# MAMMALIAN REOVIRUS:

# CHARACTERIZATION OF THE M1 GENE

# SEGMENT AND THE μ2 PROTEIN

By

Magdalena Izabela Swanson

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science

Department of Medical Microbiology and Infectious Diseases
University of Manitoba
Winnipeg, Manitoba
Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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**Mammalian Reovirus: Characterization of the M1 Gene Segment and the μ2 Protein**

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**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of**

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| Amp | ampicillin |
| Asp-N | Aspartic acid amino-terminus |
| cDNA | complementary deoxyribonucleic acid |
| CPE | cytopathic effect |
| dATP | deoxyadenosine triphosphate |
| DEPC | diethyl pyrocarbonate |
| DMSO | dimethyl sulfoxide |
| dNTP | deoxynucleoside triphosphate |
| ds | double-stranded |
| DTT | dithiothreitol |
| EDTA | ethylenediamine tetraacetate |
| EOP | efficiency of plating |
| HO buffer | homogenization buffer |
| IgG | immunoglobulin G |
| ISVP | intermediate/infectious subviral particle |
| LB medium | Luria-Bertani medium |
| MALDI | matrix-assisted laser desorption/ionization |
| MALDI QqTOF | matrix-assisted laser desorption/ionization quadrupole time of flight |
| MEM | minimal essential medium |
| MOI | multiplicity of infection |
| mRNA | messenger ribonucleic acid |
| MS | mass spectrometry/spectrometer |
| MS/MS | tandem mass spectrometry |
| m/z | mass/charge ratio |
| NTPase | nucleotide triphosphatase |
| P-value | probability value |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PFU | plaque forming unit |
| P.I. | post infection |
| PMSF | phenylmethylsulfonylfluoride |
| RdRp | RNA-dependent RNA polymerase |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RPM | rotations per minute |
| RT | reverse transcription |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| ss | single-stranded |
| T1L | type 1 Lang |

| | |
|---|---|
| T3D | type 3 Dearing |
| TBE buffer | tris/boric acid/ethylenediamine tetraacetate buffer |
| TE buffer | tris/ethylenediamine tetraacetate buffer |
| TEN buffer | tris/ethylenediamine tetraacetate/NaCl buffer |
| *ts* | temperature sensitive |
| UV | ultraviolet |
| VSP | viable serum protein |
| v/v | volume per volume |
| w/v | weight per volume |

# ABSTRACT

Mammalian reovirus is an enteric virus that contains a double-stranded RNA genome. The genome consists of 10 RNA segments that encode 8 structural and 3 non-structural proteins. The structural proteins form a double-layered structure. The innermost layer, called the core, consists of five proteins (λ1, λ2, λ3, μ2, and σ2). Protein λ3 is the RNA-dependent RNA polymerase (RdRp) and μ2 is thought to be an RdRp cofactor. Translation of most reovirus proteins is known to commence at the first start codon. Although some functions of μ2 have been determined, the protein has not been well characterized. For example, the translation initiation site of the viral core protein μ2, encoded by the M1 RNA segment, has been in dispute. Although the theoretical molecular weight of μ2 is 83,267 Da and 83,277 Da for serotypes T1L and T3D respectively, the actual molecular weight is unknown because μ2 runs aberrantly in SDS-PAGE and has resisted characterization by Edman degradation. The difficulty encountered during amino acid sequence analysis may indicate that the amino-terminus is post-translationally modified. Proteolysis coupled with matrix-assisted laser desorption/ionization quadrupole time of flight (MALDI QqTOF) mass spectrometry was used to establish that translation of serotype T1L μ2 initiates at the first AUG codon, that its actual molecular weight approximates the theoretical value of 83 kDa, and that the amino terminal methionine residue is removed and the next amino acid (alanine) is acetylated.

To allow further analysis of μ2, the protein was expressed in a baculovirus protein expression system. A recombinant baculovirus containing the serotype T1L M1 gene was created. Due to an addition of a polyhistidine tag, protein purification was successfully accomplished. This purified protein can now be utilized to perform analyses designed to uncover further information about its nature.

Hints regarding μ2 function were identified in a seemingly unrelated study. This study involved the characterization of a mammalian reovirus temperature sensitive (ts) mutant, *ts31.13*. This T3D mutant virus was crossed with a wild-type T1L virus to create reassortant viruses, a technique that is possible due to the segmented nature of the reovirus genome. These reassortants were screened to determine their ts phenotype. A mapping table was then used to determine the location of the ts lesion in *ts31.13*. Surprisingly, although no single gene was determined to contain the mutation, a four gene pattern emerged. This involved the gene segments L2, M1, M2, and S4, which encode the proteins λ2, μ2, μ1, and σ3 respectively. The mapping data was statistically analyzed in order to clarify the roles that each of the four gene segments, or proteins, have in the ts phenotype. The L2, M2, and S4 gene segments were determined to contribute significantly to the phenotype. The M1 gene segment was not found to contribute significantly to the phenotype. However, a significant interaction was identified between the M1 gene and the other three gene segments. This type of genetic, or protein, interaction, as indicated by statistical methods, has not been previously reported for reovirus.

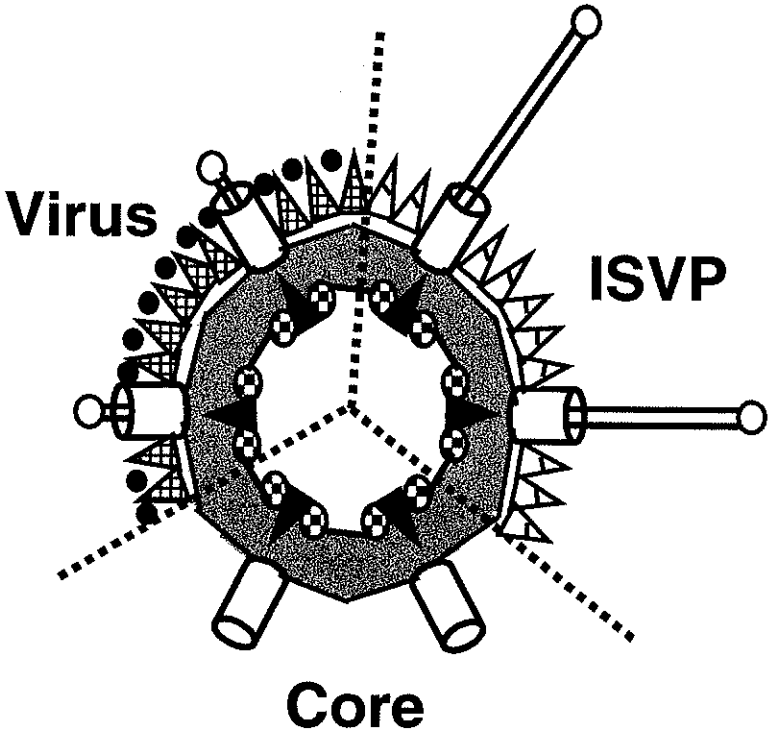
# 1. INTRODUCTION

## 1.1 The mammalian reovirus

RNA viruses account for the majority of infectious diseases worldwide, resulting in greater than eleven million deaths annually (Murray and Lopez 1996). Rotavirus in particular is responsible for a large amount of viral-induced deaths. Since rotavirus is a human pathogen, it creates special problems for researchers. Mammalian reovirus, on the other hand, has proven to be an excellent model in which to study viral functions in mammalian cells and is found in the same family as rotavirus. Reovirus is the prototype virus of the *Reoviridae* family, and the study of this virus has led to the discovery of many host cell mechanisms, such as Kozak's sequence (Kozak 1981), 5' mRNA capping (Furuichi *et al* 1975) and receptor-mediated endocytosis (Silverstein 1968), as well as a greater understanding of viral systems. Additionally, reovirus is safe and easy to work with. This is because it is not pathogenic in humans, and it can be grown to high titers and purified easily.

Mammalian reovirus is 85 nm in diameter and is a double-shelled structure (Figure 1). The outer capsid consists of proteins μ1, σ3, and σ1. The inner capsid (core) consists of proteins λ1 and σ2, both of which form the core shell, and λ2, which forms spike structures sitting on top of the core shell at the icosahedral vertices. In addition, two minor copy number proteins, λ3, the RNA-dependent RNA polymerase (RdRp), and μ2, a putative RdRp cofactor, occupy internal positions in the core (Dryden *et al* 1998).

The core plays a key role in the viral lifecycle (Figure 2). Upon viral entry into a host cell, the intact virus becomes uncoated, and the core becomes exposed. The core


Virus
ISVP
Core

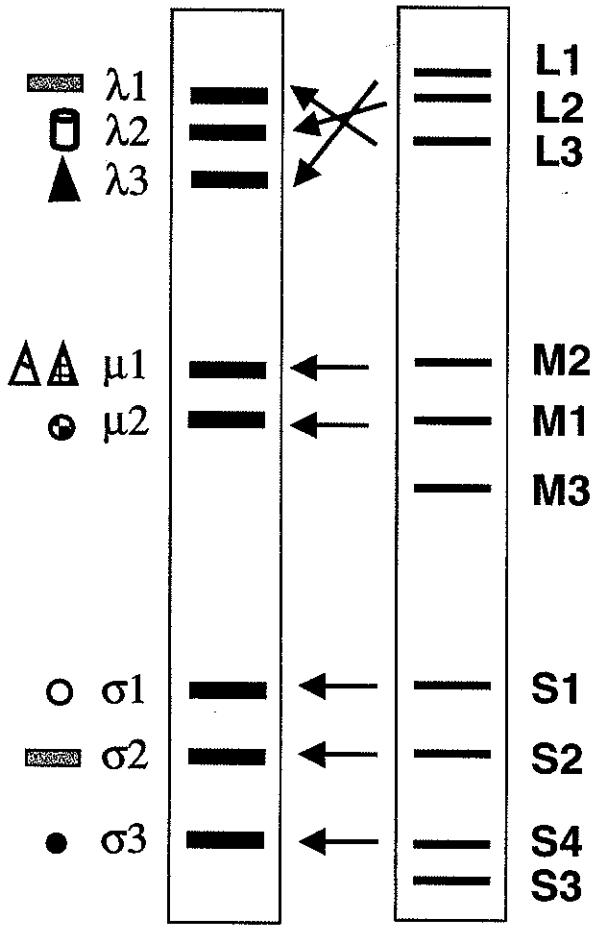

λ1
λ2
λ3
μ1
μ2
σ1
σ2
σ3
L1
L2
L3
M2
M1
M3
S1
S2
S4
S3

**Figure 1. Cartoon composite representing the genome, proteins, and structure of mammalian reovirus.** The genome consists of ten dsRNA segments (extreme right). These are the L genes, the M genes, and the S genes. The dsRNA genome encodes the λ, the μ, and the σ proteins (centre). Only the proteins present in the cartoon structure are represented here. The placing of the proteins within the virion, ISVP (infectious/intermediate subviral particle), and core structure (left) is putative, and is based on current information (Coombs 1998, Dryden *et al* 1998).

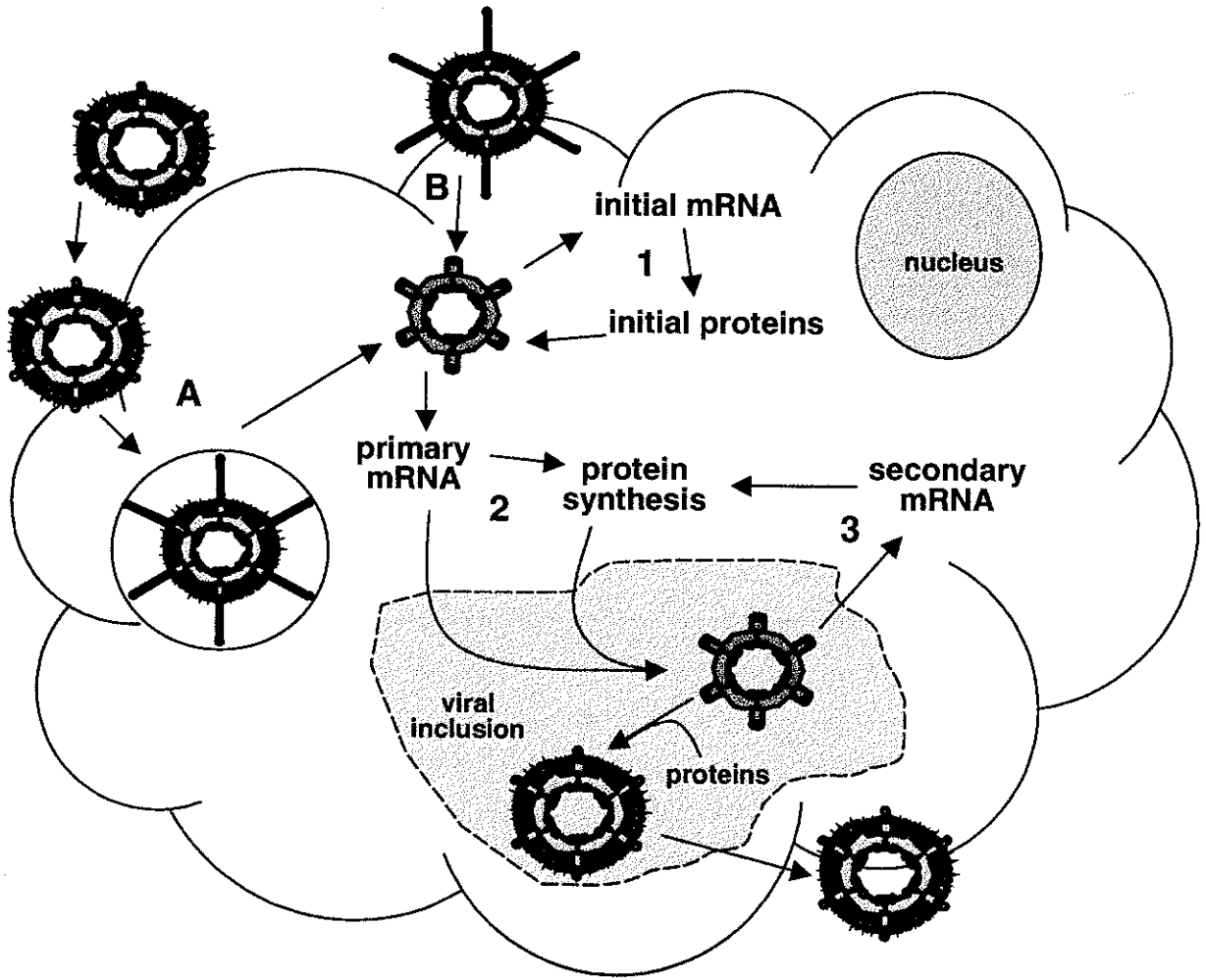

B
initial mRNA
1
initial proteins
nucleus
A
primary
mRNA
2
protein
synthesis
secondary
mRNA
3
viral
inclusion
proteins

**Figure 2. Mammalian reovirus lifecycle.** Cartoon representation of replication processes within a host cell. The virus binds to the cell surface and is internalized into an endocytic vesicle where it is exposed to proteolytic enzymes (A). Due to the cleavage and unfolding of outer capsid proteins, the spiked ISVP structure is formed. The ISVP uncoats during the process of exiting the endocytic vesicle, and reveals the core structure. Alternatively, the virion is exposed to extracellular proteolytic enzymes, resulting in the infection of a cell by the ISVP, which uncoats to form the core structure upon entry into the cell (B). The core structure carries out transcription of four initial RNA segments, which are translated to produce the initial proteins (1). These proteins activate the core to produce a full complement of primary mRNA segments (2). Primary protein synthesis takes place, and assortment of the primary proteins and mRNA segments occurs within viral inclusions. Nascent viral cores are formed, which produce secondary mRNA segments (3). Translation and assortment is repeated until enough fully assembled virion particles are present to lyse the cell.

remains intact throughout the cellular lifecycle of the virus and acts as a transcriptase complex. Activation of core activity results in production of primary transcripts. The primary transcripts are translated to produce initial proteins that stimulate a second round of transcription, which eventually leads to the expression of all of the reovirus genes. Synthesis of nascent viral genomes occurs inside viral inclusions. Through the process of assortment, nascent cores are assembled and carry out a fresh round of transcription until enough virions have been produced to lyse the cell (Coombs 2002).

## 1.2 The characterization of the μ2 protein

The reovirus genome consists of 10 double-stranded RNA segments that encode 11 proteins (Figure 1), eight of which are structural. As indicated in Figure 1, the location of most of the structural proteins within the virion is known. Additionally, the function of many of these proteins has been uncovered. For example, the outer capsid protein σ1 is known to be the cell-attachment protein (Lee *et al* 1981, Weiner *et al* 1977, Yeung *et al* 1987). λ2, a core protein, has been attributed with guanylyltransferase activity (Cleveland *et al* 1986, Fausnaugh and Shatkin 1990, Luongo *et al* 2000), and evidence gathered thus far strongly indicates that the core protein λ3 is the RNA-dependent RNA polymerase (Bruenn 1991, Drayna and Fields 1982, Starnes and Joklik 1993, Yin *et al* 1996). However, the function and location of the core protein μ2 is not as clearly understood. Although Figure 1 illustrates that μ2 is located inside the core at the vertices, its exact whereabouts are unknown. In addition, current data does not fully explain the possible function of this protein.

The μ2 protein is encoded by the M1 RNA gene segment (McCrae and Joklik 1978), which is 2304 nucleotides long for both serotypes type 1 Lang (T1L) and type 3 Dearing (T3D) (Wiener *et al* 1989, Zou and Brown 1992) (Figure 3). In reovirus infections, the M1 gene is associated with development of murine myocarditis (Sherry *et al* 1998), and with strain-specific growth differences in cultured mouse heart cells (Matoba *et al* 1991), MDCK cells (Rodgers *et al* 1997), and bovine aortic endothelial cells (Matoba *et al* 1993). M1 also is associated with liver tropism and strain-specific differences in virulence in SCID mice (Haller *et al* 1995), in the rate of viral inclusion formation in L929 cells (Mbisa *et al* 2000), and in the temperature optimum and kinetics of virus transcription *in vitro* (Yin *et al* 1996). The μ2 protein also has NTPase activity (Noble and Nibert 1997) and is a single-stranded and a double-stranded RNA binding protein (Brentano *et al* 1998). Studies also suggest that the μ2 protein may possess helicase activity, an observation based on protein domain homology. It is believed to be located internally at each of the core vertices where it is in close association with the RdRp (Dryden *et al* 1998). During the viral life cycle, μ2 is found in cellular viral inclusions, and to a lesser extent, in the nucleus and cytoplasm. It is involved in an intermediate step of virion assembly and temperature sensitive mutants, with lesions in the M1 gene, do not form mature viral inclusions (Coombs 1996). In addition, these mutants do not form any identifiable intact virion particles (Coombs 1996). However, despite characterization thus far, the exact biochemical role of μ2 in reovirus replication is not known. Initial analyses of the nucleotide sequence-predicted open reading frame and a homology search failed to match any known proteins (Wiener *et al* 1989). In

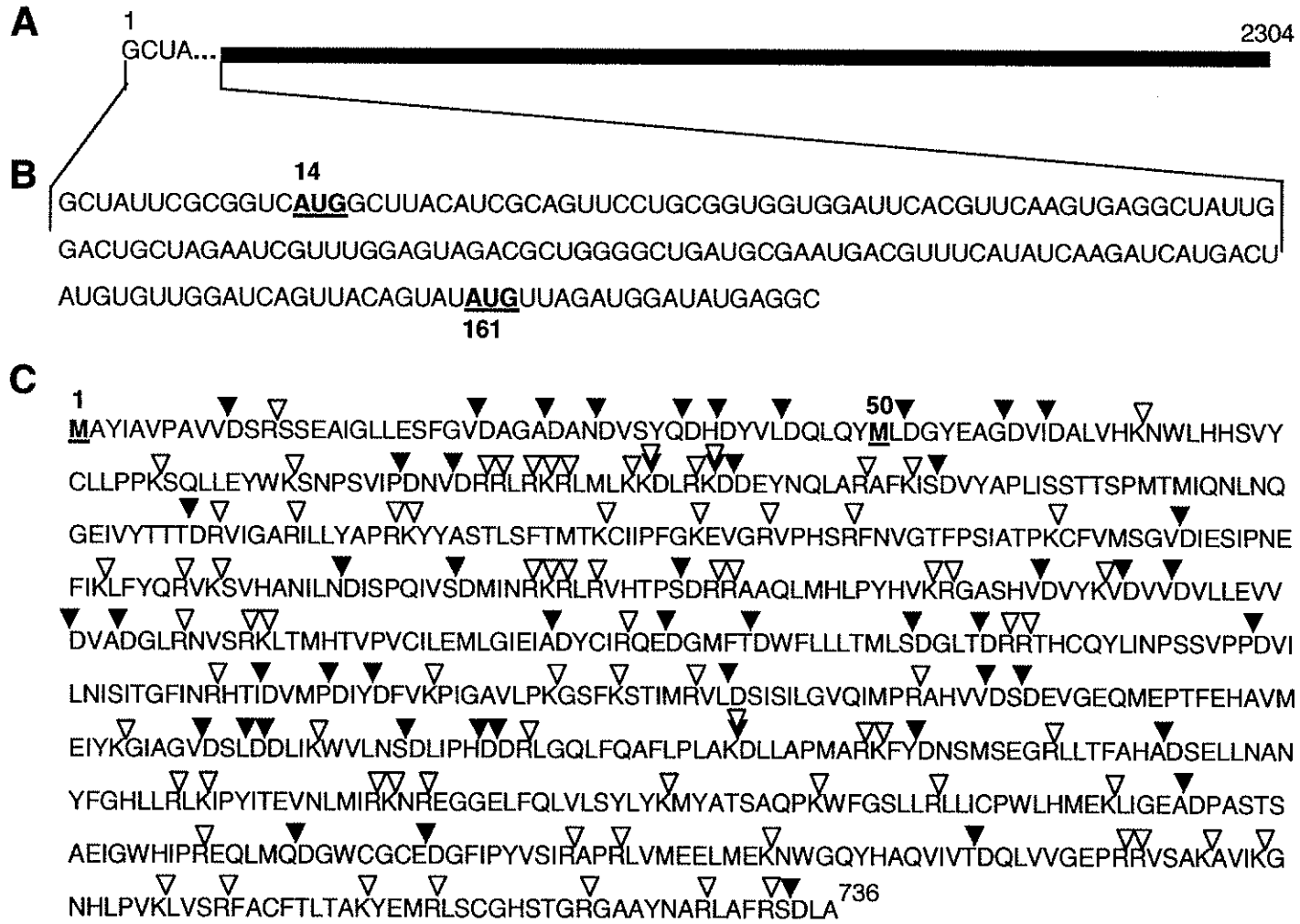

A
1
GCUA...
2304
B
14
GCUAUUCGCGGUCAUGGCUUACAUCGCAGUUCCUGCGGUGGUGGAUUCACGUUCAAGUGAGGCUAUUG
GACUGCUAGAAUCGUUUGGAGUAGACGCUGGGGCUGAUGCGAAUGACGUUUCAUAUCAAGAUCAUGACU
AUGUGUUGGAUCAGUUACAGUAUAUGUUAGAUGGAUAUGAGGC
161
C
1
MAYIAVPAVVDSRSSEAIGLLESFGVDAGADANDVSYQDHDYVLDQLQYMLDGYEAGDVIDALVHKNWLHHSVY
CLLPPKSQLLEYWKSNPSVIPDNVDRRLRKRLMLKKDLRKDDEYNQLARAFKISDVYAPLISSTTSPMTMIQNLNQ
GEIVYTTTDRVIGARILLYAPRKYYASTLSFTMTKCIIPFGKEVGRVPHSRFNVGTFPSIATPKCFVMSGVDIESIPNE
FIKLFYQRVKSVHANILNDISPQIVSDMINRKRLRVHTPSDRRAAQLMHLPYHVKRGASHVDVYKVDVVDVLLEVV
DVADGLRNVSRKLTMHTVPVCILEMLGIEIADYCIRQEDGMFTDWFLLLTMLSDGLTDRRTHCQYLINPSSVPPDVI
LNISITGFINRHTIDVMPDIYDFVKPIGAVLPKGSFKSTIMRVLDSISILGVQIMPRAHVVDSDEVGEQMEPTFEHAVM
EIYKGIAGVDSLDDLIKWVLNSDLIPHDDRLGQLFQAFLPLAKDLLAPMARKFYDNSMSEGRLLTFAHADSELLNAN
YFGHLLRLKIPYITEVNLMIRKNREGGELFQLVLSYLYKMYATSAQPKWFGSLLRLLICPWLHMEKLIGEADPASTS
AEIGWHIPREQLMQDGWCGCEDGFIPYVSIRAPRLVMEELMEKNWGQYHAQVIVTDQLVVGEPRRVSAKAVIKG
NHLPVKLVSRFACFTLTAKYEMRLSCGHSTGRGAAYNARLAFRSDLA
736

**Figure 3. RNA sequence of the M1 gene and the amino acid sequence of protein μ2.** A. Diagrammatic representation of 2304 nucleotide long M1 gene. B. Sequence of first 184 nucleotides at 5' end of serotype T1L M1 gene coding strand. C. Complete predicted sequence of 736 amino acid T1L μ2 protein. Proposed start codons and the corresponding encoded methionine residues are indicated in bold and underlined. Endoproteinase asp-N cleavage sites are indicated by black arrow heads, trypsin proteolytic cleavage sites are indicated by open arrow heads.

addition, the actual molecular weight of this protein is not known. There is controversy regarding which of two initiation codons is used.

Various attempts have been made to determine the molecular weight of μ2. The amino acid sequence of μ2, that would result from translation of an open reading frame that initiates at the first start (AUG) codon at nucleotide positions 14-16, is predicted to be 736 amino acids long, with a theoretical molecular weight of 83,267 Da and 83,277 Da for serotypes T1L and T3D respectively (Wiener *et al* 1989, Zou and Brown 1992). Other reports suggest μ2 translation initiates at a second in-frame AUG codon located 147 bases downstream of the first AUG codon (Roner *et al* 1993), which would result in a protein with a predicted molecular weight of about 78 kDa. However, SDS-PAGE analyses indicate that the μ2 protein co-migrates with the μ1C protein, which has a molecular weight of 72 kDa (Nibert and Schiff 2001). This suggests that the molecular weight of μ2 also is approximately 72 kDa. Efforts to analyze μ2 amino acid sequence by Edman degradation have failed, which indicates the protein is blocked at the amino-terminus, and suggests that the protein is post-translationally modified. Most reovirus proteins are blocked at their amino-termini (Pett *et al* 1973). The blocking group has been identified as a myristoyl group for outer capsid protein μ1 (Nibert *et al* 1991) and as an acetyl group for outer capsid protein σ3 (Mendez *et al* 2002).

When Edman degradation has proved unsuccessful, mass spectrometric analysis can be used to determine the amino acid sequence of a protein. In particular, matrix-assisted laser desorption/ionization quadrupole time of flight (MALDI QqTOF) mass spectrometry is a powerful tool (Figure 4). It provides fast and extremely accurate analysis of the size of a peptide fragment, and can be used to generate a protein sequence.

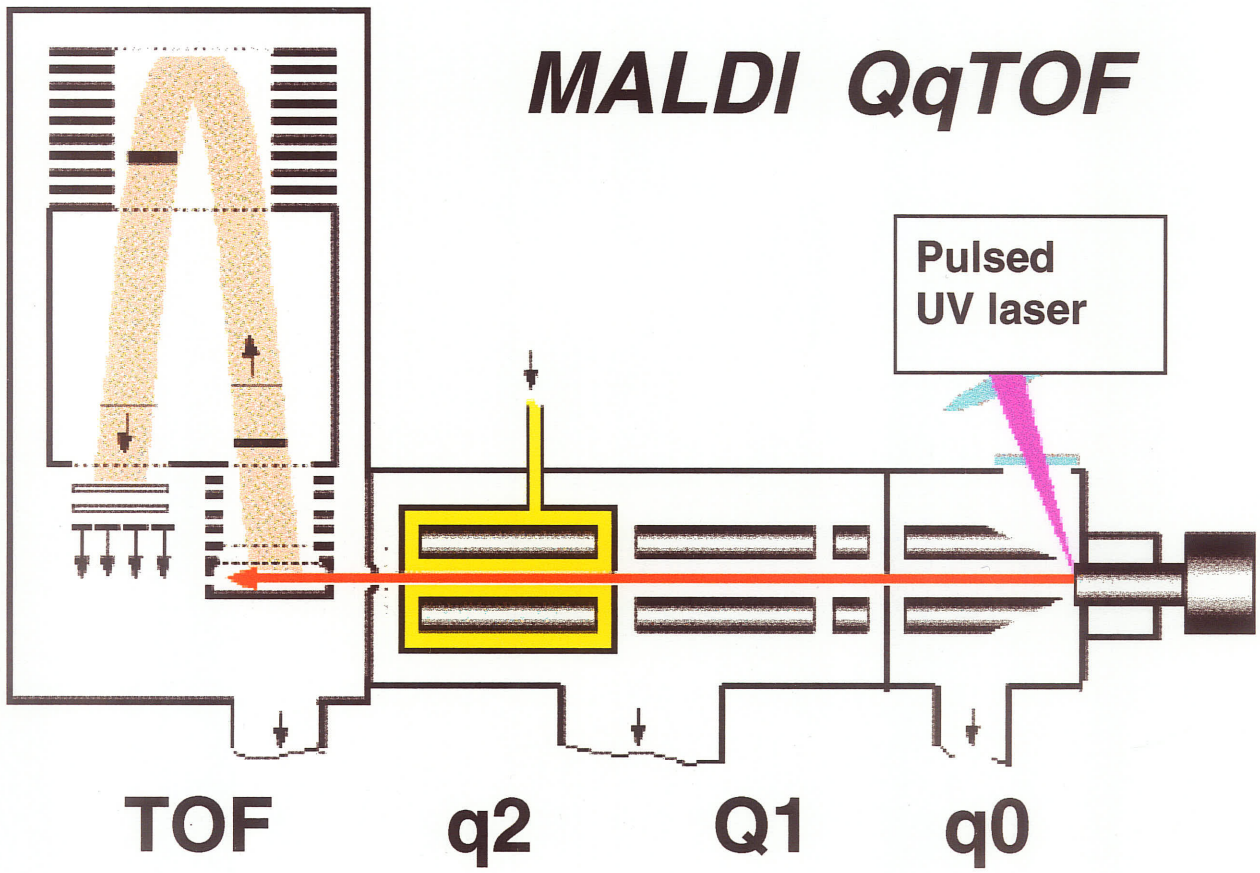

MALDI QqTOF
Pulsed
UV laser
TOF
q2
Q1
q0

**Figure 4. The MALDI QqTOF mass spectrometer.** The proteolytically cleaved protein sample is spotted onto a target and exposed to a laser beam (extreme right). The individual peptides are propagated through an electromagnetic field (q0, Q1, and q2) and enter a time-of-flight (TOF) analyzer (extreme left). The length of time required for a peptide fragment to reach the TOF detector is proportional to its mass and charge. During tandem mass spectrometry (MS/MS), a peptide of a specified mass and charge is isolated and fragmented further in the q2 quadrupole of the mass spectrometer. Its amino acid sequence is then analyzed in the TOF analyzer. This figure was provided by our collaborators in the Department of Physics and Astronomy, Drs. K. Standing and W. Ens.

Sample preparation involves the proteolytic cleavage of a protein. The resulting peptides are spotted onto a target, where they are aerosolized and given an ionic charge by exposure to a laser beam. They are then carried through an electromagnetic field towards a time of flight (TOF) analyzer. The TOF analyzer detects how long it takes for a given peptide to travel a given distance. A peptide's flight is proportional to its mass/charge ratio (m/z). The instrument is extremely sensitive and has been measured to have an accuracy of 0.1 Da within a range of 500 to 4500 Da (Loboda *et al* 2000). For each cleaved protein, a spectrum of peptide m/z ratios is produced. These ratios can be matched against a database to determine the identity or homology of the protein. In cases where the identity of a peptide needs to be confirmed, amino acid sequencing can be done. This involves the capture and further fragmentation of the peptide of interest as it is flying through the electromagnetic quadrupoles. The TOF analyzer then detects these peptide fragments, the size of which differs by a single amino acid, and an amino acid sequence ladder is produced. Amino acid sequencing by a mass spectrometer is termed tandem mass spectrometry (MS/MS). This report provides evidence gained by proteolysis and mass spectrometry that indicates the start codon of μ2, provides insight into its actual molecular weight, and identifies the modification at the amino-terminus.

### 1.3 The expression of the μ2 protein

As previously mentioned, studies carried out thus far have failed to uncover the exact biochemical role of μ2 in reovirus replication. In addition, our understanding of the protein structure of μ2 and its optimal activity parameters is poor. To make a detailed characterization of μ2 possible, a protein expression project was carried out.

Protein expression studies have been successfully utilized by researchers to purify proteins, localize them within host cells, and analyze their functions (Sambrook *et al* 1989). Mammalian proteins (Llewellyn and Roderick 1998), bacterial proteins (Stuchlik and Turna 1998), plant proteins (Luo *et al* 1997), viral proteins (Liu *et al* 1995, Funke *et al* 1995), and even dust mite allergen (Hakkaart *et al* 1998) have been successfully expressed. Both native and fusion proteins can be expressed. In the case of native protein synthesis, protein expression provides an advantage in that higher-than-normal levels of the protein can be produced. This is accomplished by the addition of a strong and regulated promoter and highly efficient ribosome binding sites upstream of the gene of interest. However, fusion proteins are easier to purify, and are ideal for the production of antibodies. Both prokaryotic and eukaryotic expression systems are available. Prokaryotic systems, such as *E. coli*, can generally produce higher amounts of protein, and are relatively simple to use. Eukaryotic systems, on the other hand, are a better choice if one is expressing a eukaryotic protein, since they are more likely to synthesize a protein with the correct post-translational modifications.

Protein expression of the μ2 protein was carried out for several reasons. The protein is present in low copy numbers within the virion, such that many liters of infected cell lysates are needed to purify enough μ2 for studies such as mass spectrometry. In addition, μ2 is a large protein, and attempts at eluting the whole protein out of a gel matrix have proved unsuccessful to date. Lastly, there is a large potential to gain a greater understanding of protein-protein interactions through co-expression studies. In particular, the co-expression of μ2 with λ3 would help uncover the role μ2 plays in reovirus transcription and translation.

Although most of the reovirus proteins have been successfully expressed and purified (Bisaillon *et al* 1997, Broering *et al* 2000, Chandran *et al* 2001, Chandran *et al* and 1999, Gillian *et al* 2000, Lemay and Millward 1986, Leone *et al* 1991, Luongo *et al* 2000, Nibert *et al* 2001), the expression of μ2 has proved difficult. The protein was eventually expressed in a baculovirus system. Our lab had obtained these virus extracts, but the protein was impossible to purify[1]. In order to avoid such difficulties in the present project, I used the Bac-To-Bac expression system (Figure 5). This baculovirus system provides high expression levels and allows the attachment of a polyhistidine tag to the protein of interest, which ensures easier purification. In addition, an insect cell line is employed to carry out the baculovirus infection, which makes the correct post-translational modification of μ2 more likely than in an *E. coli* or yeast protein expression system. With the use of this baculovirus expression system, the μ2 protein was successfully expressed as a fusion protein and purified.

## 1.4 The characterization of the reovirus temperature sensitive mutant *ts31.13*

A study that initially seemed unrelated to the subject of this thesis proved to be relevant. The study involved the mapping of a mutation in the temperature sensitive mutant *ts31.13*. Genetic mutations have proven to be extremely useful in the study of the biological processes of many eukaryotic cells, prokaryotic cells, and viruses (Coombs 1998a). Temperature sensitive mutants, which are a form of a conditionally lethal mutant, have led researchers to understand such processes as post-translational modification of viral proteins (Roussev *et al* 1998), chromosome segregation in yeast (Heo *et al* 1998), memory formation in *Drosophila* (Li *et al* 1996), and endosome

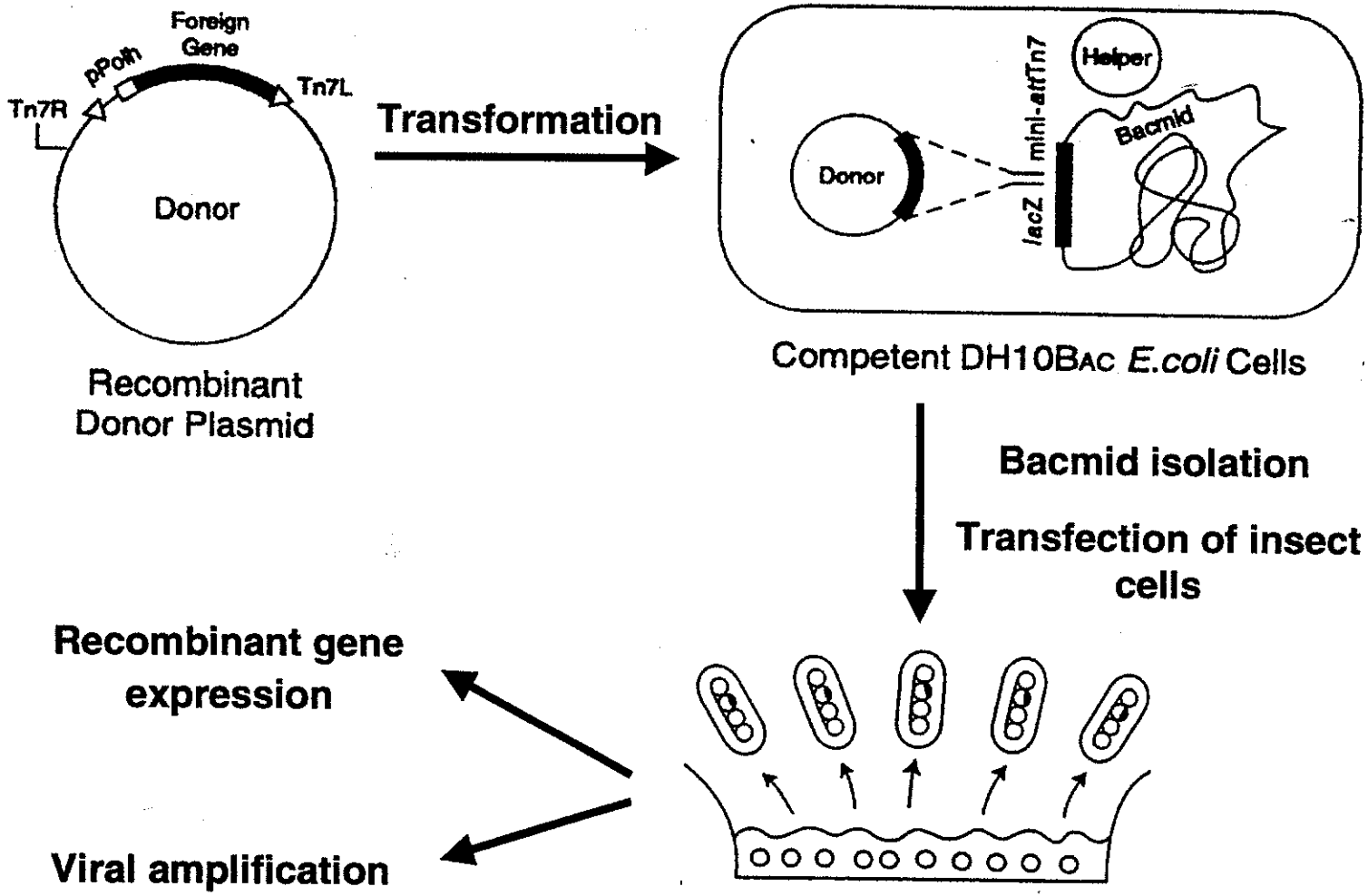

Foreign Gene
pPolh
Tn7R
Tn7L
Donor
Recombinant Donor Plasmid
Transformation
Donor
mini-attTn7
lacZ
Helper
Bacmid
Competent DH10BAC E.coli Cells
Bacmid isolation
Transfection of insect cells
Recombinant gene expression
Viral amplification

**Figure 5. The Bac-To-Bac baculovirus expression system.** Steps in the expression of recombinant proteins are represented. The gene of interest is cloned into a donor plasmid downstream of a strong baculovirus polyhedrin promoter, pPolh (upper left). The insertion site is flanked by two transposition elements, Tn7R and Tn7L. The recombinant donor plasmid is used to transform DH10Bac *E. coli* cells which contain a bacmid that encodes the baculovirus genome, and a helper plasmid that encodes a transposase (upper right). The bacmid contains the mini-*att*Tn7 transposition site inside of a *lacZ* gene for ease of identification of recombinant bacmid cells. When transposition occurs between the donor plasmid and the bacmid to produce a recombinant bacmid, the lacZ gene is disrupted, and the resultant cells are white in colour. The recombinant bacmid is isolated from the *E. coli* cells and used to transfect an insect cell line (bottom right). Recombinant baculovirus is produced, which can be amplified or used to produce the protein of interest.

function in mammalian cells (Daro *et al* 1997). In studies of the *Reoviridae* family of viruses, the mapping of temperature sensitive lesions has been used to study virus structure and assembly (Hazelton and Coombs 1999, Mansell *et al* 1994), and to gain insight on protein function (Becker *et al* 2001, Hazelton and Coombs 1995, Mansell and Patton 1990, Yin *et al* 1996).

*Ts31.13* was derived from a cross between a T3D revertant virus, *tsC447* revertant number 31, and T1L (Coombs *et al* 1994). It grows wild-type-like at permissive temperatures, but has an attenuated titer at elevated temperatures. *Ts31.13* was found to be of interest because of a recombination study. Due to the segmented nature of the reovirus genome, different viral clones that infect the same cell are able to mix their gene segments, and produce reassortant viruses that contain gene segments from both parental viruses. A recombination assay exploits this ability to assort genomes to determine whether two mutant viruses have lesions in the same or different genes. If two viruses that contain lesions in the same gene segment are crossed, then all progeny virions will retain this mutation, and the parent viruses are said to belong to the same recombination group (Figure 6). If two viruses with lesions in different genes are crossed, some of the progeny virions will be wild-type-like, i.e. they will contain no mutated genes. These two parent viruses are then said to belong to two different recombination groups. Currently, eight recombination groups are known for mammalian reovirus, out of a possible ten (there are ten gene segments). In a previous recombination study, *ts31.13* was found to recombine with representative viruses from all known recombination groups, i.e. wild-type-like progeny were produced (Coombs 1996). This implied that *ts31.13* belonged to a new recombination group, and was thus a mutant of potential

# Recombination

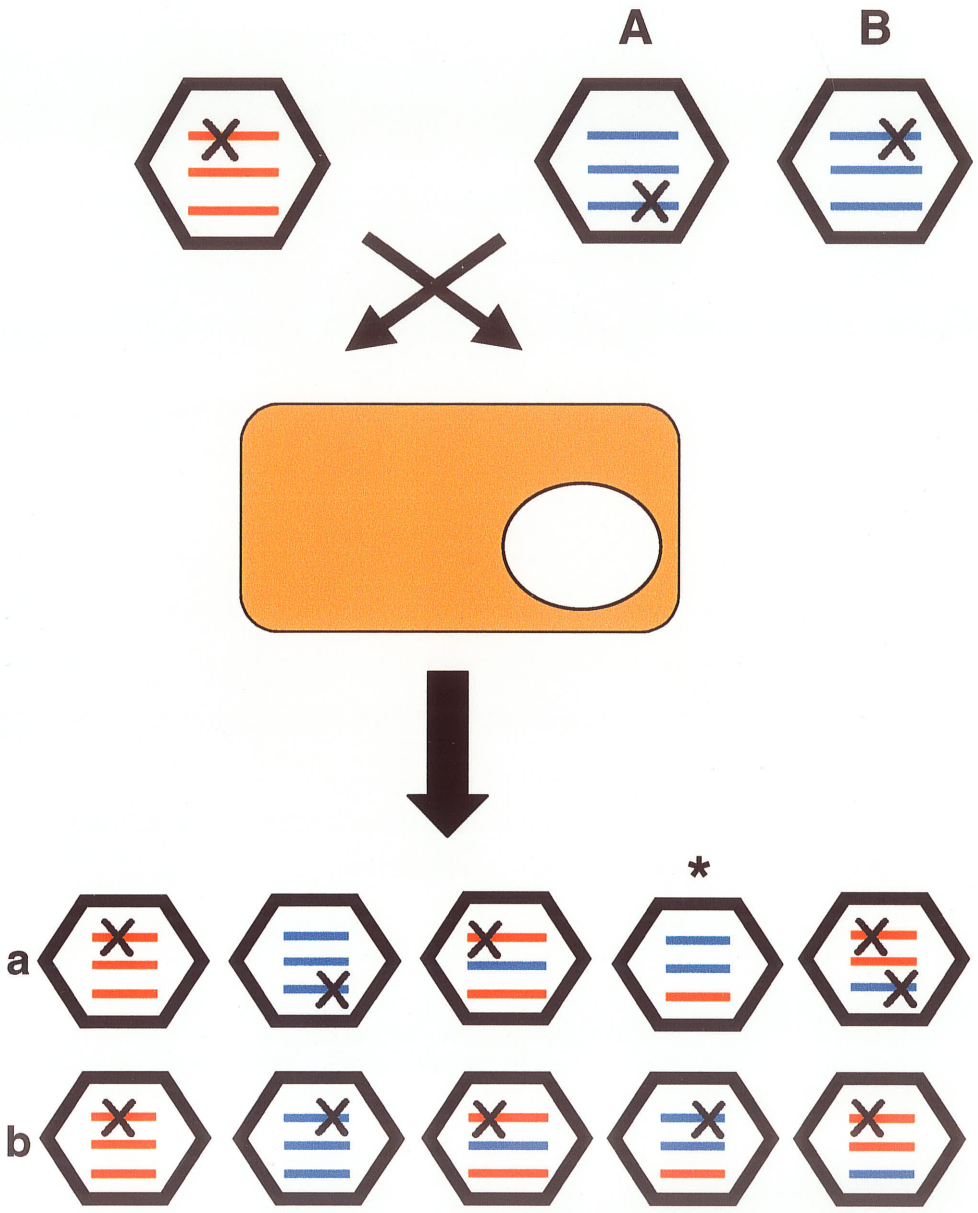


**Figure 6. Reovirus recombination assay.** When two segmented viruses are used to infect the same cell, their genomes can become intermixed. The viral progeny can have genotypes exactly like one of the parent viruses, or they can be reassortants, i.e. they can have some genes that come from one parent virus, and other genes that come from the other parent virus. When a virus with a mutation (X) in one gene (red) is crossed with a virus that contains a mutation in another gene (blue A), some of the viral progeny will be reassortants that do no contain a mutation, i.e. they will by wild-type-like (a, indicated by asterisk). If two viruses with a mutation in the same gene are crossed (red and blue B), all of the progeny virions will be mutants (b).

importance. In order to determine which gene segment(s) contained the ts lesion, *ts31.13* was subjected to further study.

Mammalian reovirus contains ten gene segments. These gene segments migrate in three groups on an SDS-PAGE: the four smallest genes are termed "S" and they have the fastest migration speed, the three genes with medium migration speed are termed "M", and the three largest genes with the slowest migration are designated with the letter "L". Each reovirus serotype has a characteristic gene migration profile (Figure 7), also termed an electropherotype, which allows for easy determination of serotype identity. Electropherotypes can be exploited to identify the parental origin of the gene segments in a reassortant virus. This is particularly important in studies that cross ts mutant viruses with a wild-type virus of an opposite serotype, a method used frequently to map the location of lesions to specific gene segments. By crossing a ts mutant with a wild-type virus of opposite serotype, two sets of reassortants can be generated. Typically, most of the reassortant viruses will display wild-type-like growth rates. A portion of the reassortants will be ts-like in their growth profiles. An efficiency of plating (EOP) value is generated for each reassortant by division of the viral titer at the non-permissive temperature by the titer at the permissive temperature. Wild-type-like viruses have EOP values approximately equal to one, while EOP values of ts-like viruses are several orders of magnitude lower. Both types of reassortants, along with the ts and wild-type parents, are arranged in a table according to their EOP values. The parental origins of the gene segments of all reassortants, which are previously determined, are then used to determine which gene segment contains the ts lesion. If a particular gene segment is of ts origin in all the ts-like reassortants, and of wild type origin in all the wild type-like reassortants,

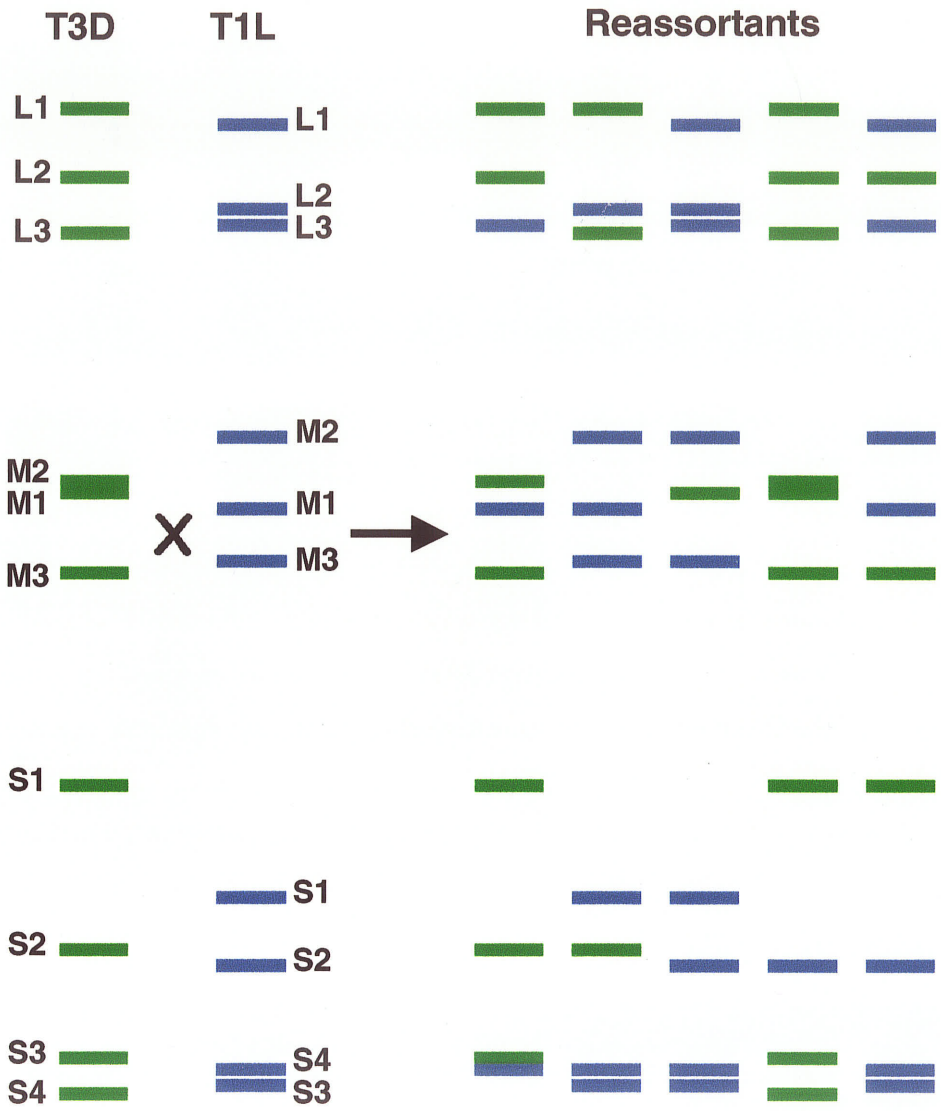

T3D
T1L
Reassortants
L1
L2
L3
L1
L2
L3
M2
M1
M3
M2
M1
M3
S1
S1
S2
S2
S3
S4
S4
S3

**Figure 7. Representative electropherotype profiles of serotype T1L and T3D reovirus, and some possible reassortants.** Serotype T3D gene segments are indicated in green, and serotype T1L gene segments are indicated in blue.

then that gene is determined to contain the lesion.

As reported in this manuscript, *ts.31.13* was subjected to the analysis described above. Initial results did not determine the location of the mutation, although a four-gene constellation effect seemed apparent. M1 was one of the gene segments involved in this possible genetic configuration. Further study identified the genes responsible for the ts phenotype, and indicated that the M1 gene was strongly correlated with three other genes, an interaction that has not been previously reported. This possible interaction between the M1 gene and other reovirus genes has relevance in the determination of the role of the protein μ2 in the viral life cycle.

# 2. MATERIALS AND METHODS

**2.1 Cells and viruses.** Mouse fibroblast L929 cells were used for passage of mammalian reovirus. The cells were grown in suspension in Joklik modified minimal essential medium (MEM medium, Gibco BRL) containing 2.5% fetal bovine serum (Gibco BRL), 2.5% VSP neonate bovine serum (Biocell), and 2mM l-glutamine in the presence of 5% $CO_2$ at 37°C. They were counted and diluted 6 days per week to 5 x $10^5$ cells/ml. The medium was supplemented with 1X penicillin-streptomycin sulfate (100U/ml penicillin, 100μg/ml streptomycin sulfate), and 1X amphotericin B (1μg/ml) when cell monolayers were used for viral infections (Coombs *et al.*, 1994).

*Spodoptera frugiperda* ovarian Sf9 cells were used for passage of baculovirus. Sf9 cells were grown in suspension in 1X Sf-900 II SFM medium (Gibco BRL) at 28°C, and were counted and diluted every 2 to 3 days to 5 x $10^5$ cells/ml.

*Escherichia coli* cells were lab stock electrocompetent DH5α cells and MAX Efficiency DH10Bac competent cells (Gibco BRL). The DH5α cells were made electrocompetent by the method described by both Fan and Dower (Fan 1994, Dower *et al* 1988). The DH5α cells were grown in LB medium (1% tryptone [w/v] [DIFCO], 0.5% yeast extract [w/v] [DIFCO], 340mM NaCl, 5mM Tris-HCl [pH 7.5]) and LB agar (LB medium with 1.5% agar [w/v] [DIFCO]), which was supplemented with 0.1mg/ml ampicilin during screening for transformants. DH10Bac cells were grown in Luria medium (1% peptone [w/v] [DIFCO], 0.5% yeast extract [w/v] [DIFCO], 170mM NaCl)

and Luria agar (Luria medium with 1.2% agar [w/v] [DIFCO]), which was supplemented with 50μg/ml kanamycin, 7μg/ml gentamicin, 10μg/ml tetracycline, 40μg/ml isopropylthio-β-galactoside (IPTG), and 10μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) during transformant selection.

Reoviruses were from prototypic lab stocks of mammalian reovirus serotypes Type 1 Lang (T1L) and Type 3 Dearing (T3D), the temperature sensitive T3D mutant *ts31.13* (Coombs *et al* 1994), and the lab stocks of reassortants *H14* (Drayna and Fields 1982, Coombs *et al* 1990) and *KC10* (generated by Kevin Coombs, reported in Nibert *et al* 1996). Viral clones resulting from a *ts31.13* X *H14* cross, and a *ts31.13* X *KC10* cross, were generated by Kevin Coombs.

Wild type baculovirus was obtained from Dr. Heinz Feldmann (Canadian Science Centre for Human and Animal Health, Winnipeg, Canada). Recombinant baculovirus was derived from transfection of Sf9 cells with a recombinant bMON14272 bacmid (Gibco BRL).

**2.2 Primers and antibodies.** Reo M1 primers for the amplification and sequence analysis of T1L M1 cDNA are standard lab primers, and have been used in the lab to amplify and sequence numerous M1 genes (Coombs 1996) (Table 1). T1LM1-5' and T1LM1-3' primers were designed and made for the present study and were used for the generation and cloning of T1L M1 cDNA (Table 1). These primers were designed to contain the Bam HI and Hind III restriction endonuclease sites, respectively.

The mouse polyclonal anti-μ2 antibody was provided by Dr. Earl Brown (University of Ottawa, Canada). The secondary antibody biotin-SP-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories. The monoclonal

# Table 1

## Reovirus M1 gene primers

| Primer | Direction | Sequence (5' to 3') | Location (ntd) | Tm (°C) |
|---|---|---|---|---|
| ReoM1-01 | forward | GCTATTCGCGGTCATGGC | 1-18 | 58 |
| ReoM1-02 | forward | CATCTCATCCACGACG | 415-430 | 50 |
| ReoM1-03 | forward | CGCGCAGTTGATGCAT | 832-847 | 50 |
| ReoM1-04 | forward | GCTGCCTAAGGGATCA | 1249-1264 | 50 |
| ReoM1-05 | forward | CGCAAGAATCGTGAGG | 1688-1703 | 50 |
| ReoM1-06 | forward | GGCTGTGATCAAGGGT | 2065-2080 | 50 |
| ReoM1-07 | reverse | GAAGCGCGTACGTAGTC | 2285-2301 | 54 |
| ReoM1-08 | reverse | CACGAGGGATATGCCA | 1871-1886 | 50 |
| ReoM1-09 | reverse | CAGCACCCACTTGATG | 1441-1456 | 50 |
| ReoM1-10 | reverse | CATCCTCTTGACGAATG | 1030-1046 | 50 |
| ReoM1-11 | reverse | CTAGAGTGAGGAACACG | 599-615 | 52 |
| ReoM1-12 | reverse | TGGACGAGTGCATCGA | 192-207 | 50 |
| T1LM1-5' | forward | GACGGATCCATGGCTTACATCGCA[1] | 5-28 | 58 |
| T1LM1-3' | reverse | GACAAGCTTTCACGCCAAGTCAGATC[1] | 2208-2233 | 57 |

Red color indicates start and stop codons
Blue color indicates BamH I restriction endonuclease cleavage site
Green color indicates HindIII restriction endonuclease cleavage site
[1]Primers were designed to replace gene ends with restriction endonuclease cleavage sites

anti-polyhistidine antibody was obtained from Sigma. The peroxidase-labeled goat anti-mouse IgG secondary antibody was obtained from Kirkegaard and Perry Laboratories Inc.

## 2.3 Viral manipulation

**2.3.1 Viral passage.** Mammalian reovirus plaques were picked (as described below) and amplified as follows: L929 cells were grown to sub-confluent monolayers. The concentration of L929 cells used to seed tissue culture plates was 4.5 x $10^5$/ml, but the total number of cells used was scaled up or down as necessary for different sizes of tissue culture flasks. For example, 2.25 x $10^6$ cells (5ml of cells at 4.5 x $10^5$cells/ml) were used to seed a T25 tissue culture flask. The overlaying media was removed and set aside. Cells were infected with virus for 1 hour at room temperature with periodic rocking. When infections were performed at 37°C, the cells were then overlaid with MEM medium (completed as described above) containing 25% pre-adapted medium, 1X penicillin-streptomycin sulfate (100U/ml penicillin, 100μg/ml streptomycin sulfate) and 1X amphotericin B (1μg/ml amphotericin B). When infections were performed at 32 or 33.5°C, the cell were overlaid with media containing 2X penicillin-streptomycin sulfate (200U/ml penicillin, 200μg/ml streptomycin sulfate) and 2X amphotericin B (2μg/ml amphotericin B). The infections were incubated at the appropriate temperature in 5% $CO_2$ until 90% cytopathic effect (CPE) was observed by light microscopy. Wild type viruses were incubated at 37°C and exhibited 90% CPE by approximately 7 days post infection (p.i.). Temperature sensitive viruses were incubated at either 32 or 33.5°C and exhibited 90% CPE in approximately 10 to 14 days p.i. To harvest virus, cells were lysed

by 3 freeze/thawing cycles at -80°C. Virus was stored at 4°C and titered by plaque assay prior to use.

Baculovirus was grown in insect Sf9 cells. A T25 tissue culture flask was seeded with 3 x $10^6$ Sf9 cells. The cells were incubated for 1 h at 28°C to permit cell attachment. The supernatant was removed and 300µl of post-transfection viral supernatant was added. The virus was allowed to adsorb for 1 h at room temperature, with periodic rocking. The cells were overlaid with 4ml of Sf-900 II SFM medium, and incubated at 28°C until approximately 85% CPE. The virus was harvested by transferring the supernatant to a 15ml tube. The cells were pelleted by centrifugation at 500 Xg for 5 min and the supernatant was transferred to a fresh tube. Fetal bovine serum (Multicell) was added to a final concentration of 2% (v/v), and the virus was stored at 4°C protected from light.

**2.3.2 Viral plaque assay.** Reovirus stocks were serially diluted in gel/saline (137mM NaCl, 0.2mM $CaCl_2$, 0.8mM $MgCl_2$, 19mM $HBO_3$, 0.1mM $Na_2B_4O_7$, 0.3% [wt/vol] gelatin). Sub-confluent L929 cell monolayers in 6 well tissue culture plates were infected by diluted virus after removal of overlaying media. The virus was typically allowed to attach for 1 hour at room temperature, with periodic rocking. The cells were overlaid with 1:1 mixture (v/v) of 2X Medium 199 (Gibco BRL) and 2% Bacto agar (Difco), supplemented with 1X penicillin-streptomycin sulfate and 1X amphotericin B, when the plaque assay was to be incubated at 37 or 39.5°C, and 2X penicillin-streptomycin sulfate and 2X amphotericin B, when incubation temperatures of 32 or 33.5°C were used, and incubated at the appropriate temperature in 5% $CO_2$. Cells incubated at 37°C were fed on day 4 p.i., and 32 and 33.5°C incubations were fed on day 6 p.i., with the same overlay mixture as above. The plates were incubated until plaques

could be visualized by the naked eye (typically 7 days p.i. for 37°C, 10 to 14 days p.i. for 32 and 33.5°C). The cells were then stained with 2ml of a 0.02% neutral red solution (Fisher) in a 1:1 mixture (v/v) of 2X phosphate buffered saline (274mM NaCl, 0.6mM KCl, 1.6mM $Na_2HPO_4$, 0.2mM $KH_2PO_4$) and 2% Bacto agar (w/v). After a 16 hour incubation, the plaques were counted.

To determine the baculovirus titer, Sf9 cells were infected with the virus. Six well tissue culture plates were seeded with 1 x $10^6$ Sf9 cells/well, and the cells were incubated for 1 h at 28°C to allow cell attachment to occur. Virus was serially diluted in Sf-900 II SFM medium. The supernatant was drawn off the cell monolayer and 100µl of virus was added. Each dilution was incubated, in duplicate, for 1 h at room temperature with periodic rocking, to allow for adsorption. The cells were overlaid with 1.5 ml/well of a 1:3 mixture (v/v) of 4% low melting agarose and 1.3X Sf-900 II SFM medium, and incubated at 28°C until plaques became visible (approximately 7 days). The plates were fed 2 days p.i. with 1 ml of the same overlay. Plaques were visualized without the aid of stain.

**2.3.3 Plaque purification.** Reovirus was purified by plaque assay as described in section 2.3.2. However, 1X penicillin-streptomycin sulfate, and 1X amphotericin B were added to the stain overlay. Plaques were picked with the use of a sterile pasteur pipet and stored at 4°C in 1ml completed MEM medium supplemented with 2X penicillin-streptomycin sulfate, and 2X amphotericin B.

**2.3.4 Concentration of virus.** For the generation of *ts31.13* x T1L reassortants, *ts31.13* viral suspensions were concentrated 14.3-fold by application onto a Centricon 50 concentrator column. The ts31.13 stock virus (6ml) with a low titer was loaded into the

column. The column was centrifuged at 5000 Xg in a Beckman J2-HS centrifuge until a small amount of the starting viral supernatant was left in the top compartment. The column was inverted and centrifuged once more to collect the supernatant which contained concentrated virus.

**2.3.5 Virus purification.** Reovirus serotypes T1L and T3D were plaque purified and grown in mouse L929 cell monolayers in Joklik MEM medium supplemented to contain 2.5% fetal calf serum, 2.5% VSP neonate bovine serum, 2mM glutamine, 100U/ml penicillin, 100$\mu$g/ml streptomycin sulfate, and 1$\mu$g/ml amphotericin-B, as previously described (Coombs *et al* 1994). Large amounts of virus were grown in spinner culture. Suspension cultures contained 6.5 x $10^8$ L929 cells/L and were infected with a multiplicity of infection (MOI) of 5 plaque forming units (pfu). The virus was allowed to adsorb for 1h at room temperature, with periodic swirling, and the infection was carried out at 33°C for 65 h. The cells were pelleted at 5000 Xg in a Beckman J2-HS centrifuge and resuspended in HO buffer (10mM Tris-HCl, pH 7.4, 250mM NaCl, 0.067% β-mercaptoethanol [v/v]). The cells were lysed by sonication (in a solution that contained 0.2% sodium desoxycholate [w/v]) (Mendez *et al* 2000). Virus was extracted with Vertrel-XF (a freon substitute), and purified in 1.2-1.45g/cc cesium chloride gradients as previously described (Mendez *et al* 2000). Virus bands were harvested and dialyzed extensively against Dialysis Buffer (D buffer: 150mM NaCl, 15mM $MgCl_2$, 10mM Tris, pH 7.4).

**2.3.6 Generation of reassortants.** A sub-confluent L929 cell monolayer in one well of a 24 well tissue culture plate was infected with a 10:10 MOI of *ts31.13*:T1L. The virus cocktail was premixed and, due to the large volume of virus inoculum used in this

instance, was added to the well 100µl at a time, allowed to adsorb for 20 minutes, and removed. Normally, virus inoculum is added all at once, and allowed to adsorb for 1 hour. This procedure was repeated until all of the virus had been adsorbed. Total adsorption time was 1 hour at room temperature. The cell layer was then overlaid with 500µl completed MEM supplemented with 1X penicillin-streptomycin sulfate, and 1X amphotericin B, and incubated at 32°C in the presence of 5% $CO_2$ for 34 hours. The plate was placed at -80°C, freeze/thawed 3 times, and sonicated for 5 seconds to lyse the cells. Three serial 1:100 dilutions of the supernatant were made in gel saline, followed by 3 serial 1:3 dilutions. Each of the final 3 dilutions were used to infect 5 P100 tissue culture plates with 300µl of virus. The plates were overlaid as described in viral plaque assay (section 2.3.2), and incubated at 32°C in the presence of 5% $CO_2$ for 17 days. The fresh nutrient agar was added on day 7 p.i. On day 17 p.i. 72 plaques separated from each other by more than 3mm were picked, as described previously, and placed in 2-dram vials overnight at 4°C in 0.5ml completed MEM medium containing 2X penicillin-streptomycin sulfate and 2X amphotericin B, to allow virus to diffuse out of the agar plug. The vials were then warmed and L929 cells in MEM medium (0.35ml) containing 2X penicillin-streptomycin sulfate and 2X amphotericin B at 4.2 x $10^5$ cells/ml were added to each of the 72 vials. The vials were incubated at 32°C in the presence of 5% $CO_2$ for 17 days. The vial caps were tightened on day 3 p.i., and the vials were periodically shaken. The vials were freeze/thawed 3 times at -80°C. The 72 virus clones were then amplified in T25 tissue culture flasks in the following manner. The supernatants from subconfluent monolayers were removed and set aside. The cells grown in T25 flasks were infected with 500µl of the amplified viral clones and overlaid

with 4ml completed MEM supplemented with 2X pen-strep and 2X amphotericin B and containing 25% pre-adapted media. The flasks were incubated at 32°C until approximately 85% CPE was observed, freeze/thawed 3 times at -80°C, and the supernatant was transferred to 2dm vials. During the incubation, the flasks were beaten on day 5 p.i. and day 12 p.i to redistribute the virus.

**2.4 Reassortant screening.**

**2.4.1 Isolation of viral genome.** Subconfluent L929 cell monolayers in P60 tissue culture plates were infected with 350µl of the doubly amplified viral clones. The plates were incubated at 32°C in the presence of 5% $CO_2$ for 6 to 8 days, until approximately 85% CPE. The dishes were scraped to harvest the cells, which were then transferred to a 15mL tube and centrifuged at 500 Xg for 10 min. The supernatant was poured off, the tube was drained of liquid, and 500µl of buffer containing 140mM NaCl, 1.5mM $MgCl_2$, 10mM Tris-HCl, pH 7.4, and 0.5% NP-40 (v/v) was added. The tube was vortexed and incubated on ice for 30 min. During incubation, the tube was vortexed every 10 min. The cell nuclei were pelleted by centrifugation at 500 Xg for 10 min, and the supernatant was transferred to a 1.5mL tube. A 10:1:20 mixture (v/v/v) (93µl) of 1M Tris (pH 7.8), 200mM EDTA (pH 8), and 10% SDS was added. The sample was vortexed, and incubated at 42°C for 15 min. To extract proteins, 14µl of 5M NaCl and 600µl of phenol/chloroform (1:1) was added, the sample was vortexed, and reheated at 42°C for 30 sec. The mixture was centrifuged at 6800 Xg for 5 min in the Eppendorf 5412 centrifuge. The top aqueous phase (0.4ml) was transferred to a fresh 1.5mL tube and placed on ice. To pellet the RNA, 36µl 3M sodium acetate and 1.0ml of cold absolute ethanol was added. The sample was vortexed and incubated at -20°C overnight.

The RNA was pelleted by centrifugation in a Baxter Canlab Biofuge A at 24 000 Xg for 30 min at 4°C. The supernatant was poured off, the tube was drained, and the viral RNA pellet was vacuum dried.

**2.4.2 Identification of reassortants.** The viral genomes were resuspended in 35µl electrophoresis sample buffer (240 mM Tris-HCl, pH 6.8, 1.5% dithiothreitol (DTT), 1% SDS) and analyzed by SDS-PAGE. A 14.5cm x 20cm x 1.5mm gel, consisting of a 10% resolving and a 4% stacking gel, was used. The gels were run at 18Amp/gel for 42 to 46 hours, stained with ethidium bromide, photographed, and the gene patterns (electropherotypes) were determined.

**2.4.3 Determination of efficiency of plating (EOP).** Viral reassortants and parent viruses were diluted as described in viral plaque assay. Cells grown in 6 well tissue culture plates were infected with virus in the following manner. One plate was infected with six sequential dilutions, typically $10^{-1}$ to $10^{-6}$, a second plate was infected with a duplicate of three sequential dilutions, typically $10^{-4}$ to $10^{-6}$. The plates were overlaid as described previously, and placed into different incubation conditions. The plate infected with six sequential dilutions was placed at the non-permissive temperature (37 or 39.5°C), and the plate infected with three sequential dilutions was placed at the permissive temperature (32 or 33.5°C). The plates incubated at the non-permissive temperature were fed on day 3 p.i., as described previously, and plates at the permissive temperature were fed on day 6 p.i. Non-permissive temperature plates were stained on day 6 p.i., and permissive temperature incubations were stained on day 14 p.i. Plaques were counted the following day, and viral titers were determined. An EOP value was determined for each virus by dividing the non-permissive titer by the permissive titer.

**2.5 Statistical analysis.** Statistical analysis was carried out by Mrs. Mary Cheang (Department of Community Health, University of Manitoba, Canada). Wilcoxon rank sum tests and Kruskal-Wallis tests were performed on the ranks of both raw and averaged EOP values (averaged EOP values were calculated from the multiple EOP assays of each parent cross). These multiple tests were adjusted for significance by using a Bonferoni correction of 0.005. This number was calculated by dividing the statistically significant P-value (0.05) by the number of tests, i.e the number of genes compared (10). Multiple regression analysis was performed on the raw EOP values as well as the logarithm of the values and the averaged values. EOP values >0.06 were defined as being "high", EOP values ≤0.06 were defined as being "not high". These were then modeled with a multiple logistic regression analysis. A backward elimination procedure was employed and significance was set at <0.05. The same test was performed on select genes, focusing on the low EOP values. A chi squared analysis was employed to determine correlations between the M1 gene and other genes.

**2.6 Mass spectrometric analysis of the μ2 protein**

**2.6.1 Purification of the μ2 protein from virions.** Outer capsid protein μ1C, a major virion structural protein, comigrates with minor core protein μ2 in SDS-PAGE. Therefore, to remove μ1C, purified dialyzed virions were digested at 37°C for 3 hours with α-chymotrypsin to generate cores, which were subsequently purified in CsCl gradients, harvested, and dialyzed against Core Buffer (1.0M NaCl, 100mM $MgCl_2$, 25mM Hepes) as described (Yin *et al* 1996). Core particles were dissolved in electrophoresis sample buffer (240 mM Tris-HCl, pH 6.8, 1.5% dithiothreitol (DTT), 1%

SDS) and proteins were resolved in 16.0 x 16.0 x 0.15mm 10% SDS-PAGE (Laemmli 1970). Gels were fixed with a 30% isopropanol/10% acetic acid solution (v/v), stained with Coomassie Brilliant Blue R-250, and visualized μ2 protein bands were excised.

**2.6.2 Western blot analysis.** Serotype T1L and T3D virions were purified as described in section 2.3.5. A portion of the infected cells was collected before virus purification. Infected cells, as well as an uninfected control, were lysed by a 5 sec sonication in electrophoresis sample buffer. Lysed cells and purified virus were analyzed in duplicate by SDS-PAGE as described in section 2.6.1. One gel was Coomassie stained to ensure equal loading of protein. The other gel was used for Western blot analysis. The protein bands were transferred onto an Immobilon-P membrane (Millipore) by a semi-dry method. The membrane was incubated in isopropanol, and stained with Ponceau S to ensure successful and equal transfer. Rabbit anti-μ2 antibody was used as the primary antibody and biotin-SP-conjugated goat anti-rabbit IgG was used as the secondary antibody. A biotin/streptavidin chemiluminescence blotting kit (Roche) was used to detect μ2 protein as described in the instruction manual.

**2.6.3 In-gel digestion.** The following steps were modified from previously published procedures (Hellman *et al* 1995, Rosenfeld *et al* 1992, Shevchenko *et al* 1996, Wilm *et al* 1996). The excised μ2 gel band was fragmented and destained by repeated washings with alternating 100mM $NH_4HCO_3$ and a 1:1 (v/v) mixture of $CH_3CN$:100mM $NH_4HCO_3$ solutions. The protein was incubated in a mixture of 10mM DTT in 100mM $NH_4HCO_3$ for 1 h at 56°C to reduce disulfide bonds, and alkylated for 45 min in the dark at room temperature by treatment with 55mM iodoacetamide in the same buffer. The sample was washed in 100mM $NH_4HCO_3$ for 10 min, and dehydrated in $CH_3CN$. This

process was repeated once more. The gel pieces were dried in a SpeedVac and reswollen with either 20µl 25mM $NH_4HCO_3$ that contained 30ng trypsin (Sigma, sequencing grade) or 20µl 10mM Tris buffer (pH 7.0) that contained 50ng endoproteinase asp-N (Boehringer Mannheim, sequencing grade) for 45 minutes at 4°C. An additional 200µl of the corresponding buffer was added to cover the gel pieces, which were then incubated at 37°C overnight. The digestion mixture was deactivated with 10µl 2% trifluoroacetic acid (TFA) and the resultant peptide fragments were eluted by sonication for 30 minutes in the presence of three successive changes in: 150µl 0.1% TFA, 150µl 1:1 mixture of acetonitrile:0.1% TFA (v/v), and 150µl 100% acetonitrile. The whole extraction procedure was repeated and the collected solutions that contained extracted peptides were pooled and dried.

**2.6.4 Mass spectrometric analysis.** Dried µ2 protein digests were redissolved in 5µl distilled $H_2O$, mixed 1:1 (v/v) with MALDI matrix solution (160mg/ml 2,5-dihydroxybenzoic acid in 1:1 (v/v) acetone:water), and analyzed by matrix assisted laser desorption ionization quadrupole time of flight (MALDI QqTOF) mass spectrometry. Monoisotopic masses of the peptide fragments were first searched against a protein database by ProFound (http://www.proteometrics.com/prowl-cgi/ProFound.exe). Selected peptide ions were further fragmented and analyzed by tandem mass spectrometry (MS/MS) and data were analyzed by Prospector MS-Tag (http://prospector.ucsf.edu).

## 2.7 Expression of the μ2 protein

### 2.7.1 Cloning

**2.7.1.1 Preparation of viral template.** The T1L virus utilized in this step was obtained from a well-characterized lab stock. The virus was amplified, gradient purified in CsCl, and dialyzed against Dialysis Buffer as described previously (Mendez *et al* 2000). Purified virions were disrupted in the presence of 50mM Tris, pH 8.0, 1mM EDTA, pH 8.0, and 0.1% SDS (w/v) for 15 min at 42°C, and extracted with phenol/chloroform, followed by a chloroform extraction. The viral template was then precipitated, washed, and resuspended in 90% DMSO/10% 10mM Tris-HCl, pH 6.8 (w/v).

**2.7.1.2 Reverse transcription polymerase chain reaction (RT-PCR).** The T1L genome (3μl) was incubated in a 1.5ml tube at 50°C for 45 min to melt apart the double stranded (ds) RNA strands, then placed in an ice-water bath. The sample was snap-cooled by the addition of 31.5μl of ice-cold 0.3% diethyl pyrocarbonate (DEPC)-treated distilled water (v/v), and returned to the ice-water bath. The following reagents were added in order: 0.53μl of Prime RNase inhibitor (30U/μl, Eppendorf), 0.5μl each of 10μM T1LM1-5' and T1LM1-3' primers, 2.5μl dNTP mix (10mM each of dATP, dCTP, dGTP, and dTTP), 10μl 5X First Strand Buffer (Gibco BRL), 0.5μl 10mg/ml bovine serum albumin, 0.5μl 100mM DTT, and 0.5μl SuperScript II RT enzyme (200U/μl, Gibco BRL). The sample was then incubated at 42°C for 2 hours, and placed on ice.

**2.7.1.3 Amplification of cDNA.** Chilled T1L M1 single stranded (ss) cDNA (16μl) was mixed in a 0.5ml tube with the following reagents: 67.5μl sterile

distilled water, 10µl 10X Expand Long Template Buffer #1 (Roche), 3.5µl 10mM dNTP, 1.0µl each of 100µM T1LM1-5' and T1LM1-3' primers, and 1.0µl Expand Long Template enzyme (3.5U/µl, Roche). The following thermocycler incubation parameters were used: initial melting at 96°C for 2 min, 30 cycles of: 92°C for 1 min, 53°C for 1 min 30 sec, 68°C for 2 min 45 sec, a final extension step at 68°C for 10 min, followed by a 4°C hold. cDNA amplification was confirmed by 0.9% agarose gel electrophoresis in TBE Buffer (45mM Tris-HCl, 45mM Boric acid, 0.9mM EDTA). The 11cm x 9.1cm x 1cm gel was stained with ethidium bromide, which was added directly to the molten agar before it was cast, run at 110 V for 1 h, and DNA was visualized with the aid of a UV light source.

**2.7.1.4 Purification of PCR product.** Amplified T1L M1 ds cDNA (20µl) was loaded onto a 0.9% 13.9cm x 12cm x 1cm agarose gel in TBE Buffer. DNA was visualized as described above. The M1 cDNA band was cut out of the gel and extracted from the agarose matrix using the QIAquick Gel Extraction kit (QIAGEN). The purified cDNA product was resuspended in 30µl 10mM Tris-HCl, pH 7.4. Purification of cDNA was confirmed by 0.9% agarose gel electrophoresis (see above).

**2.7.1.5 Restriction endonuclease digestion.** T1L M1 cDNA (1µg) was incubated in the presence of 15 units each of Bam HI (Boehringer Mannheim) and Hind III restriction endonuclease (Boehringer Mannheim), 3.0µl Buffer B (Boehringer Mannheim), and 12µl sterile distilled water at 37°C for 2 hours. pFastBac HTb donor plasmid ( 0.5µg) (Gibco BRL) was incubated under the same conditions in the presence

of 0.25µl each of Bam HI and Hind III restriction endonucleases, 2µl Buffer B, and 15µl sterile distilled water.

**2.7.1.6 Ligation.** The T1L M1 cDNA ( 30µl) digested by Bam HI and Hind III restriction endonucleases was ligated with 10µl of pFastBac HTb plasmid treated with the same enzymes. The ligation reaction mixture, which contained 3µl T4 ligase (5U/µl, Boehringer Mannheim), 5µl 10X ligation Buffer (Boehringer Mannheim), and 2µl sterile distilled water, was incubated at 14°C overnight.

**2.7.1.7 Transformation of DH5α *Escherichia coli* cells.** The pFastBac HTb/T1L M1 cDNA ligated product was concentrated by precipitation in 95% ethanol and 3M sodium acetate, resuspended in 5µl sterile distilled water, and used to transform electrocompetent DH5α *E. coli* cells with the BIO-RAD Gene Pulser electroporator. This was done in a 1mm cuvet using 1.8V, 200Ω resistance, and 25 capacitance for 5 sec. SOC medium (1ml) was added immediately, the cells were transferred to a 1.5ml tube, and incubated at 37°C for 1 hour. The cells were then spread onto an LB plate containing 0.1mg/ml ampicillin (Amp) and incubated at 37°C for 16 hours.

**2.7.1.8 Plasmid extraction.** LB + Amp medium (2ml) was inoculated with DH5α *E. coli* transformed with pFastBac HTb/T1L M1 cDNA that formed bacterial colonies on LB + Amp plates (see section 2.7.1.7). The cultures were incubated at 37°C for 16 hours with shaking, and the recombinant plasmid was extracted using the High Pure Plasmid Isolation kit (Boehringer Mannheim). Eluted DNA was resuspended in 100µl sterile distilled water.

### 2.7.2 Analysis of extracted plasmid.

**2.7.2.1 Restriction endonuclease digestion and agarose gel electrophoresis.** The presence of T1L M1 cDNA in the plasmid was determined by agarose gel electrophoresis. The extracted plasmid (5µl) was digested with 5 units of restriction endonuclease BstX I (Gibco BRL). The reaction mixture was composed of 2µl 10X React Buffer (Gibco BRL), and 12.5µl sterile distilled water, and was incubated at 55°C for 5 hours. Another 5µl of the plasmid was digested with 5 units each of restriction endonucleases BamH I and Hind III, in the presence of 2µl buffer B and 12.5µl sterile distilled water. The reaction mixture was incubated at 37°C for 5 hours. The digested DNA was analyzed by 0.8% agarose gel electrophoresis (13.9cm x 12cm x 1cm gel) and visualized as described above. The native pFastBac HTb plasmid was included in the electroporation to serve as a control.

**2.7.2.2 Southern blot analysis.** To perform Southern blot analysis on the above gel, the Reo M1-02 probe was labeled using the following procedure: 4.0µl 5X Forward Reaction Kinase buffer (GIBCO BRL), 0.5µl 100µM ReoM1-02 oligonucleotide primer, 5µl [$\gamma$-$^{32}$P] ATP (370 mBq/ml) (NEN Life Sciences Products), 1.0µl T4 Polynucleotide kinase (10U/µl, GIBCO BRL), and 9.5µl sterile distilled water were incubated at 37°C for 10 min, followed by a 5 min incubation at 55°C to inactivate the enzyme. 80µl TEN buffer (0.1M NaCl, 10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0) was added, and the reaction was passed through a Sephadex G-25 column to remove excess $^{32}$P.

After the electrophoresis, the agarose gel was exposed to UV light for 15min, and the DNA was transferred onto a nitrocellulose membrane (BIO-RAD). The membrane was exposed to UV light for 3 min and baked at 78°C for 2 hours in the presence of a vacuum. The blot was pre-hybridized overnight at 34°C with Hybridization buffer (0.2% Ficoll 400 [v/v], 0.2% polyvinylpyrrolidone [w/v], 0.2% bovine serum albumin [w/v], 1% SDS [w/v], 375mM NaCl, 25mM $NaH_2PO_4$, 2.5mM EDTA [pH 7.4]), and probed with the Reo M1-02 probe (6.9 x $10^6$ dpm) at 34°C for 5 hours. The nitrocellulose membrane was then washed, and wrapped in saran wrap. X-ray film was exposed to the blot in a cassette overnight at room temperature and developed to produce an autoradiogram.

**2.7.2.3 DNA sequencing.** Standard fluorescence dideoxy sequence analysis was performed at the Cell Biology Institute (Winnipeg, Canada) to confirm the proper sequence of the T1L M1 cDNA insert. Reactions were set up with each of the Reo M1 primers indicated in Table 1. 50ng DNA in 2.5µl was mixed with 0.5µl of 100µM Reo M1 primer.

In order to determine whether ligation of the T1L M1 cDNA and donor plasmid resulted in an in-frame insertion, the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit was used (Applied Biosystems). 8µl Terminator Ready reaction mix (Applied Biosystems), 0.5µl (325ng) pFastBac HTb/T1L M1, 1.6µl (2pmol/µl) Reo M1-12 or Reo M1-06 primer, and 9.9µl sterile distilled water was incubated in a thermocycler under the following conditions: 30 cycles of 96°C for 10 sec, 50°C for 5 sec, 60°C for 1 min 30 sec, followed by a 4°C hold. The PCR product

was precipitated with isopropanol, dried at 95°C for 2 min, and resuspended in 24µl Template Suppression reagent. The DNA was sequenced with the ABI PRISM 310 Genetic Analyzer.

**2.7.3 Transposition of DH10Bac *E. coli* cells.** The following five methods were obtained, and modified as needed, from the Gibco BRL Bac-To-Bac Baculovirus Expression System instruction manual. DH10Bac competent cells were thawed on ice. Approximately 1ng or 10ng recombinant donor plasmid (in 5µl) was added to 100µl cells and mixed gently. 1ng of a control DNA was added to 100µl of cells to serve as a standard for the transposition experiment. The samples were incubated on ice for 30 min, heat shocked at 42°C for 45 sec, and chilled on ice for 2 min. 900µl of SOC medium was added to each sample, which was then incubated in a shaking incubator at 37°C with medium agitation for 4 h. The cells were serially diluted in SOC medium: $10^{-1}$ to $10^{-3}$. 100µl of each dilution was spread evenly on Luria agar plates (10g/L peptone, 5g/L yeast extract, 10g/L sodium chloride, 12g/L agar, distilled water to a volume of 1L) supplemented with 50µg/ml kanamycin, 7µg/ml gentamicin, 10µg/ml tetracycline, 100µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), and 40µg/ml isopropylthio-β-galactoside (IPTG). The plates were incubated for at least 24 h at 37°C, then placed at 4°C overnight to ensure complete color development.

**2.7.4 Bacmid extraction.** Approximately 10 white colonies were picked from Luria plates containing DH10Bac cells transposed with recombinant pFastBac HTb/T1L M1 and streaked onto fresh Luria plates (see above) to verify the color phenotype. The plates were incubated at 37°C overnight and placed at 4°C for improved color

development. A single confirmed white bacterial colony was inoculated into 2ml LB medium supplemented with 50μg/ml kanamycin, 7μg/ml gentamicin, and 10μg/ml tetracycline, and incubated at 37°C to stationary phase (up to 24 h) with shaking at 250 rpm. 1.5ml of the culture was transferred to a 1.5ml microcentrifuge tube and centrifuged at 14 000 Xg in an IEC Micromax RF centrifuge for 1 min. The supernatant was removed by vacuum aspiration and each pellet was resuspended in 0.3ml of Solution I (15mM Tris-HCl [pH 8.0], 10mM EDTA, 100μg/ml Rnase A). 0.3ml of Solution II (0.2N NaOH, 1% SDS) was added, the mixture was gently mixed, and incubated at room temperature for 5 min. 0.3ml of 3M potassium acetate (pH 5.5) was added slowly, mixing gently during addition. The sample was placed on ice for 5 to 10 min, and centrifuged for 10 min at 14 000 Xg. The supernatant was transferred to a fresh tube containing 0.8ml absolute isopropanol, avoiding any white precipitate, mixed by inverting the tube a few times, and placed on ice for 5 to 10 min. The sample was centrifuged for 15 min at 14 000 Xg at room temperature, the supernatant was removed, and 0.5ml 70% ethanol was added. The tube was inverted several times to wash the pellet, then centrifuged at 14 000 Xg at room temperature for 5 min. The supernatant was removed, the pellet was air dried for 5 to 10 min at room temperature, and dissolved in 40μl TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0). The mixture was allowed to sit at room temperature, with occasional gentle tapping, for at least 10 min. The sample was aliquoted to avoid frequent freeze/thaw cycles and placed at -20°C for storage.

**2.7.5 Transfection of Sf9 insect cells.** Each well of a 6 well tissue culture plate was seeded with 1 x $10^6$ Sf9 cells in 2ml of Sf-900 II SFM medium (Gibco BRL)

supplemented with 0.5X penicillin-streptomycin (50 units/ml penicillin, 50μg/ml streptomycin). Cells were allowed to attach at 28°C for approximately 1 h. Transfection solutions were prepared as follows: 5μl of mini-prep recombinant bacmid DNA was diluted in 100μl Sf-900 II SFM medium. 6μl of CellFECTIN reagent was diluted in 100μl Sf-900 II SFM medium. The two solutions were combined, mixed gently, and incubated for 45 min at room temperature. 0.8ml of Sf-900 II SFM medium was added to each tube containing lipid-DNA complexes, and mixed gently. The cells were washed once with 2ml of medium without antibiotics. The media was removed, the cells were overlaid with the diluted lipid-DNA solution, and incubated at 28°C for 5 h. The transfection mixtures were removed and 3ml of medium supplemented with 0.5X antibiotics was added. The cells were incubated at 28°C for 72 h. The virus was harvested and amplified as described above.

**2.7.6 Analysis of protein expression.** Expression of μ2 was determined by Western blot analysis. Sf9 cells infected with recombinant virus in 6 well plates were lysed with 250μl SDS-PAGE gel loading buffer (200mM Tris-HCl, pH 6.8, 8% SDS [w/v], 4% Bromophenol Blue [w/v], 40% glycerol [v/v]) per well at 48 h, 72 h, 96 h, and 120 h p.i. A wild-type baculovirus infection was lysed at 120 h p.i., and mock infected cells were lysed at 48 h p.i., to serve as a control. 10μl of each sample, along with 8μl of a Kaleidoscope Prestained Standard (BIO-RAD), was analyzed by a 7.3cm x 10.2cm x 0.1cm 10% SDS-PAGE. The gel was fixed with a 30% isopropanol/10% acetic acid solution (v/v), and stained with Coomassie Brilliant Blue R-250. Gels run under the same conditions were subjected to Western blot analysis. Proteins were transferred onto Immobilon-P membranes, and stained to ensure successful transfer, as described above.

Two immunodetection methods were used. In the first one, rabbit anti-μ2 antibody was used as the primary antibody and biotin-SP-conjugated goat anti-rabbit IgG was used as the secondary antibody. The μ2 protein was detected with a biotin/streptavidin chemiluminescence blotting kit (Roche) as described in the instruction manual. In the second method, mouse monoclonal anti-polyHistidine IgG was used as the primary antibody and peroxidase-labeled anti-mouse goat IgG was used as the secondary antibody. The ECL Plus™ Western blotting kit (Amersham Pharmacia Biotech) was used to detect proteins labeled with a poly-Histidine tag, as described in the instruction manual.

**2.7.7 Recombinant protein purification.** The recombinant μ2 was purified by affinity chromatography. Ni-NTA resin (1ml) (Gibco BRL) was transferred to a centrifuge tube and centrifuged at 1 500 Xg for 5 min. The supernatant was removed and the resin was mixed with 1 volume of wash buffer (20mM Tris-HCl, pH 8.5, 500mM KCl, 20mM imidazole, 5mM β-mercaptoethanol, 10% glycerol [v/v], at 4°C). The slurry was poured into the column, which was then equilibrated with 10ml of wash buffer at 4°C at a flow rate of 0.5ml/min. A 50ml culture of 1.5 x $10^6$ Sf9 cells/ml infected with the recombinant baculovirus at an MOI of 5 was harvested 72 h p.i. by centrifugation at 500 Xg for 5 min. To prevent proteolysis of the recombinant protein, 0.1% fetal bovine serum (Multicell) (v/v) was added to the cells 48 p.i. The cell pellet was resuspended in 3ml of lysis buffer (50mM Tris-HCl [pH 8.5], 5mM 2-mercaptoethanol, 100mM KCl, 1mM PMSF, 1% Nonidet® P-40 at 4°C). The tube was inverted continually for 1 min. The cell debris was pelleted by centrifugation at 10 000 Xg for 10 min. The supernatant was tranferred to a fresh tube, and the sample was added to the pre-equilibrated column.

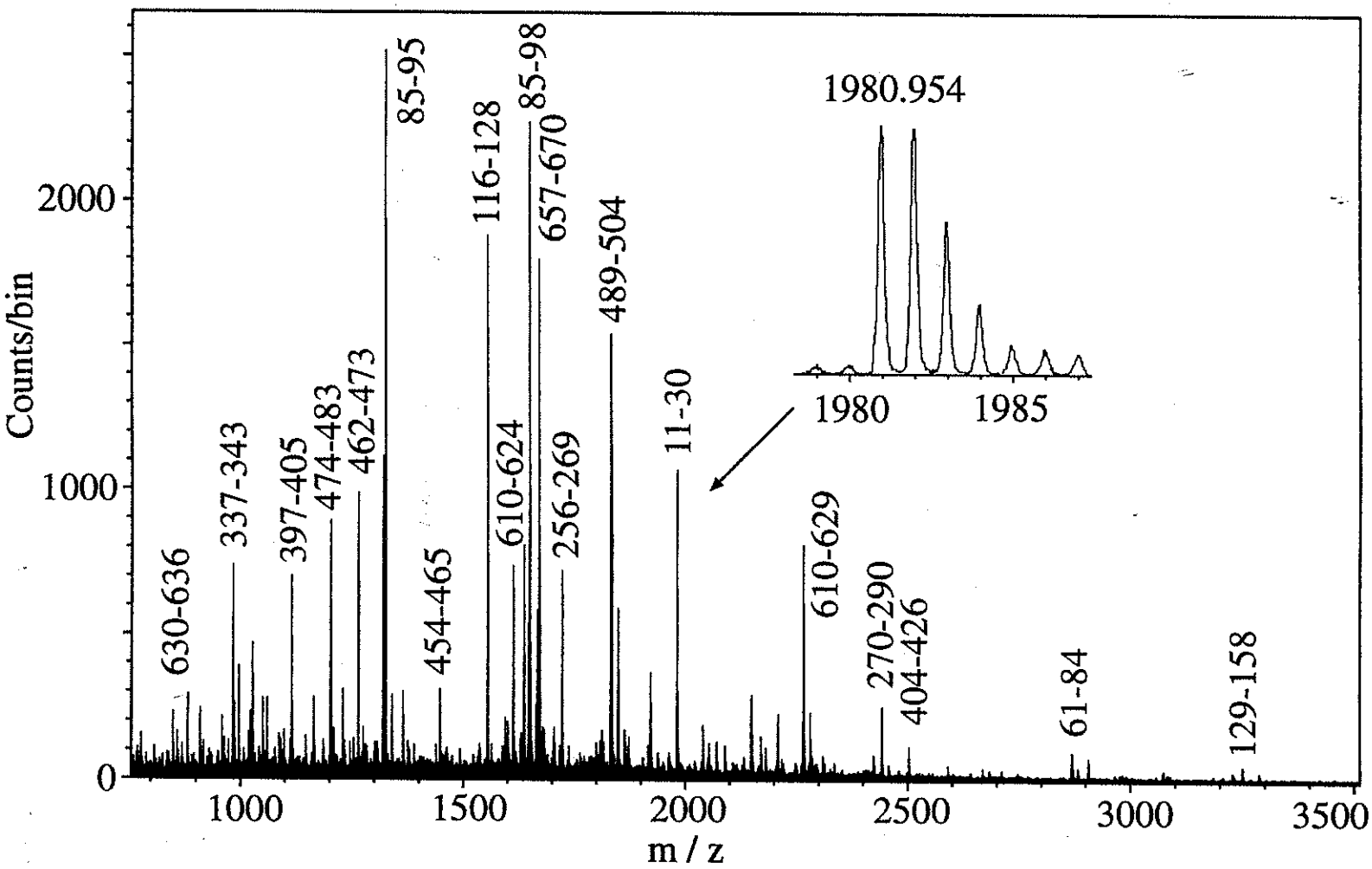
The column was washed with 10ml of Buffer A (20mM Tris-HCl, pH 8.5, 500mM KCl, 20mM imidazole, 5mM β-mercaptoethanol, 10% glycerol [v/v], at 4°C), 2ml of Buffer B (20mM Tris-HCl, pH 8.5, 1M KCl, 5mM β-mercaptoethanol, 10% glycerol [v/v], at 4°C), followed by 2ml of Buffer A. The column was eluted with 10ml of Buffer C (20mM Tris-HCl, pH 8.5, 100mM KCl, 100mM imidazole, 5mM β-mercaptoethanol, 10% glycerol [v/v], at 4°C), and 0.5ml fractions were collected.

**2.7.8 Analysis of protein purification.** Purification of μ2 was determined by Western blot analysis. Aliquots (200μl) of the fractions from the above column purification (section 2.7.7) were concentrated by 3M sodium acetate and 95% ethanol precipitation, washed in 70% ethanol, and resuspended in 20μl electrophoresis sample buffer (240 mM Tris-HCl, pH 6.8, 1.5% dithiothreitol (DTT), 1% SDS). Half the volume (10μl) of each of the concentrated samples was analyzed by SDS-PAGE. A T1L whole virus preparation in electrophoresis sample buffer served as a positive control, and electrophoresis sample buffer alone was used as a negative control. The gel was run under the same conditions, and stained, as described above (section 2.7.6). The second half (10μl) of each of the concentrated samples, as well as the T1L whole virus preparation and the electrophoresis sample buffer controls, were analyzed by Western blot. The blot analysis was carried out as described above (section 2.7.6). Rabbit anti-μ2 antibody was used as the primary antibody and biotin-SP-conjugated goat anti-rabbit IgG was used as the secondary antibody. The μ2 protein was detected with a biotin/streptavidin chemiluminescence blotting kit (Roche) as described in the instruction manual.

# 3. RESULTS

## 3.1 Mass spectrometric analysis of the μ2 protein

**3.1.1 Determination of the translation start site.** In order to settle the dispute of the translation initiation site of the μ2 protein, and due to the failure to sequence the protein by Edman degradation, the protein was proteolytically cleaved and analyzed by mass spectrometry. To obtain purified μ2 protein, a large infection of L929 cells with T1L reovirus was carried out. The virus was treated by detergent extraction, followed by solvent extraction, and purified on a CsCl gradient. The virus band was collected, dialyzed, and digested with α-chymotrypsin to generate viral cores. These cores were purified on a CsCl gradient and preparative SDS-PAGE was performed. The generation of cores was necessary to separate the μ2 protein from the outer capsid μ1C protein, which co-migrates in an SDS-PAGE of a whole virus sample. The purified μ2 band was excised and subjected to in-gel digestion. To identify the translation initiation site of μ2, the protein was analyzed for proteolytic cleavage sites with the potential to generate peptides that would differentiate between the two putative start codons (Figure 3). Initially, endoproteinase Asp-N, which cleaves proteins on the amino side of aspartic acid residues, was chosen for the higher probability of obtaining a measurable proteolytic peptide in the Asp-rich amino terminal region. The resulting peptide fragments were extracted by sonication as detailed in Materials and Methods. MALDI-QqTOF mass spectrometry (MS) was used to analyze the μ2 peptides. Many μ2 specific fragments were identified. One was a peptide fragment of 1980.954 Da (Figure 8). The peptide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1000
0
1000
1500
2000
2500
3000
3500
m / z
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337-343
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85-95
454-465
116-128
610-624
85-98
657-670
256-269
489-504
11-30
610-629
270-290
404-426
61-84
129-158
1980.954
1980
1985

**Figure 8. Mass spectrum of μ2 digested by endoproteinase Asp-N.** Virus was amplified and purified through solvent extraction, CsCl gradient, and dialysis. Viral cores were generated by α-chymotrypsin digestion of purified virions, and purified using CsCl gradient and dialysis. Core proteins were separated by SDS-PAGE. Protein μ2 was subjected to in-gel digestion with endoproteinase Asp-N followed by gel extraction. Peptides released were measured by MALDI QqTOF mass spectrometry and matched against NCBInr database using the ProFound peptide mapping tool. Highlighted peak (expanded in inset) indicates a peptide fragment at m/z 1980.954 predicted to correspond to the amino-terminal peptide 11-30 (calculated mass m/z = 1980.951) and is subsequently confirmed by MS/MS in Figure 9. Numbers above select peaks indicate the μ2 amino acid sequence that corresponds to those peaks.

mass mapping program ProFound matched this MS peak to amino acids $Asp_{11}$-$Ala_{30}$ in the μ2 protein, which have a calculated theoretical mass of 1980.951 Da. To confirm the identity of this peptide, the ion at m/z 1980.954 was subjected to MALDI QqTOF tandem mass spectrometry (MS/MS) (Figure 9) and analyzed by the Prospector MS-Tag program. The MS/MS analysis generated a series of b and y fragment ions. Both fragment ions are generated when a peptide is fragmented at the peptide bond (Siuzdak 1996). The b ion retains the charge on the amino-terminus while cleavage occurs at the carboxy-terminus. The y ion retains the charge on the carboxy-terminus and is cleaved at the amino-terminus. The amino acid sequence ladder generated from the amino terminal b fragment ions ($b_4$ to $b_{20}$) is consistent with the expected amino acid residues 14-30 in μ2, and the measured m/z values of the daughter ions indicated the parent peptide was indeed $Asp_{11}$-$Ala_{30}$ (Figure 9).

**3.1.2 Determination of the μ2 protein size.** The identification of $Asp_{11}$-$Ala_{30}$ strongly indicates translation of the M1 gene initiates at the first AUG codon to generate the full length form of μ2 found in virion and core particles. This would imply a molecular weight closer to 83 kDa. However, as previously mentioned, μ2 migrates in SDS-PAGE with an apparent molecular weight of approximately 72 kDa. To determine whether post-translational proteolytic cleavage near the carboxy-terminus may result in a truncated protein of only 72 kDa, the protein was analyzed for proteolytic cleavage sites that could generate measurable peptide fragments in the carboxy-terminus. Trypsin, which cleaves the proteins at the carboxy side of lysine and arginine residues, was chosen. In-gel tryptic digestion of purified μ2 protein was carried out and released peaks were analyzed by MALDI-QqTOF MS. A peptide fragment at m/z 892.491 was

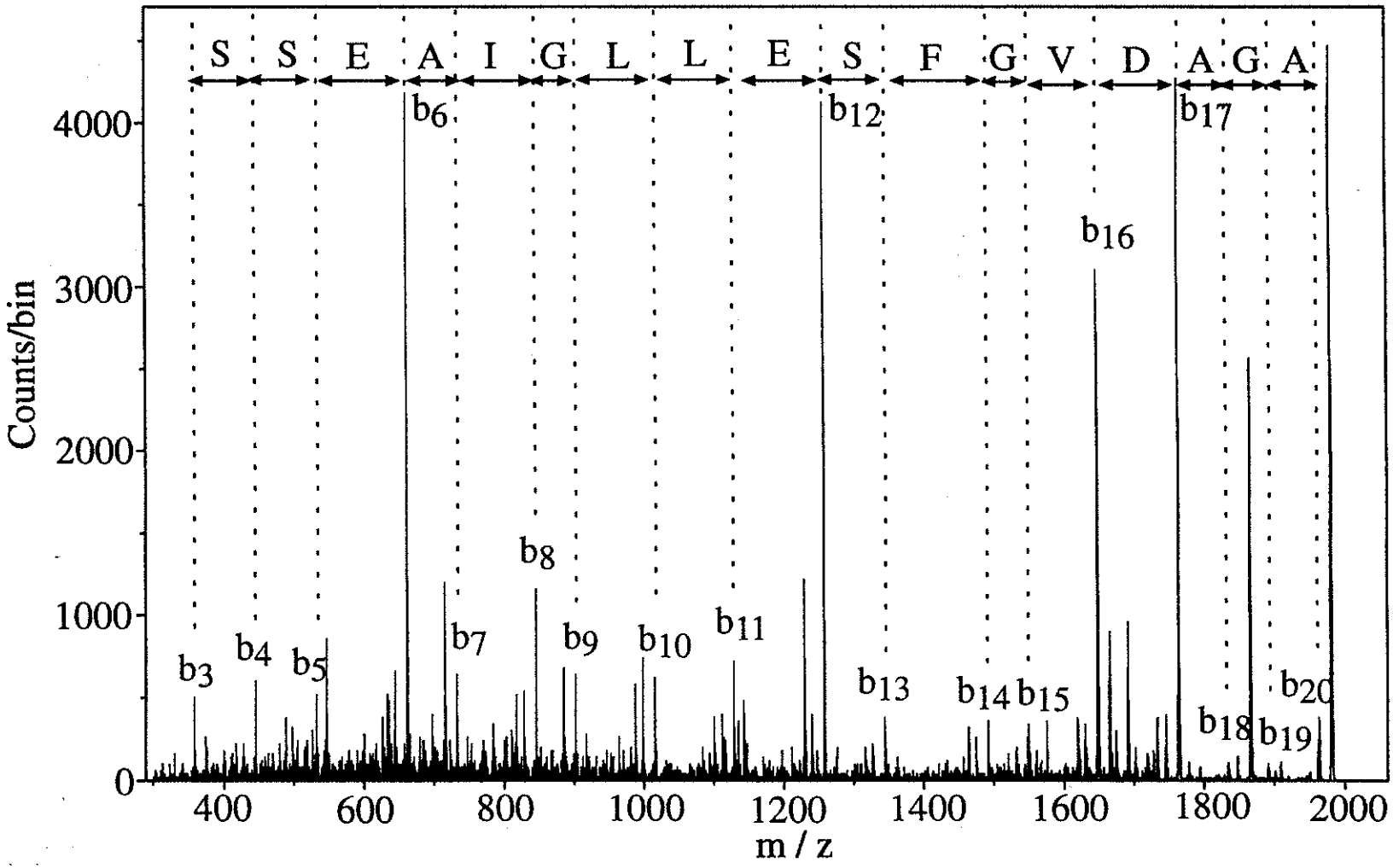
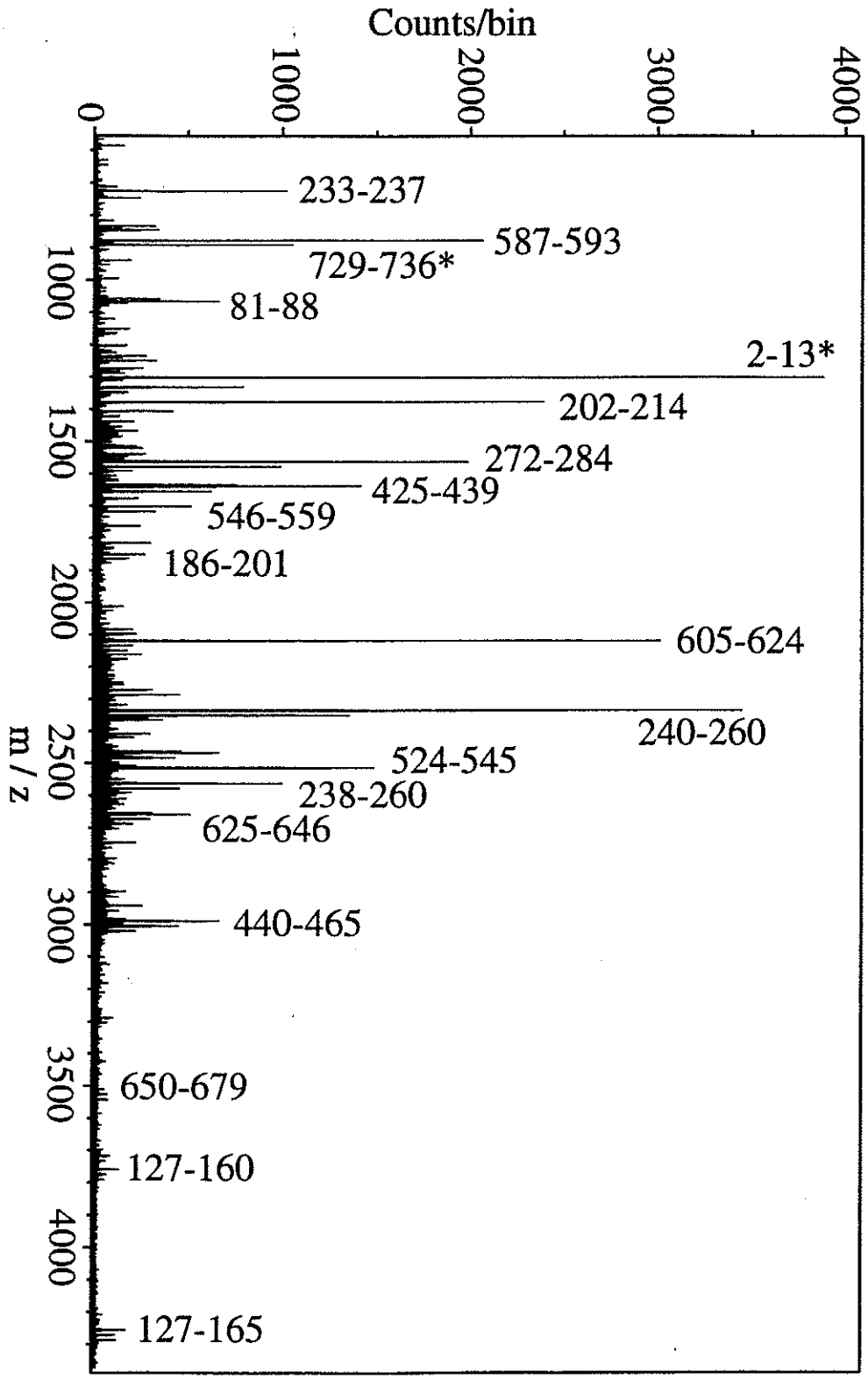

S S E A I G L L E S F G V D A G A
b6
b12
b17
b16
b8
b3
b4
b5
b7
b9
b10
b11
b13
b14
b15
b18
b19
b20
Counts/bin
0
1000
2000
3000
4000
400
600
800
1000
1200
1400
1600
1800
2000
m / z

**Figure 9. Tandem (MS/MS) mass spectrum of the ion at m/z 1980.951 Da generated by endoproteinase Asp-N digestion of μ2.** The 1980 Da peptide fragment generated by endoproteinase Asp-N digestion and identified as belonging to μ2 amino acid sequence 11-30 was subjected to MS/MS measurements and analyzed by Prospector MS-Tag to determine its amino acid sequence and probable identity. The MS/MS generated a series of b fragment ions, which occur when a peptide is fragmented at the peptide bond. Ions $b_3$ to $b_{20}$ were captured in the mass range of this spectrum, and the difference in their masses was used to generate the amino acid sequence SSEAIGLLESFGVDAGA. This sequence corresponds to the peptide fragment $Asp_{11}$-$Ala_{30}$.

identified (Figure 10) and initial analysis with ProFound suggested it to be the μ2 peptide $Leu_{729}$-$Ala_{736}$ with a predicted molecular weight of 892.489 Da, which corresponds to the carboxy-terminus. Tandem MS/MS and Prospector analysis confirmed the peptide sequence was identical to that of the carboxy-terminal amino acids 729 -736. Figure 11 revealed the prominent amino terminal b ions, and the presence of "$b_6 + H_2O$" and "$b_7 + H_2O$" rearrangment ions was caused by an Arg residue localized within the peptide sequence (LAFRSDLA). This is a common occurrence and is due to the properties of the Arg residue (Ballard and Gaskall 1992, Vachet *et al* 1996).

Peptide fragments generated by the combined trypsin and endoproteinase asp-N digestions account for 615 of 736 amino acids (83.6 %) of the μ2 amino acid sequence (Figures 8, 10, and 12). The majority of the missing amino acid sequences correspond to peptide fragments that are either too small or too large to be easily detected in the mass range of the MALDI QqTOF, which is approximately 550 to 4400 Da. Since both ends of the protein can be identified, this implies that all of the protein is present. Collectively, these data indicate that the actual molecular weight of μ2 protein found in virion and core particles is close to its theoretical value of 83 kDa, and that translation of the reovirus M1 gene can and does initiate at the first AUG codon.

**3.1.3 Comparison of purified and cellular μ2 protein.** Previous work has suggested that translation of the M1 gene may initiate at the second in-frame AUG located at nucleotide positions 161-163 (Roner *et al* 1993). To determine whether the μ2 protein purified from virions, shown above to initiate at the first AUG and to have an approximate molecular weight of 83 kDa, is the major form of the protein present in infected cells, or whether two alternate forms of μ2 are translated intracellularly, but the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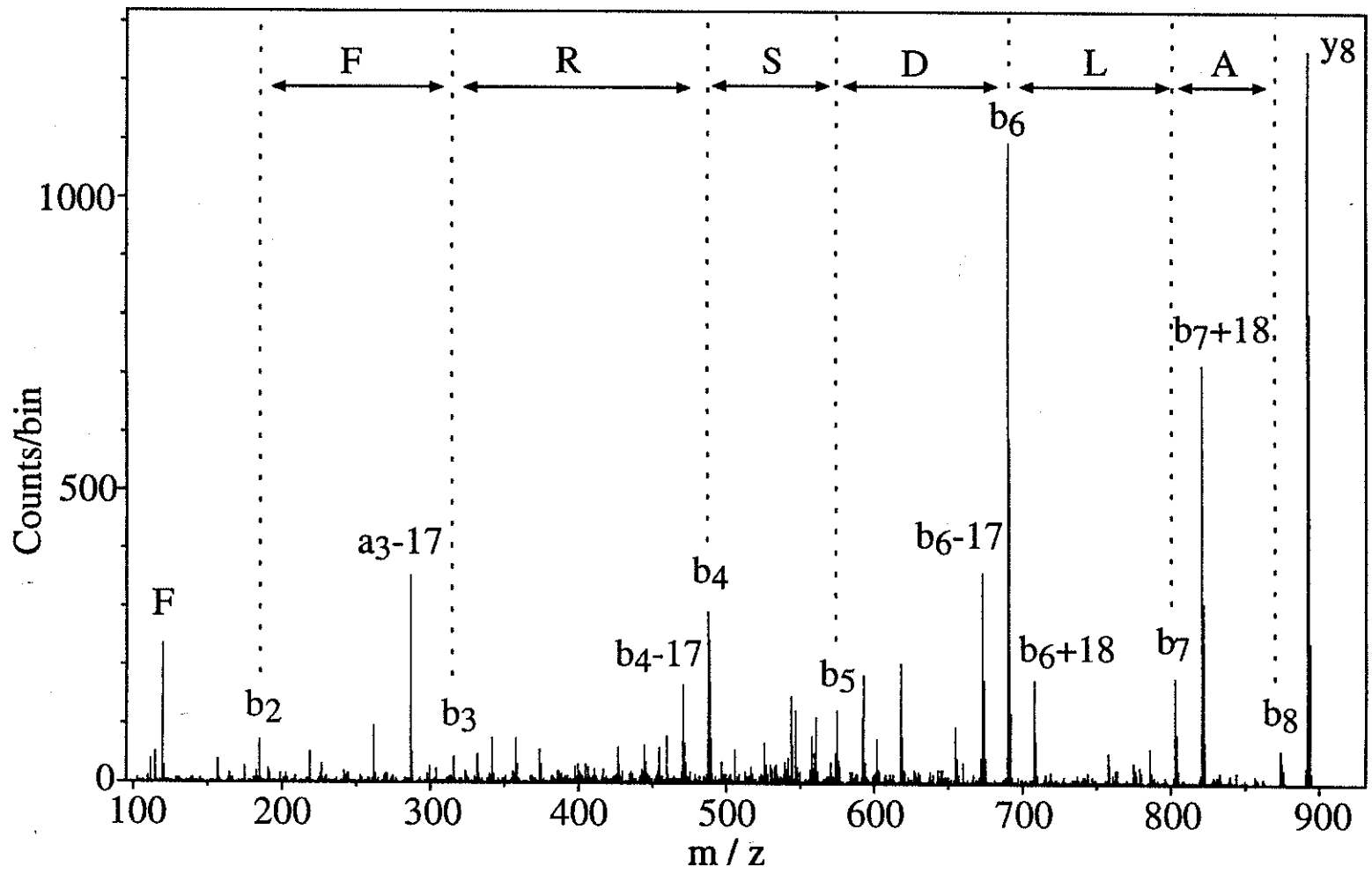
1000
2000
3000
4000
m/z
1000
1500
2000
2500
3000
3500
4000
233-237
587-593
729-736*
81-88
2-13*
202-214
272-284
425-439
546-559
186-201
605-624
240-260
524-545
238-260
625-646
440-465
650-679
127-160
127-165

**Figure 10. Mass spectrum of μ2 digested by trypsin.** μ2 protein was purified using methods described above, and subjected to in-gel digestion with trypsin. After gel extraction, the peptides were measured by MALDI QqTOF mass spectrometry and analyzed with ProFound. Peaks highlighted by asterisks (*) indicate the amino-terminal peptide fragment 2-13 (m/z 1302.704), and the carboxy- terminal peptide fragment 729-736 (m/z 892.491).


F
R
S
D
L
A
y8
b6
1000
b7+18
Counts/bin
500
a3-17
b4
b6-17
F
b4-17
b6+18
b7
b2
b5
b3
b8
0
100
200
300
400
500
600
700
800
900
m / z

MAYIAVPAVVDSRSSEAIGLLESFGVDAGADANDVSYQDHDYVLDQLQYMLDGYEAGDVID
ALVHKNWLHHSVYCLLPPKSQLLEYWKSNPSVIPDNVDRRLRKRLMLKKDLRKDDEYNQLA
RAFKISDVYAPLISSTTSPMTMIQNLNQGEIVYTTTDRVIGARILLYAPRKYYASTLSFTMTKCII
PFGKEVGRVPHSRFNVGTFPSIATPKCFVMSGVDIESIPNEFIKLFYQRVKSVHANILNDISPQIV
SDMINRKRLRVHTPSDRRAAQLMHLPYHVKRGASHVDVYKVDVVDVLLEVVDVADGLRNV
SRKLTMHTVPVCILEMLGIEIADYCIRQEDGMFTDWFLLLTMLSDGLTDRRTHCQYLINPSSVP
PDVILNISITGFINRHTIDVMPDIYDFVKPIGAVLPKGSFKSTIMRVLDSISILGVQIMPRAHVVDS
DEVGEQMEPTFEHAVMEIYKGIAGVDSLDDLIKWVLNSDLIPHDDRLGQLFQAFLPLAKDLLA
PMARKFYDNSMSEGRLLTFAHADSELLNANYFGHLLRLKIPYITEVNLMIRKNREGGELFQLV
LSYLYKMYATSAQPKWFGSLLRLLICPWLHMEKLIGEADPASTSAEIGWHIPREQLMQDGWC
GCEDGFIPYVSIRAPRLVMEELMEKNWGQYHAQVIVTDQLVVGEPRRVSAKAVIKGNHLPVK
LVSRFACFTLTAKYEMRLSCGHSTGRGAAYNARLAFRSDLA

**Figure 12. Approximation of the size of protein μ2.** The peptides detected by MS are indicated in black. The peptides not detected by MS are indicated in green.

full-length form is preferentially packaged into virions, serotype T1L and T3D infected cell lysates and T1L and T3D purified virus were resolved, in duplicate, in SDS-PAGE and analyzed by Western blot (Figure 13). Coomassie staining of one set demonstrated an approximately equal loading of viral protein in the infected cell lysates and purified virus sample when analyzed visually (Figure 13A). A single major band was detected in all virus and viral-infected samples with anti-μ2 antiserum that had the same molecular weight and which corresponded to the form of the protein identified by MS in purified virions (Figure13B). The anti-μ2 antiserum also reacted with a slightly larger band in the T3D-infected cell lysates. This band was not detected in the T1L-infected cell lysates, even after longer exposures (data not shown). Despite apparently equal viral protein loading (Figure 13A) there was significantly more immunoreactive μ2 in the infected cells compared to the purified virions, indicating that most synthesized μ2 remains cell-associated and is not packaged into virion particles. No shorter form of the μ2 protein, that would correspond to initiation from an alternate AUG, was identified.

**3.1.4 Identification of the μ2 protein post-translational modification.** As previously mentioned, attempted Edman degradation analyses of the μ2 protein indicated that the amino terminus is blocked. In order to identify this post-translational modification, the mass spectra resulting from both the endoproteinase asp-N and tryptic cleavages of μ2 were manually analyzed. This was necessary since the protein databases used for the identity match-up could not recognize the mass of a post-translationally modified peptide fragment belonging to the amino-terminus of the μ2 protein. In the manual analysis, a peptide peak that would correspond to a combined mass that included both the amino-terminus of μ2 plus any of a number of common modifications, including

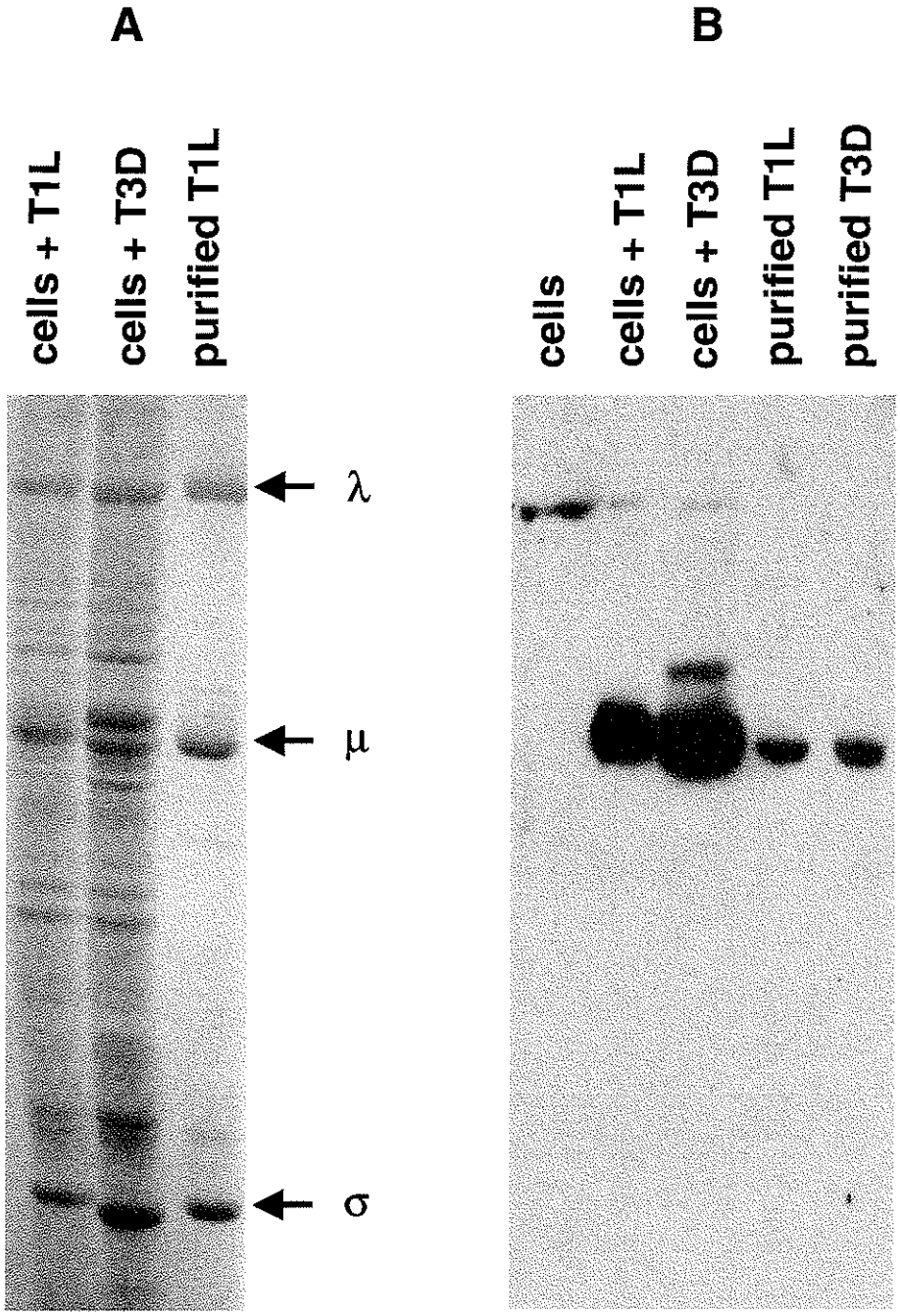

A
cells + T1L
cells + T3D
purified T1L
λ
μ
σ
B
cells
cells + T1L
cells + T3D
purified T1L
purified T3D

**Figure 13. SDS-PAGE and Western blot analysis of T1L and T3D infected cells and purified virions.** T1L and T3D cell lysates, as well as T1L and T3D purified virions were doubly analyzed by SDS-PAGE and Western blotting. A. SDS-PAGE showing equal loading of cell lysate and purified virion samples. B. Western blot probed with α-μ2 antibody. Lane 1 is an uninfected cell lysate control.

acetylation, phosphorylation, or glycosylation was not identified. However, a prominent peak in the tryptic cleavage mass spectrum (Figure 10) with an m/z value of 1302.704 Da was identified. This peak did not correspond to any unmodified μ2 peptides predicted to be released by either protease by a database search with Profound. However, this anomalous fragment ion is 42.009 Da larger than the predicted mass of a peptide fragment that would correspond to amino acid residues 2-13 (assuming the amino terminal methionine was removed; calculated mass m/z = 1260.695). The MS/MS spectrum of this ion (Figure 14) shows a complete set of carboxy terminal y ions ($y_1$ to $y_{11}$), which is consistent with the amino acid sequence of residues 3-13, suggesting a modification at the amino terminus. The 113 Da mass difference between the parent ion (i.e. $y_{12}$) and the daughter ion $y_{11}$, which is 42 Da in excess of the 71 Da mass of an Ala residue, indicated the amino terminal Ala was acetylated, which is in agreement with the mass increments of all amino terminal b ions observed (Table 2). Identification of these peaks further confirms that the μ2 protein initiates at the first AUG codon.

### 3.2 μ2 protein expression.

In order to make functional and structural analyses of a purified μ2 protein easier, protein expression was carried out. The baculovirus expression system Bac-To-Bac was selected because the virus infects insect cells, which makes proper post-translational modification of the μ2 protein more likely than in an *E. coli* or yeast expression system, and because it offers the option of the addition of a poly-histidine tag to the protein to

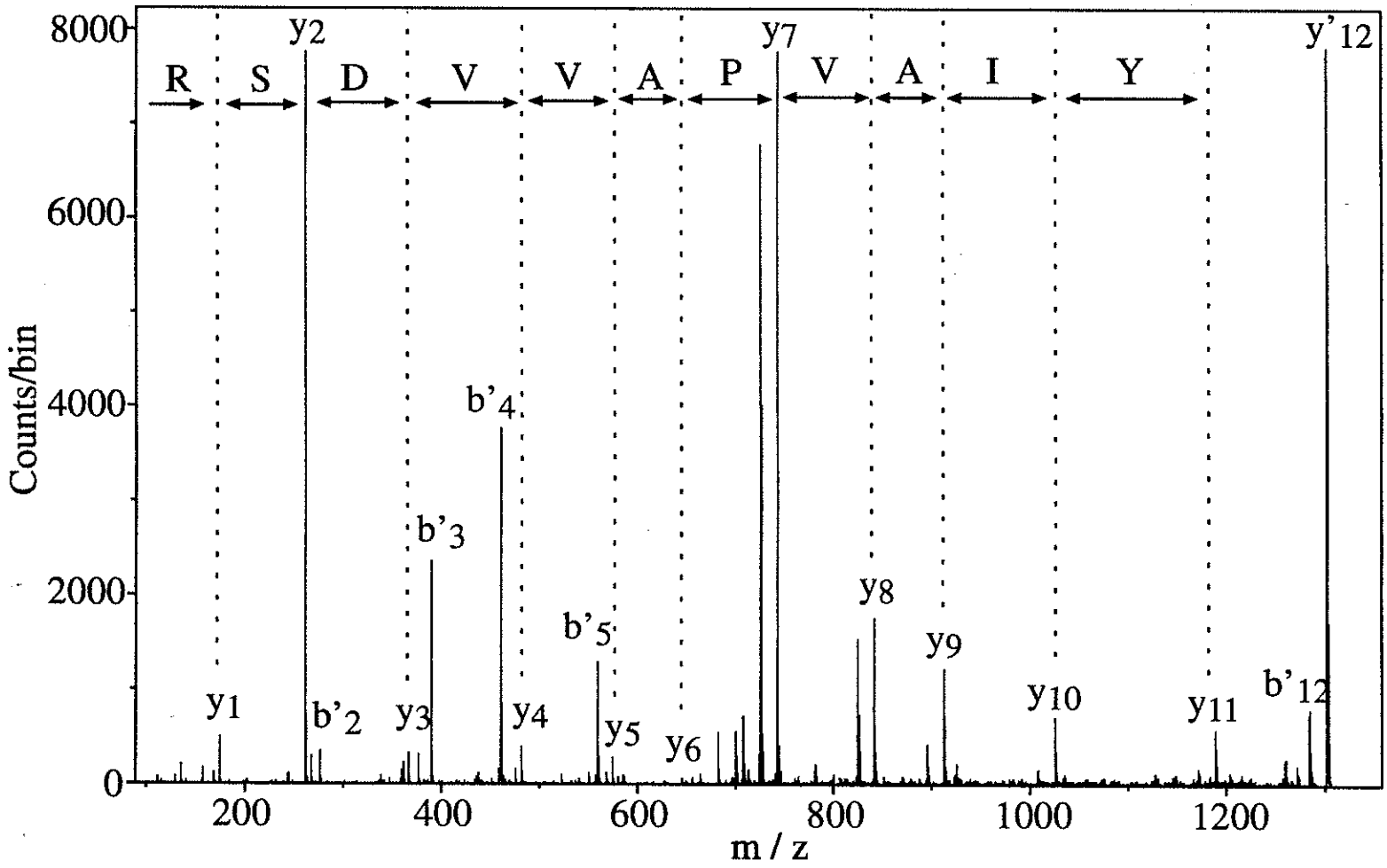

8000
6000
4000
2000
0
Counts/bin
200
400
600
800
1000
1200
m / z
R
S
D
V
V
A
P
V
A
I
Y
y2
y7
y'12
b'4
b'3
b'5
y8
y9
y1
b'2
y3
y4
y5
y6
y10
y11
b'12

**Figure 14. MS/MS spectrum of the ion at m/z 1302.704.** The 1302 Da peptide fragment generated by trypsin digestion was subjected to MS/MS and analyzed by Prospector MS-Tag to determine its amino acid sequence and probable identity. An apostrophe denotes an acetylated ion (b'n), the measured mass of which is 42 Da heavier than the value predicted by the nucleic acid sequence.

## Table 2

## Observed MS/MS fragments of the m/z 1302.704 ion from the μ2 tryptic digest vs mass values calculated assuming the acetylated N-terminus i.e. AYIAVPAVVDSR

| b ion | m/z (Obs.) | $MH^+$ (Calc.) | Δ (Da) | y ion | m/z (Obs.) | $MH^+$ (Calc.) | Δ (Da) |
|---|---|---|---|---|---|---|---|
| b'1 | - | 114.056 | - | y1 | 175.111 | 175.119 | -0.008 |
| b'2 | 277.117 | 277.116 | -0.001 | y2 | 262.151 | 262.151 | 0.000 |
| b'3 | 390.200 | 390.203 | -0.003 | y3 | 377.179 | 377.178 | -0.001 |
| b'4 | 461.236 | 461.240 | -0.004 | y4 | 476.242 | 476.247 | -0.005 |
| b'5 | 560.306 | 560.308 | -0.002 | y5 | 575.317 | 575.315 | 0.002 |
| b'6 | - | 657.361 | - | y6 | 646.350 | 646.352 | -0.002 |
| b'7 | - | 728.398 | - | y7 | 743.406 | 743.405 | 0.001 |
| b'8 | 827.463 | 827.467 | -0.004 | y8 | 842.475 | 842.474 | 0.001 |
| b'9 | 926.533 | 926.535 | -0.002 | y9 | 913.508 | 913.511 | -0.003 |
| b'10 | - | 1041.562 | - | y10 | 1026.594 | 1026.595 | -0.001 |
| b'11 | - | 1128.594 | - | y11 | 1189.659 | 1189.658 | 0.001 |
| b'12 | 1284.695 | 1284.695 | 0.000 | y12 | 1302.704 | 1302.706 | -0.002 |

ensure easier purification. In addition, other reovirus proteins have been successfully expressed in a baculovirus system.

**3.2.1 Cloning.** T1L reovirus was grown to high amounts (2-6 x $10^{13}$ virions) and its ds RNA genome was purified. The purified genome was reverse-transcribed withT1LM1-5' and T1LM1-3' primers to obtain the M1 ss cDNA. The ss cDNA product was then amplified by PCR using the same primers, and amplification of a product of the appropriate molecular weight was confirmed by agarose gel electrophoresis (Figure 15A). Preparative agarose gel electrophoresis, followed by excision and elution of the M1 cDNA from the gel matrix was carried out to purify the cDNA. The sample was then analyzed by agarose gel electrophoresis to confirm its purity (Figure 15B). The M1 cDNA and the pFastBac HTb donor plasmid DNA were prepared for cloning by digestion with restriction endonucleases Bam HI and Hind III. The digested M1 cDNA was ligated into the digested vector, and the ligated product, pFastBac HTb/T1L M1, was washed by precipitation in ethanol and sodium acetate. DH5α *E. coli* cells were transformed with the washed recombinant plasmid by electroporation to amplify the DNA. Cells electroporated without DNA were used as a negative control. The recombinant plasmid was extracted after selection for transformed cell colonies on LB agar plates supplemented with ampicillin.

**3.2.2 Analysis of cloned DNA.** Recombinant pFastBac HTb/T1L M1 DNA was analyzed by agarose gel electrophoresis. Figure 16 clearly shows that the recombinant plasmid DNA (lanes 2 to 6) is larger in size than the uncloned pFastBac HTb plasmid DNA (lane 17), indicating an insert is present. Furthermore, digestion of the recombinant plasmid DNA with restriction endonucleases Bam HI and Hind III (lanes

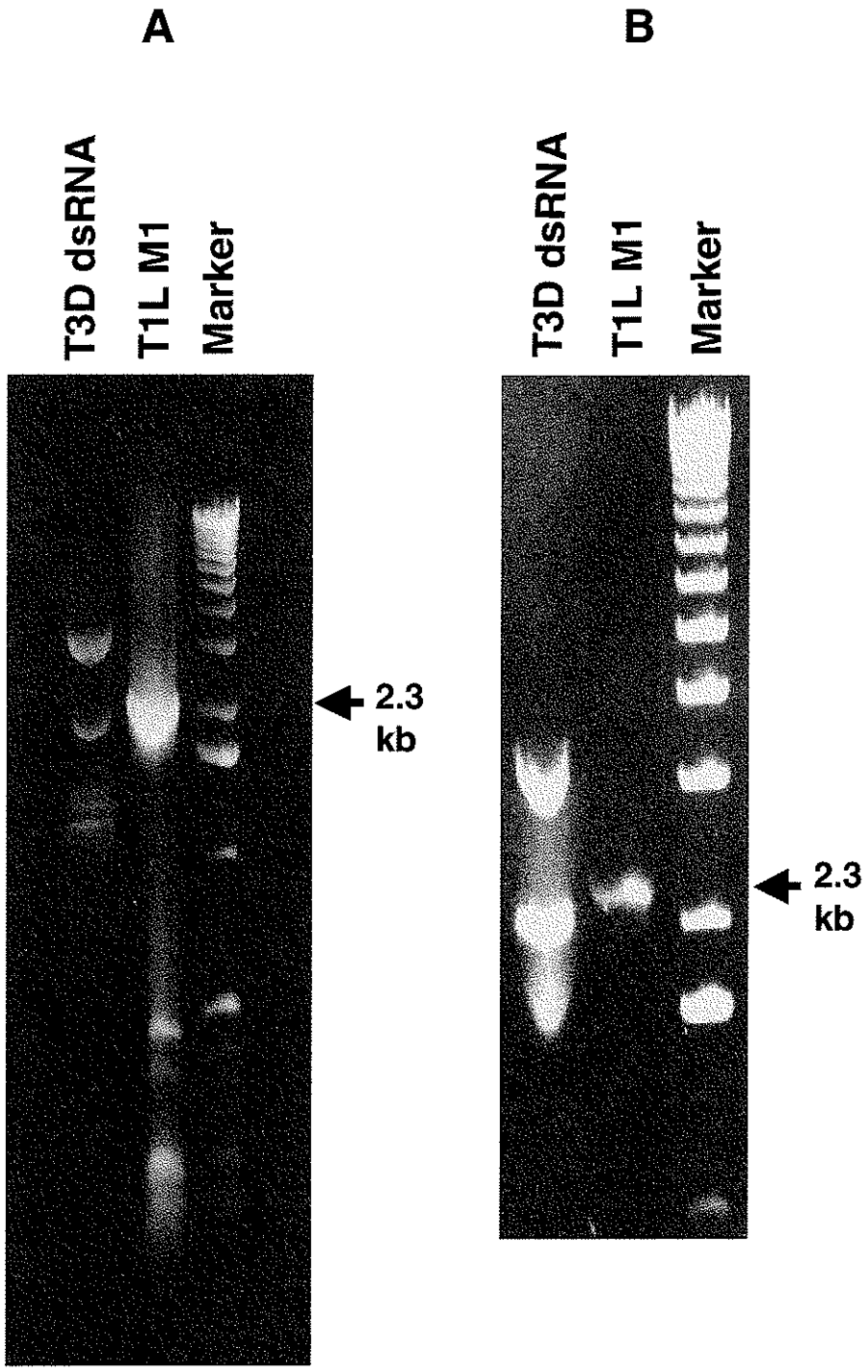

A
T3D dsRNA
T1L M1
Marker
2.3
kb
B
T3D dsRNA
T1L M1
Marker
2.3
kb

**Figure 15. Confirmation of T1L M1 PCR product.** A. Agarose gel electrophoresis analysis which confirms amplification of the T1L M1 cDNA. T3D dsRNA (lane 1) was used as a molecular size standard. B. The amplified T1L M1 cDNA was purified by agarose gel electrophoresis and extracted from the agarose matrix using the QIAquick Gel Extraction kit (QIAGEN). Agarose gel electrophoresis was used to confirm the purification of the T1L M1 cDNA. T3D dsRNA once again served as the molecular size standard.

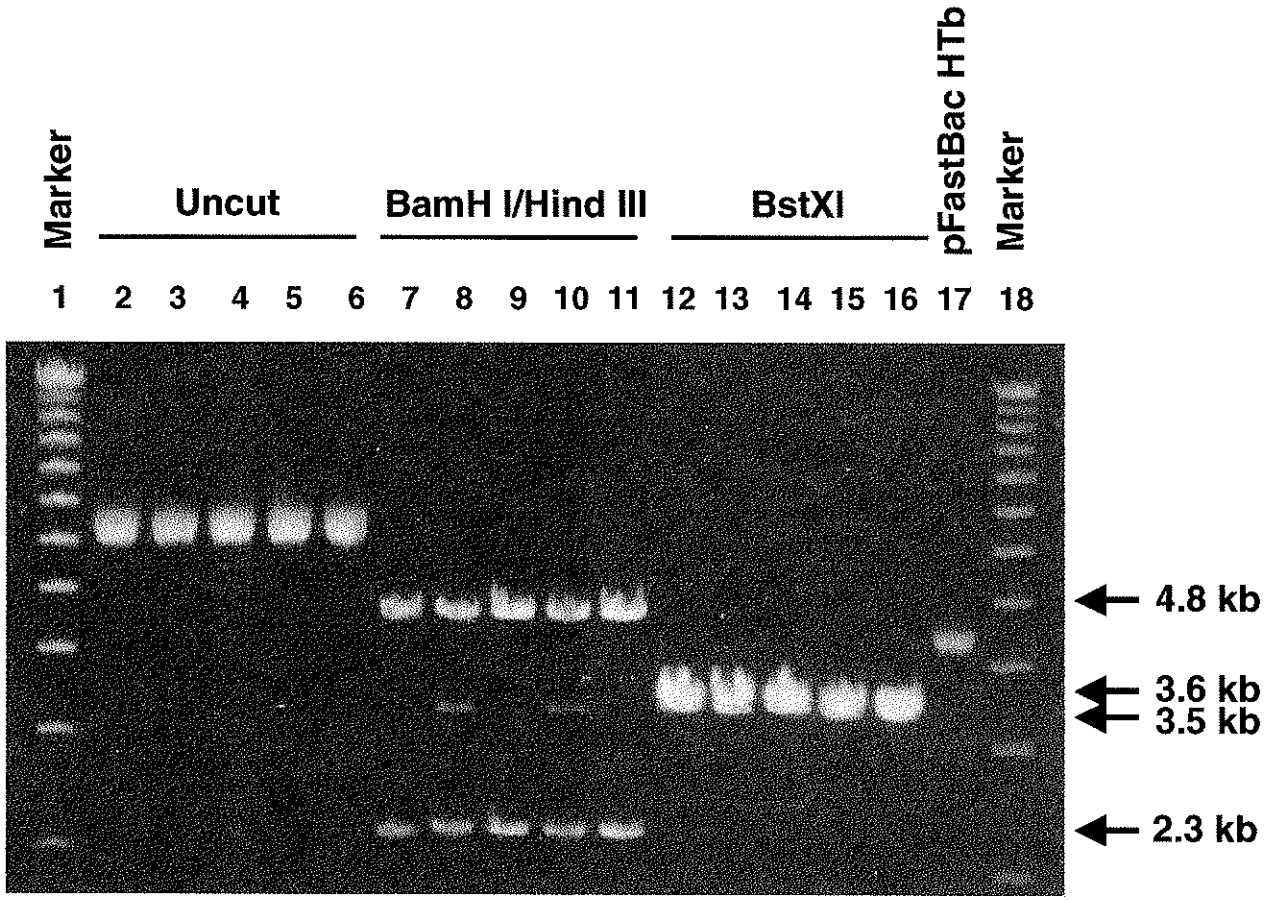

Marker
Uncut
BamH I/Hind III
BstXI
pFastBac HTb
Marker
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
4.8 kb
3.6 kb
3.5 kb
2.3 kb

**Figure 16. Agarose gel of T1L M1 insertion into pFastBacHTb.** Recombinant plasmids were digested with restriction endonucleases and analyzed by agarose gel electrophoresis. Lanes 2 to 6 were loaded with undigested recombinant plasmids. Lanes 7 to 11 contain recombinant plasmids digested with BamH I and Hind III restriction endonucleases. Recombinant plasmids digested with BstXI restriction endonuclease are shown in lanes 12 to 16. Lane 17 contains a pFastBacHTb plasmid that was not ligated with T1L M1 cDNA.

7 to 11), the same enzymes used to prepare T1L M1 cDNA for insertion into the plasmid, produced two DNA bands of a size corresponding to the pFastBac HTb plasmid and the T1L M1 gene insert. Digestion with restriction endonuclease Bst XI (lanes12 to 16) was predicted to produce two DNA bands of 3488 and 3582 base pairs (bp) if the T1L M1 cDNA was correctly inserted into the donor plasmid DNA. An incorrect, or backwards, insertion would yield 1558 bp and 5512 bp DNA bands. Although it is less clear in this reproduction of the gel, two DNA bands of the appropriate size are present. The cut plasmid DNA in lanes 7 to 11 migrates at a slower rate than the uncut, supercoiled plasmid in lane 17 due to its linear nature.

To definitively identify the DNA inserted into the plasmid, the recombinant pFastBac HTb/T1L M1 DNA, cleaved with the same restriction endonucleases as above, was subjected to Southern blot analysis (Figure 17). A [$\gamma$-$^{32}$P]-labeled ReoM1-02 probe was used to identify M1 cDNA. The blot indicates that the T1L M1 cDNA is present in all of the sample lanes, and that it is absent from lane 16, which contains uncloned donor plasmid DNA. In addition, in lanes 6 to 10, it is the lower 2300 bp band that corresponds to the T1L M1 cDNA, a result that further emphasizes that the donor plasmid contains the correct insert.

Lastly, DNA sequencing was performed to rule out the possibility of mutations and to ensure that the T1L M1 cDNA was inserted correctly into the plasmid reading frame. A preparative agarose gel of a pFastBac HTb/T1L M1 sample digested with Bam HI and Hind III was performed. The T1L M1 cDNA band was excised, and purified using the High Pure Plasmid Isolation kit (Boehringer Mannheim). 50ng of the purified cDNA was used in each sequencing reaction, the results of which are shown in Figure 18.

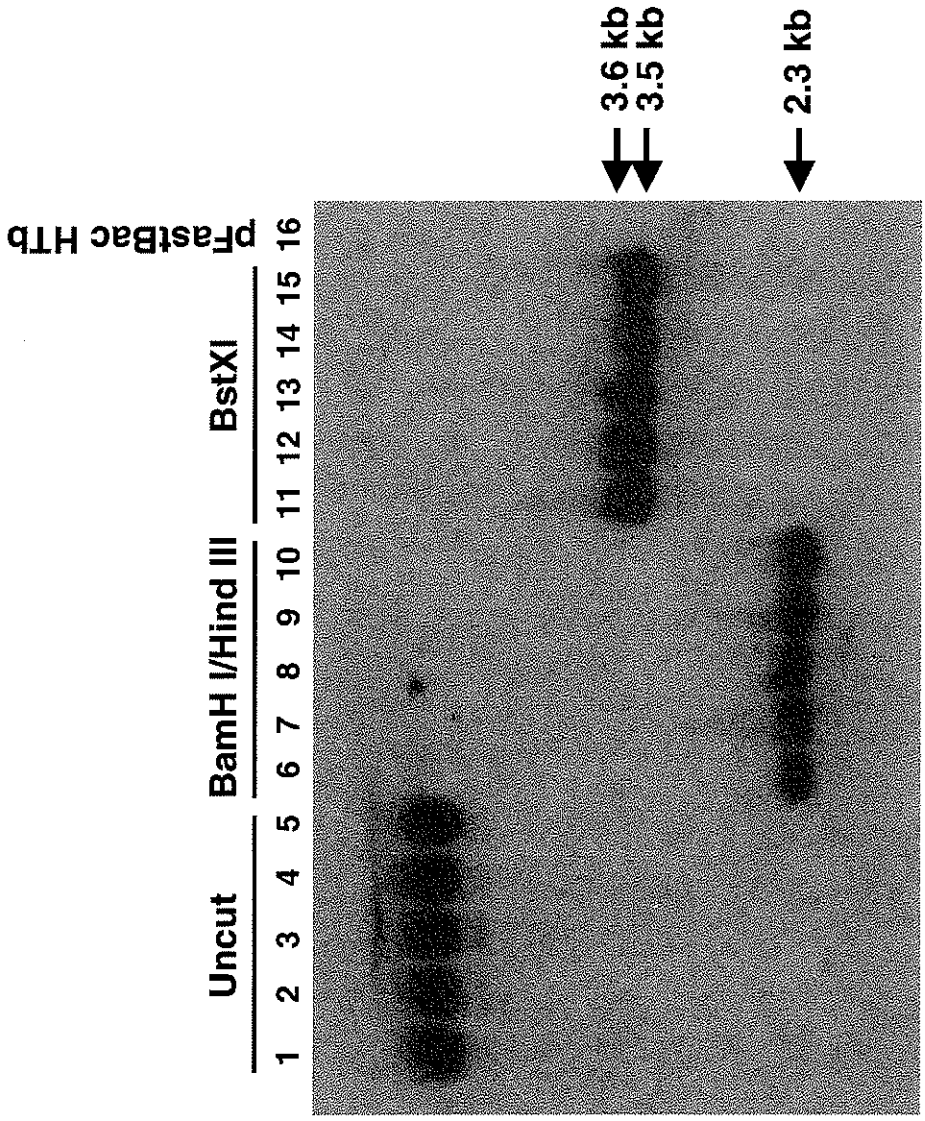

pFastBac HTb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Uncut
BamH I/Hind III
BstXI
3.6 kb
3.5 kb
2.3 kb

**Figure 17. Southern blot analysis of T1L M1 insertion into pFastBacHTb.** Restriction endonuclease digested recombinant pFastBacHTb DNA analyzed by agarose gel electrophoresis (Figure 16) was transferred onto a nitrocellulose membrane. [$\gamma$-$^{32}$P]-labeled Reo M1-02 probe was used to detect the presence of T1L M1 cDNA. Lanes 1 to 5 contain undigested recombinant plasmids, lanes 6 to 10 contain recombinant plasmids digested with restriction endonucleases BamH I and Hind III, and lanes 11 to 15 contain recombinant plasmids digested with restriction endonuclease BstXI. A pFastBacHTb plasmid DNA negative control is present in lane 16.


1
20
211
230
T1L M1 GCUAUUCGCG GUCAUGGCUU.....GAAUUGGUUA CAUCACUCCG....
T3D M1
U
T1L M1 cDNA
T
371
400
901
....CAACUAGCGC GUGCUUUCAA GAUAUCGGAU.....UGUUGUAGAC
T3D M1
G
T1L M1 cDNA
T
930
2301
2304
T1L M1 GUGUUGUUAG AGGUAGUGGA.....GUACGCGCUU CAUC
T3D M1 A
C
T1L M1 cDNA
C

**Figure 18. DNA sequencing of the T1L M1 cDNA insert.** Sequence alignment of serotype T1L and T3D M1 RNA, and T1L M1 cDNA, which was cloned into pFastBacHTb donor plasmid. Wild-type T1L M1 RNA sequence is indicated in black. Wild-type T3D M1 RNA sequence is indicated in blue. The sequence of the T1L M1 cDNA is indicated in green.

Three nucleotide base changes were detected when compared to the published T1L sequence (Zou and Brown 1992), two of which were conservative, and a third that resulted in an amino acid change from Leu to Phe. However, our available T1L wild-type lab stock also has this change as compared to the published sequence. 325ng of undigested pFastBac HTb/T1L M1 DNA was amplified in each of two PCR reactions, one with the M1-06 primer, the other with the M1-12 primer, in order to sequence the insertion site. The resulting amplicons were sequenced, and the T1L M1 cDNA insert was determined to be positioned correctly within the plasmid transcription frame (Figure 18).

**3.2.3 Generation of recombinant virus.** DH10Bac *E. coli* cells were transformed with 10ng of the pFastBac HTb/T1L M1 recombinant plasmid DNA, according to the Gibco BRL Bac-To-Bac manual. Ten white colonies were selected and their color was confirmed by incubation on fresh plates. Recombinant bacmids were isolated and used to transfect Sf9 cells. The supernatant was harvested 72 h p.i., the recombinant virus was amplified, and the viral titer was determined. The presence of the μ2 protein was determined by both SDS-PAGE and Western blot analysis (Figure 19 and data not shown). The SDS-PAGE (Figure 19) did not appear to indicate the presence of an 83 kDa protein that was unique to the recombinant baculovirus cell lysate lanes (lanes 2-5). However, lanes 2-5 contain a protein band not present in either the mock infected (lane 1) or the wild-type baculovirus infected (lane 6) cell lysate. This band, indicated with black arrows, is approximately 65 kDa.

When the same samples were subjected to Western blot analysis and detected with an anti-μ2 antibody, several bands were detected (data not shown). However, these

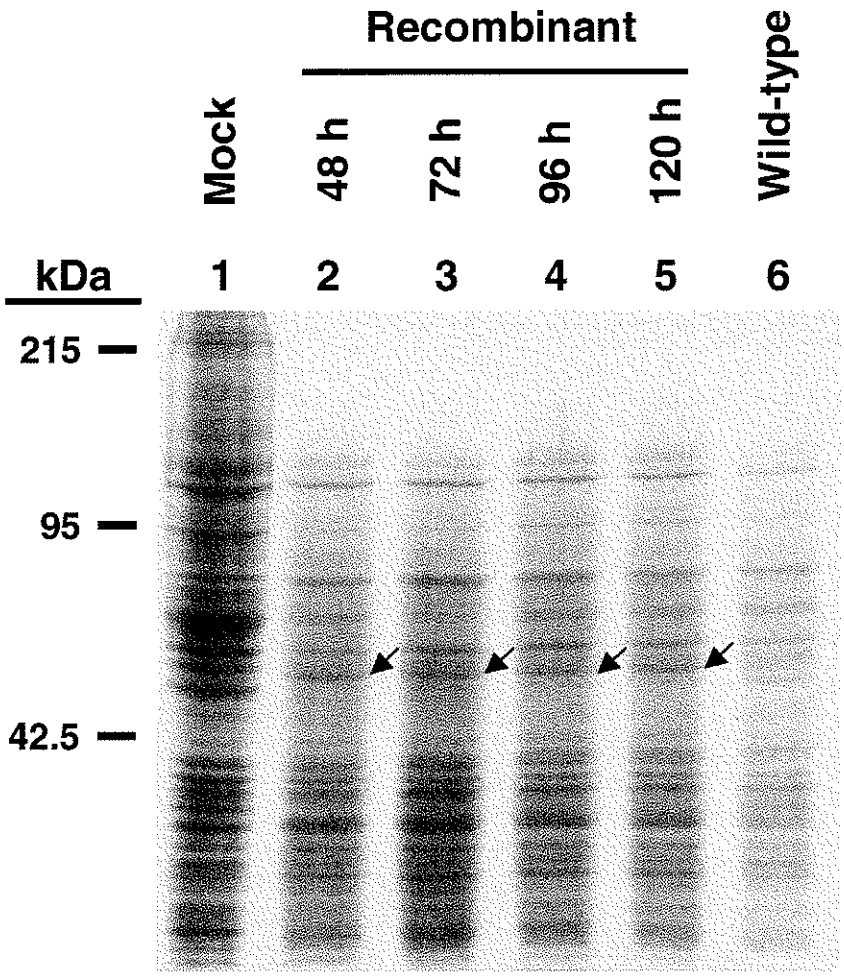
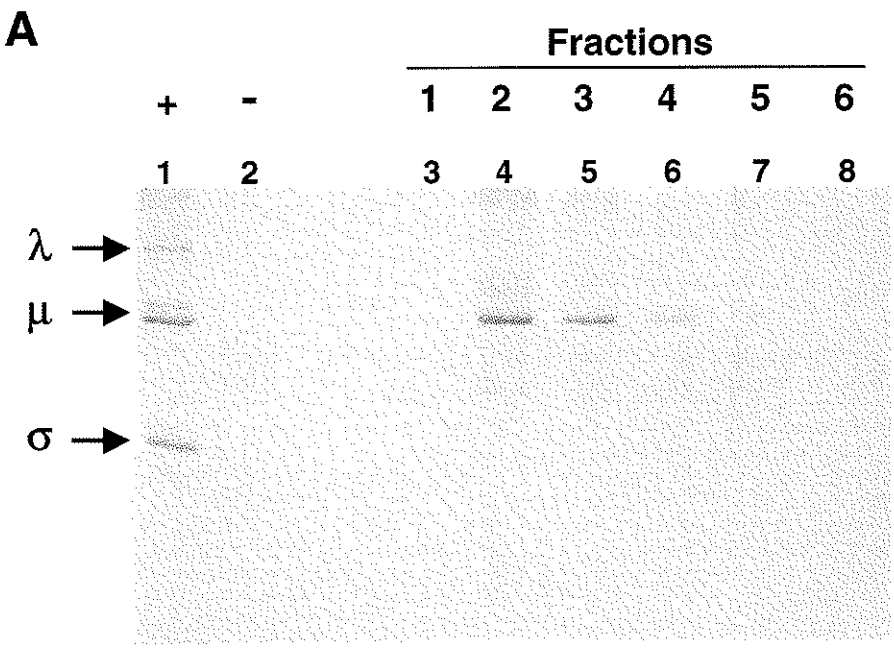

Recombinant
Mock
48 h
72 h
96 h
120 h
Wild-type
kDa
1
2
3
4
5
6
215
95
42.5

**Figure 19. SDS-PAGE analysis of μ2 protein expression by recombinant baculovirus.** Sf9 cells infected with recombinant baculovirus were lysed 48 h, 72 h, 96 h, and 120 h p.i. (lanes 2 to 5). A wild-type baculovirus infection was lysed at 120 h p.i. (lane 6), and mock infected cells were lysed at 48 h p.i. (lane 1), to serve as a control. The cell lysates were analyzed by SDS-PAGE. Black arrows indicate a protein band in the recombinant baculovirus cell lysates that is not found in the control lanes (lanes 1 and 6).

bands were not unique to the lanes that contained recombinant baculovirus cell lysates, but were also visible in the control lanes. A protein band of approximately 85 kDa was detected in recombinant baculovirus cell lysates on a blot detected with an anti-polyHistidine antibody (data not shown). This same band was not visible in the mock infected or wild-type baculovirus infected lanes.

**3.2.4 μ2 protein purification.** Sf9 cells were infected with recombinant baculovirus at an MOI of 5 for subsequent protein purification. The protein was purified using a ni-NTA resin column, and 0.5ml fractions were collected. The fractions were analyzed for the presence of the μ2 protein by SDS-PAGE (Figure 20A). Purified T1L virus was added as a positive control (lane 1), and electrophoresis sample buffer served as the negative control (lane 2). A prominent protein band, which migrated at the same rate as the μ2 protein in the positive control, was seen in column fractions 2, 3, and 4 (lanes 5 to 7, indicated by arrow).

To confirm the presence of purified μ2 protein, Western blot analysis was carried out (Figure 20B). The same controls were used as above, and the presence of μ2 was detected with an anti-μ2 antibody. Column fractions 2, 3, 4, and 5 (lanes 4 to 7) contain a protein band that reacted positively with the antibody (indicated by arrow). The size of the band corresponds to the one seen in the positive control (lane 1). In addition, several protein bands of a larger size were present in column fractions 2 and 3 (lanes 4 and 5).


A
Fractions
+
-
1
2
3
4
5
6
1
2
3
4
5
6
λ
μ
σ

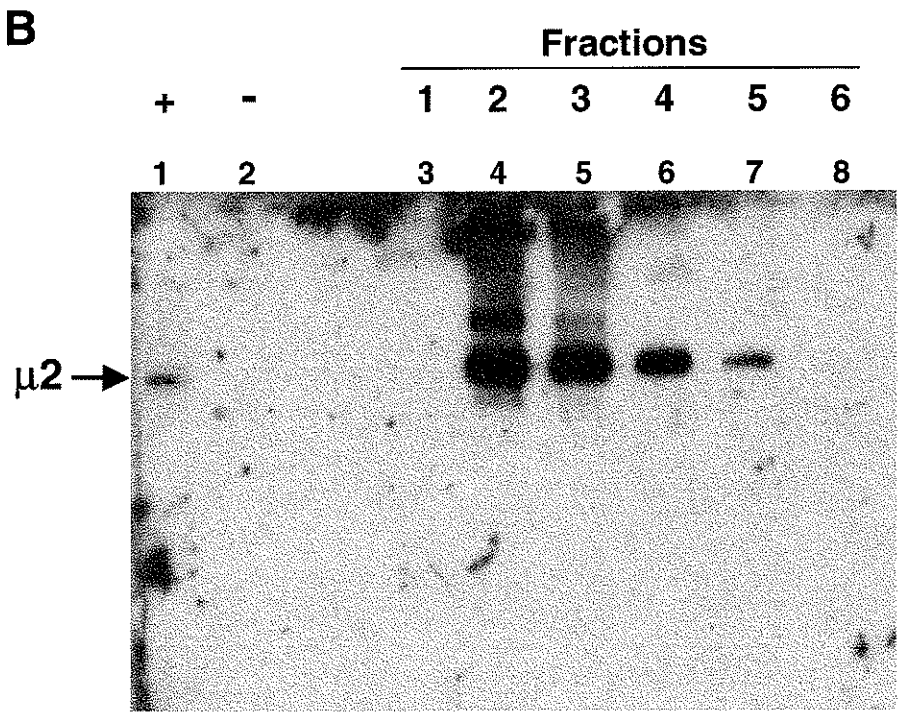

B
Fractions
+
-
1
2
3
4
5
6
1
2
3
4
5
6
μ2

**Figure 20. Analysis of purified μ2 protein.** A. Recombinant baculovirus- infected cell lysates that contained recombinant μ2 protein were subjected to purification by affinity chromatography. Fractions eluted from the column were analyzed by SDS-PAGE for the presence of μ2. SDS-PAGE analysis of the first six fractions is presented here (lanes 3 to 8). Electrophoresis sample buffer was used as a negative control (lane 2), and serotype T1L virus was used a positive control (lane 1). B. The six column fractions analyzed by SDS-PAGE were transferred onto an Immobilon-P membrane for Western blot analysis (lanes 3 to 8). The presence of purified μ2 protein was detected by rabbit anti-μ2 antibody. Lane 1 contains serotype T1L virus positive control, and lane 2 contains the electrophoresis sample buffer negative control.

## 3.3 Analysis of the *ts31.13* temperature sensitive mutant.

### 3.3.1 Screening of *ts31.13* x KC10 and *ts31.13* x H14 viral progeny.

*Ts31.13* was previously crossed with two reassortants, KC10 and H14. All the KC10 gene segments are T1L in lineage, except for the S2 gene segment which came from a T3D parent. The L3, S2, and S3 gene segments of H14 have a T3D electropherotype, while the rest are T1L. Due to the results from the recombination analyses, which indicated a possible lesion in the M3 or S1 gene segment, it was originally believed that the use of the KC10 and H14 reassortants in the place of a T1L wildtype parent would not affect the outcome of the mapping for the *ts31.13* lesion. In order to identify which of the *ts31.13* x KC10 and *ts31.13* x H14 progeny virions were new reassortants, the viral genomes of each of the plaque purified progeny clones were isolated by a cytoextraction method. This involved lysing the cells with detergent so that the nuclear DNA was retained within the lysed cells and did not contaminate the viral genomes. The viral dsRNA was purified away from contaminating proteins by phenol/chloroform extraction and precipitation in 95% ethanol/sodium acetate. The final purified viral RNA was resuspended in Laemmli buffer and analyzed by SDS-PAGE. Reovirus serotypes T3D, the wildtype electropherotype of *ts31.13*, and T1L, the wildtype electropherotype of most of the KC10 and H14 gene segments, were used as standards to which each viral clone gene segment was compared. The parental origin of each gene segment was then determined, and reassortant viruses were identified. Ten reassortants were identified in the *ts31.13* x KC10 cross, and four reassortants were identified in the *ts31.13* x H14 cross (Table 3). This panel of reassortants was considered to be somewhat small in numbers, therefore a new cross was carried out with wildtype T1L.

# Table 3

## Electropherotypes and mapping results for the *ts31.13* x KC10 and *ts31.13* x H14 reassortant crosses

| Clone | L1 | L2 | L3 | M1 | M2 | M3 | S1 | S2 | S3 | S4 | Average EOP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***ts31.13*** | **3** | **3** | **3** | **3** | **3** | **3** | **3** | **3** | **3** | **3** | **0.006979** |
| K51[1] | 3 | 3 | 1 | 3 | 3 | 1 | 1 | 3 | 1 | 3 | 0.025448 |
| K54 | 1 | 3 | 1 | 1 | 1 | 1 | 3 | 3 | 3 | 1 | 0.284642 |
| K14 | 3 | 3 | 1 | 3 | 3 | 1 | 1 | 3 | 3 | 1 | 0.325868 |
| H56[2] | 1 | 3 | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 1 | 0.356553 |
| K27 | 3 | 1 | 1 | 3 | 3 | 3 | 1 | 3 | 3 | 1 | 0.435485 |
| K34 | 1 | 1 | 1 | 1 | 1 | 3 | 1 | 3 | 3 | 1 | 0.474353 |
| H18 | 1 | 1 | 3 | 1 | 1 | 3 | 1 | 3 | 3 | 1 | 0.713846 |
| H48 | 1 | 1 | 3 | 1 | 1 | 3 | 1 | 3 | 3 | 3 | 0.718769 |
| K72 | 1 | 1 | 1 | 1 | 3 | 1 | 1 | 3 | 1 | 1 | 0.737396 |
| K74 | 1 | 3 | 1 | 1 | 1 | 3 | 1 | 3 | 3 | 1 | 0.965768 |
| K35 | 1 | 3 | 1 | 3 | 1 | 1 | 1 | 3 | 3 | 1 | 1.067236 |
| K61 | 3 | 3 | 1 | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 1.179985 |
| **H14** | **1** | **1** | **3** | **1** | **1** | **1** | **1** | **3** | **3** | **1** | **1.193050** |
| **KC10** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **3** | **1** | **1** | **1.407775** |
| H21 | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 1 | 1.724450 |
| K58 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 1 | 3 | 11.580965 |

[1]Reassortants designated with 'K' come from the *ts31.13* x KC10 cross

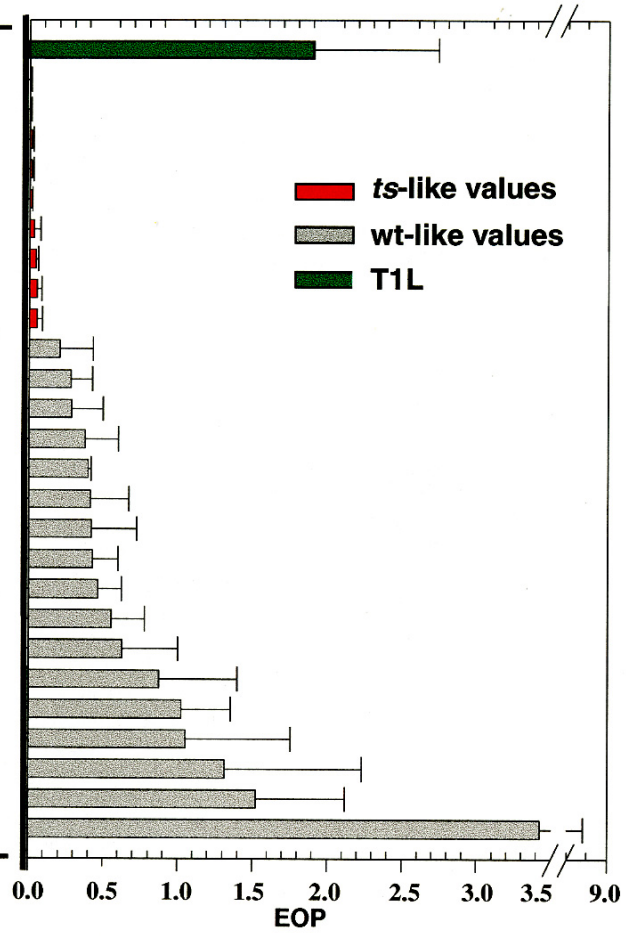
[2]Reassortants designated with 'H' come from the ts31.13 x H14 cross

**3.3.2 Generation of *ts.31.13* x T1L reassortants.** Wildtype T1L virus was crossed with a concentrated stock of *ts.31.13* at a multiplicity of infection (MOI) of 10:10, as outlined in Materials and Methods. Post infection, the viral supernatant was diluted, and used to infect P100 tissue culture plates, which were overlaid with agar. 72 plaques were picked and each plaque was amplified twice in L929 cells.

**3.3.3 Screening of *ts31.13* x T1L viral progeny.** In order to identify reassortants among the 72 viral clones generated from the *ts31.13* x T1L cross, the viral progeny was subjected to cytoextraction and purification by phenol/chloroform and ethanol/sodium acetate. The purified dsRNA viral genomes were resuspended in Laemmli buffer and analyzed by SDS-PAGE. Wildtype T1L and T3D viral genomes were used as standards to aid in assignment of electropherotypes. 25 reassortants were identified among the viral progeny (Figure 21). However, the electropherotypes of reassortants number 17, 18, 21, and 36 could not be fully assigned as two attempts at analysis by SDS-PAGE failed to clearly identify parental origin.

**3.3.4 Screening of reassortants for temperature sensitive phenotype.** The accepted method for identification of temperature sensitivity of a reovirus is to carry out an efficiency of plating (EOP) assay (Ramig and Fields 1983, Coombs 1998a). The EOP value is determined by dividing the viral titer obtained at a non-permissive temperature, such as 39°C, by the viral titer obtained at a permissive temperature, such as 32°C. Under non-permissive temperature conditions, a temperature sensitive phenotype would become evident, resulting in a lower viral titer. This in turn would result in a low EOP value. A typical EOP value for a temperature sensitive virus is 0.001 or lower. A wildtype virus would grow equally well at both the non-permissive and the permissive

| Clone | L1 | L2 | L3 | M1 | M2 | M3 | S1 | S2 | S3 | S4 | EOP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| T1L | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1.8966 |
| *ts31.13* | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0.0070 |
| 7 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0.0054 |
| 43 | 3 | 3 | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 3 | 0.0139 |
| 34 | 3 | 3 | 3 | 3 | 3 | 1 | 3 | 1 | 3 | 3 | 0.0142 |
| 13 | 3 | 3 | 1 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 0.0150 |
| 25 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0.0323 |
| 29 | 3 | 3 | 1 | 3 | 3 | 3 | 1 | 3 | 3 | 3 | 0.0432 |
| 33 | 3 | 3 | 1 | 3 | 3 | 3 | 1 | 3 | 3 | 3 | 0.0505 |
| 24 | 1 | 3 | 1 | 3 | 3 | 3 | 1 | 3 | 1 | 3 | 0.0517 |
| 39 | 1 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 0.2046 |
| 9 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 1 | 0.2798 |
| 18 | 1 | 1 | 1 | 1 | 3 | 1 | 1 | 3* | 1 | 1 | 0.2870 |
| 59 | 3 | 1 | 1 | 1 | 3 | 1 | 1 | 3 | 3 | 1 | 0.3761 |
| 23 | 1 | 3 | 1 | 1 | 1 | 3 | 3 | 3 | 3 | 3 | 0.3964 |
| 72 | 3 | 3 | 1 | 3 | 1 | 3 | 1 | 3 | 3 | 3 | 0.4111 |
| 61 | 3 | 3 | 1 | 1 | 1 | 1 | 3 | 3 | 3 | 3 | 0.4183 |
| 8 | 3 | 3 | 1 | 3 | 1 | 1 | 1 | 3 | 1 | 1 | 0.4266 |
| 36 | 1 | 3 | 1* | 1 | 1 | 3 | 1 | 1 | 3 | 3 | 0.4642 |
| 5 | 1 | 3 | 1 | 3 | 1 | 3 | 1 | 1 | 1 | 1 | 0.5537 |
| 6 | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 0.6260 |
| 70 | 3 | 3 | 1 | 3 | 1 | 3 | 1 | 1 | 3 | 3 | 0.8731 |
| 2 | 1 | 1 | 1 | 3 | 1 | 1 | 1 | 3 | 3 | 1 | 1.0230 |
| 56 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 1 | 3 | 1.0517 |
| 17 | 1 | 1 | 1 | 3 | 3* | 1 | 3 | 1 | 3 | 3 | 1.3136 |
| 21 | 3 | 1 | 3* | 1 | 1 | 3 | 1 | 3* | 1 | 1 | 1.5251 |
| 19 | 1 | 3 | 1 | 3 | 1 | 1 | 1 | 1 | 3 | 3 | 3.4234 |



temperatures. Its EOP would be approximately equal to 1. Typically, an EOP value is not exactly 1 or 0.001. A variation of one order of magnitude is deemed acceptable by reovirus researchers. The reassortants generated by the *ts31.13* x KC10 cross were analyzed by an EOP assay carried out at 39°C, the non-permissive temperature, and 33.5°C, the permissive temperature. This EOP assay was repeated three more times. EOP values for reassortants 51, 61, and 72 were successfully obtained three times. One temperature sensitive reassortant was identified in this group (Table 4). An EOP assay at 39°C and 33.5°C for *ts31.13* x H14 reassortants was repeated three times. No temperature sensitive reassortants were identified (Table 4). An EOP assay for *ts31.13* x T1L reassortants was carried out once at 32 and 39°C, and twice at 33.5 and 39°C. A 32°C permissive temperature was initially chosen due to concern that virus growth might be slightly attenuated at 33.5°C. The permissive temperature was changed to 33.5°C upon repetition of the assay as tests determined it was a safe temperature and it allowed the assay to be concluded in a shorter time frame due to faster virus growth (results not shown). Eight reassortants were identified as being temperature sensitive (Figure 21).

**3.3.5 Mapping of the temperature sensitivity of *ts31.13*.** The identified reassortants were arranged into two panels. The top panel includes the reassortants that exhibit the temperature sensitive phenotype, the bottom panel of reassortants exhibit the wildtype phenotype (Table 3 and Figure 21). A reassortant was deemed to be temperature sensitive if its average EOP value (an average of all its EOP assay trials) was within one order of magnitude of the *ts31.13* average EOP value. If a reassortant had an average EOP value that was within one order of magnitude of the wild-type average EOP value, it was considered to have a wildtype phenotype. Frequently, this type of

# Table 4

## Efficiency of plating results for the *ts31.13* x KC10 and *ts31.13* x H14 reassortants

| Clone | Average EOP | Standard Error |
|---|---|---|
| KC10 | 1.407780 | 0.768643 |
| 14 | 0.325868 | 0.210740 |
| 27 | 0.435485 | 0.118486 |
| 34 | 0.474353 | 0.068945 |
| 35 | 1.067240 | 0.633045 |
| 51 | 0.025448 | 0.012642 |
| 54 | 0.284642 | 0.129408 |
| 58 | 11.58100 | 9.688951 |
| 61 | 1.179990 | 0.994310 |
| 72 | 0.737385 | 0.672989 |
| 74 | 0.965768 | 0.375349 |
| H14 | 1.193050 | 0.422363 |
| 18 | 0.713847 | 0.165224 |
| 21 | 1.724450 | 1.568090 |
| 48 | 0.718769 | 0.164474 |
| 56 | 0.356553 | 0.165753 |

arrangement of EOP values allows the identification of the gene segment that contains the temperature sensitive lesion. Typically, one gene segment in the temperature sensitive panel will come only from the ts-like parent, while in the wild-type panel, this same gene segment is only from the wild-type parent. This clearly indicates that the temperature sensitive lesion is located in this gene segment. However, this type of clear mapping result is not seen in the *ts31.13* mapping tables (Table 3 and Figure 21). There is no gene segment that is clearly T3D in one panel, and T1L in the other. Despite this, a pattern was identified. In the temperature sensitive panel, the L2, M1, M2, and S4 gene segments were T3D. In the wildtype panel, there were cases in which three, two, or one of the gene segments were T3D, but not all four.

**3.3.6 Statistical analysis of the *ts31.13* mapping.** The pattern evident in the above mapping experiment indicated that four gene segments, L2, M1, M2, and S4, were associated with the temperature sensitive phenotype seen in *ts31.13* and the ts-like reassortants. Non-parametric tests were carried out for all gene distributions on EOP values from 3 EOP assays of the *ts31.13* X T1L reassortants, 4 EOP assays of *ts31.13* X *KC10* reassortants, and 3 EOP assays of *ts.31.13* X *H14* reassortants to determine whether the gene electropherotype distributions were significant. Nonparametric tests were used as a mapping table does not have a normal distribution (i.e. a bell-shaped curve). Instead, a curve of a mapping result may contain two peaks, and the wildtype peak would be larger due to a greater amount of wildtype-like reassortants. A probability value (P-value) was calculated for each gene segment that described the probability that its particular electropherotype distribution could have occurred by chance. This

information can be used to indicate whether a particular gene segment may contribute to the temperature sensitive phenotype. P-values of <0.05 were considered to be significant.

**3.3.6.1 Distribution tests for individual gene segments.** In order to test the distribution of electropherotypes, a Wilcoxon rank sum test and a Kruskal-Wallis test were carried out, using the individual and unaveraged (raw) EOP values, that looked at each gene one at a time. A Wilcoxon rank sum test is a nonparametric test used to analyze the distribution of paired data, such as the distribution of the T1L and T3D gene segments. As its name implies, it ranks the subject by the degree of phenotype. A Kruskal-Wallis test is also nonparametric, and is used to compare the averages of several independent samples. The three parent crosses were combined together, and with a Bonferoni correction the P-value considered to be significant in this case was <0.005. A Bonferoni correction was utilized as it sets more stringent standards for individual comparisons to prevent apparently significant differences from being obtained by accident in the comparisons. The results of this test are in Table 5. The L1, L2, M1, M2, S1, and S4 gene segments were proved highly significant. The same test was also performed using the averages of the EOP values (Table 6). The results are similar to that of the previous test, with the exception that the M1, S1, and S4 gene segments are not highly significant.

**3.3.6.2 Collective distribution tests.** To test all of the gene segments together, multiple logistic regression analysis was carried out on the EOP values (Table 7). Multiple regression analysis determines which of the various independent variables has a genuine relationship with the dependent variable, the strength and nature of the relationship, and it makes sense of the tangle of influences that may be present. Logistic

# Table 5

## Distribution analysis results for the raw EOP values of *ts31.13* reassortants[a]

| | T1L Genes | | T3D Genes | | |
|---|---|---|---|---|---|
| Gene | Median | Range | Median | Range | P-value |
| L1 | 0.541 | 0.007-9.590 | 0.088 | 0.002-1.974 | <0.0001 |
| L2 | 0.848 | 0.112-2.447 | 0.185 | 0.002-9.590 | <0.0001 |
| L3 | 0.348 | 0.002-9.590 | 0.094 | 0.003-1.974 | 0.3599 |
| M1 | 0.515 | 0.007-2.447 | 0.090 | 0.002-9.590 | <0.0001 |
| M2 | 0.569 | 0.212-9.590 | 0.035 | 0.002-2.353 | <0.0001 |
| M3 | 0.515 | 0.004-9.590 | 0.185 | 0.002-1.974 | 0.0275 |
| S1 | 0.395 | 0.003-9.590 | 0.120 | 0.002-2.353 | <0.0001 |
| S2 | 0.569 | 0.004-9.590 | 0.212 | 0.002-1.974 | 0.0400 |
| S3 | 0.565 | 0.022-2.447 | 0.235 | 0.002-9.590 | 0.0132 |
| S4 | 0.590 | 0.112-2.447 | 0.124 | 0.002-9.590 | 0.0002 |

[a]The *ts31.13* x T1L, x KC10, and x H14 crosses were analyzed together

# Table 6

## Distribution analysis results for the averaged EOP values of ts31.13 reassortants[a]

| | T1L | | T3D | | |
|---|---|---|---|---|---|
| Gene | Median | Range | Median | Range | P-value |
| L1 | 0.728 | 0.052-11.581 | 0.326 | 0.005-1.724 | 0.0033 |
| L2 | 1.023 | 0.287-11.581 | 0.341 | 0.005-3.423 | 0.0009 |
| L3 | 0.435 | 0.005-11.581 | 0.491 | 0.007-1.724 | 0.5881 |
| M1 | 0.716 | 0.205-11.581 | 0.357 | 0.005-3.423 | 0.0284 |
| M2 | 0.919 | 0.285-11.581 | 0.052 | 0.005-1.314 | <0.0001 |
| M3 | 0.737 | 0.014-11.581 | 0.423 | 0.005-1.724 | 0.1295 |
| S1 | 0.554 | 0.014-11.581 | 0.282 | 0.005-1.724 | 0.046 |
| S2 | 0.873 | 0.014-3.423 | 0.415 | 0.005-11.581 | 0.1143 |
| S3 | 0.737 | 0.025-11.581 | 0.415 | 0.005-3.423 | 0.1126 |
| S4 | 0.634 | 0.280-1.897 | 0.396 | 0.005-11.581 | 0.0363 |

[a]The *ts31.13* x T1L, x KC10, and x H14 crosses were analyzed together

# Table 7

## Multiple logistic regression results for the raw EOP values of ts31.13 reassortants[a]

| Gene | Point Estimate | Confidence Limits | P-value |
|---|---|---|---|
| L1 | 3.382 | 0.177-64.508 | 0.0749 |
| L2 | 19.634 | 0.600-642.992 | 0.4180 |
| L3 | 1.629 | 0.183-14.531 | 0.6619 |
| M1 | 1.461 | 0.042-51.023 | 0.8344 |
| M2 | 224.155 | 11.516->999.999 | 0.0004 |
| M3 | 0.048 | 0.001-1.955 | 0.1082 |
| S1 | 1.268 | 0.182-8.837 | 0.8103 |
| S2 | 13.043 | 0.026->999.999 | 0.4184 |
| S3 | 0.237 | 0.009-5.905 | 0.3799 |
| S4 | 33.106 | 1.889-580.279 | 0.0166 |

[a]The high EOP values were focused on

regression tests the probability of a variable to belong to one category where it is seen as either being present or absent. In this case, the categories are "high" or "not high", and each reassortant belongs to either one category or the other. A backward elimination procedure was employed in which gene segments were eliminated from the set of ten. If an EOP value was >0.06, it was set to be "high". All other EOP values (i.e. ≤0.06) were set to be "not high". The significance was set at P-value <0.05. The M1 gene segment was eliminated first, due to a high P-value. The L2 and M3 gene segments were deemed borderline significant, and the M2 and S4 gene segments were considered significant. In the context of the mapping experiment, the L2, M2, M3 and S4 gene segment electropherotype distributions are independently significant according to this test. This means that they give independent information that is used to separate the high EOP values (wild-type-like) from the low EOP values (ts-like).

In a multiple regression analysis in which the logarithm of EOP values was used, the S2 gene segment was considered borderline significant, while the L2, M2, and S4 gene segments were significant (Table 8). The same test was repeated using the averages of the EOP values (data not shown). The L1, L2, M1, M2, and S4 gene segments were significant. The S1 gene was borderline significant. When the same test was repeated once more, this time only the significant genes were included: L1, L2, M1, M2, S1, and S4 (Table 9). The focus was on the low EOP values and the raw EOP values were used. The L2, M2, and S4 gene segments remained significant. Their influence on the temperature sensitive phenotype was found to be additive. The M2 gene segment contributed the most, with 113 to 1 odds that a low EOP would be generated if the M2 gene had a T3D electropherotype (Table 10). The L2 gene segment added 18 to 1 odds,

# Table 8

## Multiple regression results for the logarithm EOP values of ts31.13 reassortants[a]

| Gene | Parameter Estimate | Standard Error | P-value |
|---|---|---|---|
| L1 | 0.344 | 0.289 | 0.2355 |
| L2 | 1.254 | 0.313 | 0.0001 |
| L3 | 0.158 | 0.277 | 0.5678 |
| M1 | -0.220 | 0.321 | 0.4935 |
| M2 | 2.029 | 0.252 | <0.0001 |
| M3 | -0.036 | 0.258 | 0.8886 |
| S1 | 0.189 | 0.293 | 0.5194 |
| S2 | 0.512 | 0.330 | 0.1233 |
| S3 | -0.165 | 0.294 | 0.5754 |
| S4 | 0.558 | 0.252 | 0.0285 |

[a]The high EOP values were focused on

# Table 9

## Multiple regression results for the raw EOP values of select genes of *ts31.13* reassortants[a]

| Gene | Point Estimate | Confidence Limits | P-value |
|---|---|---|---|
| L1 | 4.689 | 0.832-26.424 | 0.0798 |
| L2 | 13.065 | 1.085-157.365 | 0.0430 |
| M1 | 0.450 | 0.050-4.056 | 0.4767 |
| M2 | 135.711 | 13.825->999.999 | <0.0001 |
| S1 | 0.608 | 0.137-2.697 | 0.5125 |
| S4 | 9.904 | 1.918-51.131 | 0.0062 |

[a]The low EOP values were focused on

# Table 10

## Odds ratio estimates calculated for significant genes from the multiple logistic regression analysis of *ts31.13* reassortants[a]

| Gene | Point Estimate | Confidence Limits | P-value |
|---|---|---|---|
| L2 | 17.557 | 1.913-161.172 | 0.0113 |
| M2 | 112.658 | 13.400-947.181 | <0.0001 |
| S4 | 7.870 | 1.892-32.737 | 0.0046 |

[a]The original analysis used the unaveraged EOP values and focused on the low values

and the S4 gene segment added 8 to 1 odds. The total odds of a temperature sensitive phenotype being exhibited in the presence of all three gene segments, if they are of a T3D origin, was 139 to 1.

**3.3.6.3 Role of the M1 gene segment in the mapping experiment.** In the mapping experiment described above, four gene segments appeared to be involved in the separation of the temperature sensitive EOP panel from the wild-type EOP panel. These included the L2, M1, M2 and S4 gene segments. Statistical tests revealed that the L2, M2, and S4 gene segments were significant in their contribution to the temperature sensitive phenotype. To better understand the role of the M1 gene segment in the mapping distribution, a chi squared analysis was employed to determine if the M1 gene segment correlates with any of the other gene segments (Table 11). A P-value of 0.05 was considered significant. The M1 gene segment was found to highly correlate with the L1, L2, and M2 gene segments. Other significant correlations included the M3, S1, S3, and S4 gene segments.

# Table 11

## Chi-square analysis of the EOP values of *ts31.13* reassortants: M1 gene vs all other genes

| Genes | Chi-Square Value[a] | P-value[a] |
|---|---|---|
| L1 | 43.820 | <0.0001 |
| L2 | 42.127 | <0.0001 |
| L3 | 0.101 | 0.7504 |
| M2 | 22.455 | <0.0001 |
| M3 | 5.044 | 0.0247 |
| S1 | 4.604 | 0.0319 |
| S2 | 2.036 | 0.1537 |
| S3 | 10.101 | 0.0015 |
| S4 | 8.274 | 0.0040 |

[a]Calculated with unaveraged EOP values

# 4. DISCUSSION

## 4.1 Characterization of the μ2 protein by mass spectrometry

Results indicate that translation of minor reovirus core protein μ2 initiates at the first AUG codon. This corresponds to currently known reovirus translation events and definitively settles the dispute of the M1 transcription start site. The first AUG codon is in the context of a strong eukaryotic translation initiation sequence, while the sequence that surrounds the second AUG is weak (Kozak 1981). Each of the reovirus proteins initiate at the first start codon identified in the RNA sequence, with the exception of the σ1s protein which initiates from a downstream start codon on the S1 RNA segment (the only RNA segment to encode two proteins). Roner *et al* indicated that a 78 kDa protein that resulted from initiation at the second AUG codon is more likely to occur *in vivo* (Roner *et al* 1993). In their *in vivo* studies, the M1 gene was cloned into various vectors and the region upstream of the M1 coding region was varied. Such addition of unrelated sequences may disrupt normal translation initiation events *in vivo* (Zou and Brown 1996, and references therein). Translation initiation may be modulated by folding and/or host proteins and any change in the size and/or content of the upstream sequence may result in translation initiation occurring at the second AUG codon. In addition, *in vitro* translation of M1 transcripts routinely generates lower molecular weight products (Brentano *et al* 1998, Roner *et al* 1993, Zou and Brown 1996). *In vitro* studies carried out by Roner *et al*, that involve the use of a rabbit reticulocyte lysate system, indicated that translation initiation could occur from either of the two AUG codons. Rabbit reticulocyte lysates are

known to initiate translation at internal sites not used under physiological conditions *in vivo* (Roner *et al* 1993).

The mass spectra identified peptide fragments that, when interpreted as a whole, indicated that the actual molecular weight of the serotype T1L μ2 found in virions is closer to its theoretical molecular weight of 83 kDa. Additionally, the immunoblot in Figure 13 clearly indicated that only the 83kDa form of μ2, which results from translation initiation at the first AUG codon, is produced in infected L929 cells. Furthermore, the immunoblot revealed that the μ2 protein present in purified T1L and T3D virions, and in T1L and T3D infected cells, are the same size, indicating no serotypic differences in translation of the M1 gene. No detectable μ2 proteins of smaller molecular weight were present in either the purified virions or the infected cells. The Western blot does contain bands of higher molecular weight in the cell lysate lanes that migrate slower than the 83kDa μ2 protein. Since the uninfected cell lysate contains the same size band, it is highly likely that the band corresponds to a host cell protein that cross-reacts with the anti-μ2 antibody. In addition, T3D-infected cells appear to contain an anti-μ2 reactive band slightly larger than the 83 kDa μ2 protein band that does not appear in the T1L infected cell lysates, even with longer film exposures. Although it can be speculated that this protein is a differently modified form of μ2, its identity is yet to be determined.

There is strong evidence that post-translational modifications occur in reovirus replication. The μ1 protein is myristoylated (Nibert *et al* 1991), and the σ3 protein has been recently shown to be acetylated (Mendez *et al* 2002). The avian reovirus counterpart of μ2, the μ1(μA) protein, is glycosylated (Varela *et al* 1996), and several rotavirus proteins are post-translationally modified (Both *et al* 1994). This data, along with the

observation that the amino-terminus is blocked to Edman degradation, indicate that a post-translational modification of the μ2 protein is highly likely. The above MS and MS/MS results indicate that the μ2 amino terminus is post-translationally cleaved at $Met_1$ and the next amino acid residue (alanine) is acetylated. Methionine cleavage and amino-terminal acetylation are both extremely common post-translational modifications present on a wide variety of proteins in eukaryotes (Polevoda and Sherman 2000).

In conclusion, the results indicate that the μ2 protein found in reovirus virions initiates from the first AUG codon in the viral M1 gene and is post-translationally modified by cleavage of the N-terminal methionine residue and the addition of an acetyl group. In addition, results indicate that within infected cells the reovirus M1 gene appears to exclusively be translated from the first AUG and most μ2 is not incorporated into virions. These results contradict reports that μ2 initiates from a second in-frame AUG codon, and add to our understanding of the structure of μ2. They will also aid future researchers to uncover the role of μ2 in the reovirus life cycle, which, in turn, will contribute to increased knowledge of virus-host cell interactions. This is essential since virus-host cell interaction is a poorly understood process.

## 4.2 Expression and purification of the μ2 protein

In order to allow further characterization of the μ2 protein in its purified form, a baculovirus protein expression system was employed. Although analysis of the unpurified cell lysates infected with recombinant baculovirus proved inconclusive, column purification of infected cell lysates yielded definitive results. The results indicate that a protein band of approximately 80 kDa is present in column fractions from Sf9 cells

infected with a recombinant baculovirus containing the reovirus T1L M1 gene. Although the native μ2 protein was reported to migrate at 72 kDa in SDS-PAGE analysis (Nibert and Schiff 2001), it may be reasonably argued that the μ2 fusion protein, which contains a poly-histidine tag, would have a different migration pattern. In addition, Western blot analysis revealed that this protein band is of approximately the same molecular size as the band in the positive control (Figure 20B). Collectively, the results indicate that the μ2 protein is expressed, and that the correct size of the protein is synthesized. However, the anti-μ2 antibody also reacted with other protein bands (Figure 20B). These reactions are likely with aggregates of μ2, or possibly μ2 proteins that contain alternative modifications due to synthesis within Sf9 cells. In order to definitively prove the identity of the expressed protein, and the other proteins that reacted with the anti-μ2 antibody, their amino acid sequences need to be determined.

In conclusion, the serotype T1L reovirus μ2 protein was successfully expressed in a baculovirus system and purified. The protein can now be utilized in studies whose goal is to increase our understanding of the optimal activity parameters, the structure, and most importantly, the function of μ2. Since μ2 was not found to be homologous to any other known proteins in protein databases, an opportunity is at hand to gain insight into a possibly new, unexplored class of proteins. Such a feat would increase our current knowledge of protein biochemistry, and biological processes in general.

## 4.3 *Ts31.13* and the M1 gene segment

Protein analysis is one method by which the function of μ2 can be determined. Genetic analysis is another. When a project was undertaken to determine the location of the mutation(s) in the temperature sensitive (ts) mutant, *ts31.13*, the lesion(s) were not successfully mapped to any one gene segment. Due to the large size of the reassortant set, it is highly unlikely that this unclear mapping resulted due to a lack of data. The genetic mapping was followed by statistical analysis. Taken together, the multiple regression analyses indicate that gene segments L2, M2, and S4 are significant in their independent contribution to the ts phenotype. This implies that mutations are present in these gene segments. To identify the nature and location of these mutations, the genes in question must be sequenced.

The gene segment first eliminated from the possibility of any contribution to the ts phenotype was the M1 gene segment. Despite this, a four-gene pattern is evident in the mapping table. The question remains whether the M1 gene somehow interacts with any of the other three genes? The statistical data appears to support this hypothesis. The M1 gene correlated highly with the L1, L2, and M2 gene segments, which encode the λ3, λ2, and μ1 proteins, respectively. This type of interaction, in which the gene in question does not contribute significantly to the phenotype, has not been previously reported for reovirus. It is not clear at this point whether the perceived interaction is at the gene or at the protein level. Site-directed mutagenesis might be able to determine the nature of any interaction. However, although one attempt has been made to design a reverse genetics system for reovirus, it appears to be extremely cumbersome and is not generally favored (Roner *et al* 2001).

The highly statistically significant interaction detected between the M1 gene and the L1, L2, and M2 genes may have its roots in protein interactions. The μ2 protein is known to contribute to viral inclusion formation. This implies a role in viral assembly. Scientists now understand that assembly of proteins to form a virion is a largely sequential process. It seems that several proteins congregate, followed by the addition of other proteins, and so on. The μ2 protein could be a link in this assembly line responsible for the aggregation of the λ3, λ2, and μ1 proteins. As illustrated in Figure 1, the μ2 protein is located inside the viral core, most probably at the vertices (Coombs 1998b, Dryden *et al* 1998). λ3, the RNA-dependent RNA polymerase (RdRp), is also located within the core at the vertices. The λ2 protein in located at the vertices and forms the spike. The μ1c protein is located in the capsid and is in contact with the λ2 spike. There is some evidence that λ2 and μ2 interact, and that μ2 is actually located within the pocket inside the vertices (Hazelton and Coombs 1999). Due to their proximity in the virion particle, it is highly probable that these four proteins interact during the assembly process.

An alternative protein interaction that may be reflected in the statistical data is the process of transcription or replication. During transcription, the nascent mRNA is synthesized within the core by λ3, the RdRp, and most probably by μ2, the putative RdRp co-factor. It is then extruded through the spike, where primary transcripts acquire a cap (Luongo *et al* 2000). It is clear that transcription involves interactions between the μ2, λ3, and λ2 proteins. Any role that μ1 would play in transcription and replication is unclear at this time. It is known that the replicase particle responsible for replication is not a closed shell. It is highly likely that other proteins, such as host cell proteins or

possibly μ1, are allowed to interact with the core proteins to aid in the synthesis of the nascent genome.

A genetic interaction is also possible, but is less likely to occur. Although it is known that the viral RNA is contained within the core, not much is known about how the gene segments are packaged inside. It is possible that during core assembly specific interactions occur between gene segments. Alternatively, gene interaction may occur during the transcription or replication processes.

Although initially undertaken as a separate project, this study contributed to the growing data on the M1 gene and the μ2 protein it encodes. Statistical analysis tested the hypothesis that an interaction of four gene segments was responsible for the ts phenotype and helped to delineate the role of these gene segments. To determine if a genetic or a protein interaction occurs that involves the M1 gene segment or the μ2 protein, more needs to be understood about reovirus protein functions. Some of these questions may be answered by further study of the μ2 protein (see section 4.5).

Mammalian reovirus has frequently served to act as a model for all viruses that infect eukaryotic cells. Any research that increases our current knowledge of reovirus genetic or protein interactions will significantly impact the study of the assembly and replication of other viruses. When viral assembly is understood at the molecular level, therapies can be developed to target viral replication. This has enormous implications for efforts to combat viral disease.

## 4.4 Conclusions

In this study, mass spectrometric analysis was carried out that proved reovirus μ2 protein initiates from the first AUG codon, and contradicted past reports that initiation occurs at the second in-frame AUG codon. The post-translational modification of the amino-terminus of μ2 was identified, and insight was gained on the molecular size of the protein. In addition, western blot analysis indicated that the μ2 protein found in virions is the same molecular size as the one located within infected host cell lysates, and that there is a potential serotypic difference in the types of μ2 protein produced in T1L and T3D-infected cells. The analysis also suggested that most of the synthesized μ2 protein is not incorporated into virions, but remains within the host cell. All of these results can now be used to help uncover the role that μ2 plays in viral replication and virus-host cell interactions.

Reovirus μ2 protein expression was carried out with the use of a baculovirus expression system. More importantly, the μ2 protein was purified with the aid of a poly-histidine tag. This is a first since no reports of the successful expression and purification of μ2 are found in the literature. Previously unfeasible steps can now be taken to uncover the structural and functional properties of μ2.

Lastly, the temperature sensitivity phenotype of the *ts31.13* mutant virus was mapped to three gene segments, L2, M2, and S4. The M1 gene segment, which encodes the μ2 protein, was found to significantly interact with the L1, L2, and M2 gene segments. This type of interaction has not been previously reported for reovirus. As a result, this study has indicated a new avenue of reovirus analysis that can be undertaken,

which has the potential to help us understand the protein interactions that occur during the viral life cycle.

## 4.5 Future directions

In many cases, the solution to a problem uncovers more questions. This study is no exception. Some of the results reported here indicate unexpected areas that require further analysis, while others require routine follow-up studies. One such study would be to identify the protein band that reacted with the anti-μ2 antibody, that appeared in the cell lysates of serotype T3D-infected cells, but not in the T1L-infected cell lysates (Figure 13B). Mass spectrometric analysis is a likely tool to aid in the determination of the amino acid sequence of this protein, and ultimately, its identity. Another such study involves the definitive determination of the size of μ2. The whole μ2 protein can be analyzed by a MALDI QqTOF mass spectrometer, which has the capacity to detect molecules with larger masses. However, high accuracy at the 83 kDa range is still difficult to achieve with this machine. A second possibility is to employ alternate enzymatic cleavage strategies of μ2 to identify a complete set of amino acid sequences.

The Western blot portrayed in Figure 13B suggests that more μ2 remains in host cells than is incorporated into virion particles. Pulse-chase studies can be carried out to determine the percentage of μ2 that is incorporated into virions, as well as the location of the μ2 that remains within the host cell. In addition, pulse-chase experiments can be used to confirm the acetylation of μ2 that was detected by MS in this study, and to uncover other possible post-translational modifications.

MS and MS/MS can be used to determine the amino acid sequences and identify any mutations or alterations in post-translational modification of the recombinant μ2 protein. However, mutations in the μ2 amino acid sequence are not expected since only conservative mutations were identified in the cloned M1 cDNA nucleotide sequence. In addition, it would be beneficial to determine if the infection conditions can be improved upon in order to boost the level of protein expression. This could include the optimization of the viral titer used to infect the cells, and the use of alternate insect cell lines. It also would be beneficial to determine whether protein expression can be carried out in mammalian cells to lay to rest any doubts of proper post-translational modification.

Subsequent to positive identification, the expressed recombinant protein can be employed in a variety of studies. The first logical analysis to perform would be an assay of enzymatic activity. The μ2 protein has been shown to contain NTPase activity (Noble and Nibert 1997), the ability to bind single-stranded and double-stranded RNA (Brentano *et al* 1998), and may contain helicase activity. An electrophoretic mobility shift assay, or a UV-crosslinking assay would determine if the expressed μ2 protein is still able to bind RNA. A dsRNA unwinding assay would determine if the protein retains helicase function, and hydrolysis of NTP to NDP would indicate NTPase function. These analyses will help to establish the feasibility of future functional studies.

A second avenue of analysis would be the determination of protein structure. X-ray crystallography is a tool used to delineate the structure of many proteins. It is highly likely that μ2 will yield to this type of analysis. The interaction of μ2 with other core proteins as well as viral RNA is a little known process. Co-crystallization with reovirus

RNA is one study that can be attempted to understand the interaction of μ2 with the virus genome. Protein-protein interactions, however, need to be analyzed by another approach.

Since baculovirus expression has proved successful for μ2, as well as other reovirus proteins, co-expression of μ2 with other core proteins can be attempted. Any protein interactions can be tested using studies such as UV cross-linking or enzymatic cross-linking. Figure 13 suggests that the majority of the synthesized μ2 protein is not included in the assembled virions but is left behind in the host cell cytoplasm. The interaction of the protein μ2 with host proteins can be tested using a surface plasmon resonance system designed to carry out binding assays, such as the one produced by Biacore. The μ2 protein can be immobilized on the sample surface and uninfected, as well as infected cell lysates, can be floated across this surface. The affinity, kinetics, specificity, and level of the binding event can be measured. Any unidentified protein interaction can be subjected to analysis by a linked MALDI QqTOF mass spectrometer, and identity of the proteins involved can be determined. The Biacore system has been deemed to be an accepted method to identify and measure protein interaction, as evidenced by recently published manuscripts (Ahmad *et al* 2002, Katahira *et al* 2001, Okumura *et al* 2001). Since μ2 has been determined to play a role in the formation of viral inclusions (Coombs 1996), experiments that test the interactions between μ2 and any host proteins would help to uncover its intracellular function. This type of study would also aid researchers to understand the role of μ2 in viral assembly.

As mentioned in section 4.3, the mutations in *ts31.13* need to be identified. This can be accomplished by sequencing the three gene segments that have been shown to contribute to the temperature sensitive phenotype, L2, M2, and S4. To further

characterize the *ts31.13* mutant, several studies may be undertaken. The growth curve of the mutant can be compared to that of the wild type virus to determine if there are differences in their respective growth rates. Electron microscopy can be utilized to compare the synthesis of the virion and core particles between the mutant and the wild-type virus. Additionally, *in situ* metabolic labeling of viral RNA and proteins can be employed to identify differences in transcription and replication.

Reovirus provides a simple model to study the complex interaction of multiple gene products that give rise to a single well-characterized phenotype. In most biological systems, such as mammalian cells, this is very difficult to study. Data analysis can be extremely complicated for the determination of processes such as signal transduction, or the etiology of a disease such as cancer and Alzheimer's disease. In contrast, reovirus is a relatively uncomplicated system, and interpretations of multiple gene product interaction studies can be reasonably straightforward. Techniques such as DNA microarrays can be used to monitor the expression of the reovirus gene segments, and western blot analysis can be used to measure protein quantity. Protein-protein interactions can be studied with a yeast two-hybrid system, as well as the Biacore system. To sum up, the ts reovirus can provide a valuable model to study complex *in vivo* structure and function interactions.

The experiments outlined in this section have the potential to add to the growing data on the μ2 protein, and on the reovirus life cycle in general. Reovirus can then be used as a model for other viruses, and ultimately, more can be understood about how viruses carry out transcription, assort their genomes, assemble component parts to make whole viruses, and interact with the host cell. This base of information will aid future

researchers in their common goal to understand viral and host cell processes, and possibly, in the development of anti-viral therapies. Additionally, ts reoviruses can be invaluable models in the study of multiple gene product interactions. Through these models, we may come to understand intricate biochemical processes, and find answers to such puzzles as complex disease etiologies.

# 5. REFERENCES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## FOOTNOTES

[1]Coombs, K.M., personal communication