformation of DH5α with the PCR product and MN-F+ (1-3) and MN-M2+ (3-3) linearized by Bsm I.

pT7MNdf clones 3, 5, 6 have the Neo gene fused to the AUG at nucleotide 14 of the M1 5'-terminus.

pT7MNdm clones 47, 3, 8 have the 4 AUGs in the M1 gene 5'-terminal 135 nucleotides mutated to GUGs.

pT7MNdf' clone 30 has the M1-Neo fusion without mutation of AUGs to GUGs but has reversed pgk polyadenylation signal.

(pT7MNdf clone 6 has an extra G after nucleotide 11 of the Δ ribozyme and pT7MNdm clone 47 has a TCC deletion after nucleotide 22 of the Δ ribozyme.)

15. pT7-ß-Gal

Blunt-end ligation of a T7 promoter fragment (from Dr. Earl Brown's "Utility Vector" which contains the standard T7 promoter plus several restriction sites) into pSV-ß-Galactosidase (Promega) cut by Nco I and Hind III.

Clones 10, 11, 12, 13 are correct by restriction digestion but sequencing showed a 8-nt deletion in the T7 promoter.

16. pT7mßGal

PCR of clone B6 from Jane Bailey which contains T7 promoter in pGEM7Zf(+) with primers T7M and M13 reverse primer, digestion of the PCR product with Nsi I and blunt-end ligation into pSV-ß-Gal cut by Nco I and Hind III.

Clones 2, 12 are correct as confirmed by sequencing.

17. pGEM-MD

Digestion of pT7MNdf clone 3 with Hga I, recovery of a 1.1-kb fragment and blunt-end ligation into Bam HI-cut pGEM7Zf(+).

Clone 8 is correct but SP6 RNA polymerase should be used for transcription and Bbv-1 site digestion for run-off transcription.

18. pT7MNd'

PCR of the Δ-ter fragment from pIV-catδter by primers SZ13+/SZ14- and cotransformation of DH5α with the PCR product and MN-F/M2+ (clones 1-3 and 3-3)

linearized by Bsm I.

pT7MNd'f clones 38, 76 and pT7MNd'm clones 44, 49, 50, 53 are correct.

19. pT7Puro

a. Blunt-end ligation of the PCR-amplified T7 fragment (got in preparation of pT7mßGal) into Sma I-cut pUC19.

Clones 27, 37 have Xba I site downstream of the T7 promoter and clones 18, 21 have Kpn I downstream of the T7 promoter.

b. Blunt-end ligation of Puro (from plasmid pPGKPuro by Hind III digestion) into Xba I-cut clone 27.

clones 1, 5, 14 are correct.

20. pT7MNdw

PCR of the Δ-ter fragment and the Δ-pgk poly A signal-ter fragment with primers SZ17+/SZ14- and cotransformation of DH5α with the PCR product and the Bsm I-linearized MN-F/M2+ clones 1-3 & 3-3.

The following clones are correct:

pT7MNdwf 1, 6, 7, 9, 14 18 (forward pgk 3', M1-Neo fusion)

pT7MNdwf' 23, 26, 28 (reversed pgk 3', M1-Neo fusion)

pT7MNdwm 53, 78, 80, 85, 96 (forward pgk 3', AUGs mutated)

pT7MNdw'f 33, 37 (No pgk 3', M1-Neo fusion)

21. M1CN2 (constructs b)

PCR of nucleotides 145-445 of the M1 5'-terminus with primers SZ15+/SZ16-, digestion of the PCR product with Xho I, sticky-end ligation into M1CN T1-11 and T3-15 cut by Xho I completely and by Bgl II partially, and transformation of DH5α.

T1M1 clones 6, 42 and T3M1 clones 13, 46, 58 are correct.

22. M1CN3 (constructs c and d)

PCR of the Xho I-cut PCR products (of M1CN T1-11/T3-15 with primers SZ15+/SZ16-) with primers SZ19+/SZ16- (for constructs c) or SZ20+/SZ16- (for constructs d) and cotransformation of DH5α with the PCR product and M1CN T1-11/T3-15 cut by Xho I and Bgl II.

Constructs c T1 clones 4, 7 and T3 clones 15, 21, 22 are correct (mutation of the -3 position of AUG_{161} in the M1 gene).

Constructs d T1 clone 12 and T3 clones 28, 32, 35 are correct (mutation of the -3 and +4 positions of AUG_{161} in the M1 gene).

23. M1CN4 (constructs e, f, g, h)

PCR of M1CN (constructs a), M1CN2, M1CN3 with primers SZ22+/SZ21- using the Taq Extender from Stratagene, to delete the 80-nt 3' UTR of the M1 gene, followed by Klenow treatment, kinase reaction and ligation.

The following clones are correct by Nde I/Apa I digestion:

Constructs e T1 clones 3, 5, 34, 50 (from M1CN T1-11)

Constructs e T3 clones 52, 56 (from M1CN T3-15)

Constructs f T1 clones 13, 14, 15, 18 (from M1CN2 T1-6)

Constructs f T3 clones 19, 20, 23 (from M1CN2 T3-13)

Constructs g T1 clones 25, 26, 30 (from M1CN3 T1-4)

Constructs g T3 clones 36, 40, 41, 42 (from M1CN3 T3-15*)

Constructs h T1 clones 33, 63, 65, 68 (from M1CN3 T1-12)

Constructs h T3 clones 43, 47, 48 (from M1CN3 T3-28)

24. M1CN5 (constructs i, deletion of the 160 nucleotides of M1 5')

PCR of Xho I-cut M1CN3 T1-12/T3-28 with primers SZ25+/SZ16- and cotransformation of DH5α with the PCR product and the M1CN T1-11/T3-15 cut by Xho I and Bgl II.

T1 clones 152, 158, 165, 168 and T3 clones 182, 184, 202, 203 are correct.

25. M1CN5+CITE (constructs k)

Digestion of pool of clones 152, 158, 165, 168 and pool of clones 182, 184, 202, 203 with Xho I, PCR of CITE with primers SZ23+/SZ24-, and cotransformation of DH5α.

T1 clones 3, 5, 10, 12 and T3 clones of 29, 38, 40, 41 are correct.

26. M1CN5-3' (constructs j, deletion of the 3' UTR of M1)

PCR of clones 158 and 203 with primers SZ22+/SZ21-, Klenow treatment, kinase reaction and ligation.

T1 clones 1, 8, 17 and T3 clones 22, 23, 25 are correct.

27. M1CN6, M1CN6c, M1CN7, M1CN7c, M1CN8, M1CN8c

Digestion of M1CN T1-11/T3-15 with Cla I and ligation (M1CN6).

Digestion of M1CN5+CITE T1/T3 with Cla I & ligation (M1CN6c).

Digestion of M1CN T1-11/T3-15 with Nsi I, treatment with T4 DNA polymerase and blunt-end ligation (M1CN7, a UGA occurs after 7 aa from the rejoining point).

Digestion of M1CN5-CITE T1/T3 with Nsi I, treatment with T4 DNA polymerase and blunt-end ligation (M1CN7c).

Digestion of M1CN T1-11/T3-15 with Nsi I/Sac II, treatment with T4 DNA polymerase (to restore the reading frame) and blunt-end ligation (M1CN8).

Digestion of M1CN5-CITE T1/T3 with Nsi I/Sac II, treatment with T4 DNA polymerase and blunt-end ligation (M1CN8c).

The following clones are correct:

M1CN6: T1-2, 4, T3-10, 11 M1CN6c: T1-21, 24, T3-27, 28

M1CN7: T1-60, 62, T3-53, 54 M1CN7c: T1-42, 44, T3-34, 36

M1CN8: T1-86, 88, T3-80, 81 M1CN8c: T1-74, 75, T3-66, 67

28. pATH2-Neo

Sticky-end ligation of the Neo gene (amplified from pMAMneo by PCR and cut by Bcl I/Bam HI) into Bam HI-cut pATH2.

Clones 3, 4, 6, 7, 9, 13 are correct.

29. pCMV-M1CN

a. Digestion of pCDNA3 (Invitrogen) with Tth111 I/Bssh II to delete the Neo gene sequence) and blunt-end ligation.

Clones 7, 16, 17, 21 are correct.

b. Blunt-end ligation of the M1-CITE-Neo fragment (from M1CN by Xho I/Bam HI digestion) into Bam HI/Xho I-cut clone 16.

pCMV-M1CN T1 clones 3, 7, 22 and T3 clone 13 are correct.

30. M1CNm (mutation of 5 AUGs to GUGs in the M1 5'-terminus and modification of the -3/+4 positions of AUG_{161})

PCR of the fragment cut out from pGEM-98B11-1-Neo M clone 6 (by Kpn I/Sac I) with SZ26-/SZ27+ and cotransformation of DH5α together with Xho I-cut and Klenow-treated M1CN5.

T1 clones 36, 41 and T3 clones 4, 46 are correct.

31. pGEM-M1 (-144 nt)

Sticky-end ligation of mixture of Xho I/Bgl II-cut pGEM-M1 T1-15 & Xho I/Bgl II/Sca I-cut M1CN2 T1-6 or that of pGEM-M1 T3-18/M1CN2 T3-13.

T3 clones 43, 55, 59, 69, 78 are correct.

32. pSEG7M1CN (insertion of influenza segment 7 DNA upstream of M1CN)

Blunt-end ligation of the segment 7 (from pSEG7/3-2 by Apa I/Kpn I digestion) into

Xho I-cut M1CN T1-11/T3-15.

T1 clones 1, 2, 3, 4, 11, 12, 13, 16 and T3 clones 20, 23, 28, 31, 32 have a segment 7 in forward orientation.

T1 clones 5, 6, 7, 8, 9, 15, 17 and T3 clones 19, 21, 24, 27, 33 have a segment 7 in backward orientation.

33. pGEM-SEG7-M1 (pGEM-S7-M1)

Blunt-end ligation of segment 7 DNA (from pSEG7/3-2 by Apa I/Kpn I digestion) into pGEM-M1 T1-15 and T3-18 cut by Apa I/Kpn I.

T1 clone 5 (T1-5F), T3 clones 14 (T1-14F), 23 (T3-23F) have segment 7 in forward orientation.

T1 clones 1, 2 (T1-1R, T1-2R), T3 clones 19, 20 (T3-19R, T3-20R) have segment 7 in backward orientation.

34. pT7M1CN(s) and pT7SEG7M1CN (pT7S7M1CN)

-cut M1CN, M1CN2, M1CN3, M1CN5, M1CN5+CITE, pSEG7M1CN with Xho I/Sac I

-kinase treatment of primer SZ28- (T7 promoter & Not I site)

-ligation of SZ28- and the cut vector

-Klenow treatment

-transformation of DH5α and growth of the transformed pool

-prepare DNA, cut with Not I and separate on low-melting agarose gel

-ligation and transformation

pT7M1CN T1-42, T3-48, pT7M1CN2 T1-2, T3-3, pT7M1CN3 T3-18, pT7M1CN5 T3-26, pT7M1CN5+CITE T1-36 are correct.

pT7S7M1CN clones T1-2, 14 and T3-32, 35 have a forward T7 promoter and T1-12R, T3-21R have a reversed T7 promoter.

APPENDIX FOUR

Publications Originating from Thesis Research

Referred papers:

Zou, S. and Brown, E.G. (1995). Control of reovirus $\mu 2$ expression in transfected cells. Proceedings of the Second Academic Conference of Young Scientists, Life Science Session. pp.182-188. Science & Technology Press of China, Beijing, China.

Zou, S. and Brown, E.G. (1992). Nucleotide sequence comparison of the M1 genome segment of reovirus type 1 Lang and type 3 dearing. Virus Research. 22, 159-164.

Zou, S. and Brown, E.G. (1992). Identification of sequence elements containing signals for replication and encapsidation of the reovirus M1 genome segment. Virology. 186, 377-388.

Abstracts:

Zou, S. and Brown, E.G. (1995). Control of reovirus $\mu 2$ expression in transfected cells. American Society for Virology 14th Annual Meeting.

Zou, S. and Brown, E.G. (1993). Constitutive expression of reovirus $\mu 2$ protein in mouse L cells. American Society for Virology 12th Annual Meeting.

Zou, S. and Brown, E.G. (1991). Identification of the minimum essential sequences for replication and assembly of the reovirus M1 genome segment. American Society for Virology 10th Annual Meeting.

Manuscripts submitted:

Zou, S. and Brown, E.G. (1995). Stable expression of reovirus $\mu 2$ protein complemented the growth of a reovirus ts mutant with a defect in its M1 gene. Virology.

Zou, S. and Brown, E.G. (1995). Translation of reovirus M1 gene initiates from the first AUG codon in both infected and transfected cells. Virus Research.