CHARACTERIZATION OF A NOVEL REOVIRUS AND ANTIVIRAL ACTIVITY AGAINST MAMMALIAN REOVIRUS

By

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In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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CANADA

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LIST OF ABBREVIATIONS

aa	amino acid	**DTT**	dithiothreitol
ADP	adenosine diphosphate	**EM**	electron micrscopy
AMP	adenosine monophosphate	**ESB**	electrophoresis sample buffer
ARV	avian reovirus	**FCS**	fetal calf serum
ATP	adenosine triphosphate	**Freon**	1,1,2-trichloro-1,2,2-trifluoroethane
BSA	bovine serum albumin	**GDP**	guanosine diphosphate
cDNA	complementary DNA	**GMP**	guanosine monophosphate
CNS	central nervous system	**GTP**	guanosine triphosphate
CPE	cytopathic effect	**HAI**	hemagglutination-inhibition
CSF	cerebrospinal fluid	**HIV**	human immunodeficiency virus
dADP	deoxyadenosine diphosphate	**HO buffer**	homogenization buffer
dATP	deoxyadenosine triphosphate	**hpi**	hours post-infection
D-buffer	dialysis buffer	**IMP**	inosine monophosphate
ddH_2O	double-distilled water	**IMPDH**	inosine monophosphate dehydrogenase
DEPC	diethyl pyrocarbonate	**ISVP**	intermediate subviral particle
dGDP	deoxyguanosine diphosphate	**MEM**	minimum essential media
dGTP	deoxyguanosine triphosphate	**MOI**	multiplicity of infection
DMSO	dimethylsulfoxide	**MPA**	mycophenolic acid
DNA	deoxyribonucleic acid	**mRNA**	messenger RNA
DOC	deoxycholate	**NT**	neutralization
dsRNA	double-stranded RNA	**ORF**	open reading frame

PAGE	polyacrylamide gel electrophoresis	**T1L**	Type 1 Lang
PBS	phosphate-buffered saline	**T2J**	Type 2 Jones
PCR	polymerase chain reaction	**T2W**	Type 2 Winnipeg
PFU	plaque forming units	**T3D**	Type 3 Dearing
pi	post-infection	**TBE**	Tris/borate/EDTA buffer
RMK	rhesus monkey kidney cells	***ts***	temperature sensitive
RNA	ribonucleic acid	**UV**	ultraviolet
rpm	revolutions per minute	**Vertrel**	1,1,1,2,3,4,4,5,5,5-decafluoropentane
RT	reverse transcriptase	**VSP**	viable serum protein
SDS	sodium dodecyl sulphate		

ABSTRACT

The role of reoviruses (respiratory, enteric, orphan virus) in human disease remains uncertain and it is difficult to provide evidence to link reovirus infection to specific human disease processes. Most identified cases are sporadic in nature and appear to be asymptomatic or produce minor upper respiratory or gastrointestinal symptoms that are indistinguishable from other illnesses seen in infancy or early childhood.

In November 1997, a reovirus clone was isolated from the cerebrospinal fluid of a severe combined immunocompromised infant with respiratory, gastrointestinal and central nervous system symptoms. Preliminary serological and electron microscopy examination identified the isolate to be a reovirus serotype 2 strain. There are only a few cases presented in the literature that associate reovirus with central nervous system disease. None have identified a reovirus serotype 2 clone; hence it was hypothesized the clone isolated in this work was unique and the clone was named Type 2 Winnipeg (T2W). Further characterization of T2W included evaluation of growth and sequencing studies of the S gene segments. Results confirmed the hypothesis that T2W was indeed novel.

Since reovirus infections are generally asymptomatic, there has been little evaluation of antiviral compounds on reovirus replication. In the past decade, knowledge of viral replication has allowed for the development of a variety of antiviral compounds that are either broad spectrum (e.g. ribavirin) or target a specific step in a particular virus' life cycle (e.g. HIV protease inhibitors). A variety of antiviral compounds were tested for their ability to inhibit reovirus replication. Two inosine monophosphate dehydrogenase inhibitors, ribavirin and mycophenolic acid, were shown to have antiviral activity against reovirus. Ribavirin is a broad spectrum antiviral agent that has been used for many years. Mycophenolic acid is used clinically for immunosuppression in kidney and heart transplants and its antiviral properties have only recently been examined in any detail. Mycophenolic acid is a more potent antiviral agent than ribavirin against reovirus replication. It was shown to inhibit T2W replication *in vitro*, which suggests it could have been used to treat infection with T2W.

1. *INTRODUCTION*

1.1. Mammalian reovirus introduction. The family *Reoviridae* currently contains ten genera (van Regenmortel et al. 2000;Attoui et al. 2000) (Table 1). Five of these genera, *Orthoreovirus*, *Orbivirus*, *Rotavirus*, *Coltivirus*, and *Seadornavirus* infect humans. The others infect plants, insects and fish. The common characteristic features of all these genera are genomes composed of ten to twelve segments of double stranded RNA (dsRNA), non-enveloped infectious particles 60 to 85nm in diameter, proteins arranged in two or three concentric layers with icosahedral symmetry and synthesis of viral messenger RNAs (mRNAs) by virally encoded enzymes within partially uncoated viral particles (Table 2). Despite similarities, viruses within different genera exhibit significant genetic, biochemical and biological differences.

The *Orthoreovirus* genus contains the prototype members of the *Reoviridae* family, the nonfusogenic mammalian orthoreoviruses (reoviruses). It also includes the fusogenic avian orthoreoviruses and an assortment of other orthoreoviruses (e.g. Nelson Bay virus, baboon reovirus, etc...). Viral isolates are assigned to the *Orthoreovirus* genus based on similar traits. These include a genome made up of ten dsRNA segments, size and morphology of particles by electron microscopy, characteristic protein profile with three large (λ), three medium (μ) and four small (σ) primary translation products, serologic reactivities and the ability of these viruses to assort their genome segments upon co-infection of cells to yield reassortant progeny. Assortment suggests a practical approach for the designation of species within the genus, i.e. if two isolates can assort their genome segments to produce infectious progeny, then they are said to belong to the same species (Duncan 1999).

Mammalian reoviruses are ubiquitous in nature. They are easily found in the environment – in rivers, stagnant water and sewage (Stanley 1967;Matsuura et al. 1993). They infect a variety of mammalian species including humans, monkeys, rats, mice, insects, fish and reptiles (Tyler and Fields 1996). The name 'reovirus' (**r**espiratory, **e**nteric, **o**rphan) was proposed by Sabin (Sabin 1959) to describe this group of viruses typically isolated from human respiratory and gastrointestinal tracts, but not associated with serious disease (hence orphan designation). Four serotypes of mammalian reoviruses have been identified by hemagglutination-inhibition (HAI) and neutralization studies

Table 1. The *Reoviridae*

Genus	Number of Genome Segments	Hosts
Turreted[a]		
Orthoreovirus	10	mammals, birds, reptiles
Aquareovirus	11	fish, molluscs
Cypovirus	10	insects
Fijivirus	10	plants, insects[b]
Oryzavirus	10	insects[b]
Nonturreted[a]		
Rotavirus	11	mammals, birds
Orbivirus	10	mammals, birds, arthropods[b]
Coltivirus	12	mammals, arthropods[b]
Seadornavirus	12	mammals, arthropods[b]
Phytoreovirus	12	plants, insects[b]

[a] Proposed division based on presence or absence of "turret-like" protein projection from vertices.

[b] Serve as vectors for transmission to other hosts.

* From (Attoui et al. 2000;Nibert and Schiff 2001).

Table 2. General Characteristics of the Reoviridae

Structure	Size: 60 to 85nm Shape: nearly spherical icosahedral symmetry Non-enveloped Two or three distinct capsid shells
Genome	Double-stranded RNA 10 to 12 gene segments present in equimolar amounts Mostly monocistonic, some segments have additional ORF 12 to 30 kbp
Replication	Fully cytoplasmic Incomplete uncoating of virions Possesses all enzymes required for dsRNA transcription Full-length, 5' capped, nonpolyadenylated mRNA transcripts

* Modified from (van Regenmortel et al. 2000;Nibert and Schiff 2001).

(NT) (Sabin 1959;Rosen 1962;Stanley 1967). Field strains were initially isolated and characterized by Rosen and colleagues. An isolate from a healthy child is the prototype for reovirus serotype 1 (Ramos-Alvarez and Sabin 1958;Sabin 1959) (strain Lang; abbreviated as T1L); an isolate from a child with diarrhea is the prototype virus for serotype 2 (Sabin 1959) (strain Jones, T2J); isolates from a child with diarrhea (Ramos-Alvarez and Sabin 1958;Sabin 1959) (strain Dearing, T3D) and from an anal swab taken from a baby (Rosen et al. 1960b) (strain Abney, T3A) are prototypes for serotype 3 and an isolate from the liver and brain of *Mus musculoides* is the prototype for serotype 4 (Attoui et al. 2001) (strain Ndelle). The prototype virus isolates for serotype 1, 2 and 3 are the most extensively studied, but in recent years, other isolates have been increasingly studied.

Avian reoviruses are less well characterized than their mammalian counterparts. They can be isolated from both domestic and wild birds, in which they cause several diseases such as arthritis/tenosynovitis, a gastrointestinal maladsorption syndrome and runting (Nibert and Schiff 2001). This makes ARV a major economic loss in the poultry industry. Neutralization tests have divided avian reoviruses into five or more different serotypes; however, avian reovirus (ARV) 138 and ARV 176 are the most commonly studied. Important differences between avian and mammalian reoviruses include the ability of ARV to form multinucleated synctia and the inability to agglutinate red blood cells (Martinez-Costas et al. 1997).

The understanding of a number of fundamental concepts in virology and molecular biology has been increased by the study of dsRNA viruses. In particular, reoviruses provided one of the first systems for *in vitro* studies of RNA synthesis (Borsa and Graham 1968;Shatkin and Sipe 1968;Skehel and Joklik 1969), evidence for the 5' cap on eukaryotic mRNAs (Furuichi et al. 1975) and early evidence for a consensus sequence for translation in eukaryotic mRNAs (Kozak's sequence) (Kozak and Shatkin 1978). Several early studies of reovirus pathogenesis revealed the role of animal virus receptor-binding proteins in tropism and/or virulence (Weiner et al. 1980). Studies also showed the role of M cells in viral penetration of the mucosal barrier during entry into the animal host (Wolf et al. 1981). Recently reoviruses have also been explored as novel oncolytic

agents (Coffey et al. 1998;Norman and Lee 2000;Norman et al. 2002;Shah et al. 2003;Hanel et al. 2004).

1.2. Reovirus structure. Mammalian reoviruses are non-enveloped, icosahedrally symmetric particles with two radially concentric, separable protein shells (inner and outer capsid) which surround a centrally condensed dsRNA genome (Vasquez and Tournier 1962;Dales et al. 1965;Mayor et al. 1965;Luftig et al. 1972) (Figure 1). Ten unique genome segments are present in equimolar amounts and are distinguished in polyacrylamide gels (Shatkin et al. 1968). There are three large (L1, L2, L3), three medium (M1, M2, M3) and four small genome segments (S1, S2, S3, S4). Each RNA segment is monocistronic (i.e. one gene encodes a single protein), with the exception of the S1 and M3 gene segments. The S1 gene segment encodes a second protein in a separate, overlapping open reading frame (ORF) (Sarkar et al. 1985;Jacobs and Samuel 1985;Ernst and Shatkin 1985), while the M3 gene segment encodes an additional protein, which initiates at the second or third in-frame AUG in the ORF of the dominant protein (McDowell et al. 1972;McCutcheon et al. 1999). Together, the ten gene segments encode twelve proteins, four proteins are non-structural while the other eight are packaged, with a defined copy number and position, into mature virus particles and can be separated by electrophoresis (Both et al. 1975;McCrae and Joklik 1978) (Table 3).

Reoviruses exist in three forms: virion, intermediate subviral particle (ISVP) and core (Figure 1), which differ in morphology, protein composition, physicochemical and biological properties (Table 3). The virion is the mature reovirus particle and is the predominant form released from infected cells. It contains all eight structural proteins and has a diameter of 85nm shown by cryoelectron microscopic analysis (Metcalf et al. 1991;Dryden et al. 1993) and x-ray diffraction (Harvey et al. 1981). Virions have a T = 13 icosahedral symmetry formed by 600 copies each of the outer capsid proteins σ3 (encoded by S4) and μ1 (encoded by M2) (Dryden et al. 1993). The μ1 protein undergoes proteolytic cleavage almost immediately after assembly of complete progeny virus particles. The cleavage fragments, μ1N and μ1C, are both found in virions (Nibert et al. 1991b). Five monomers of λ2 (encoded by L2) form a pentameric complex that looks like a "turret" or "spike" and projects 9.5nm from the surface with a central channel 5 to 8nm in diameter. This yields a total of 60 copies at each of the 12 vertices

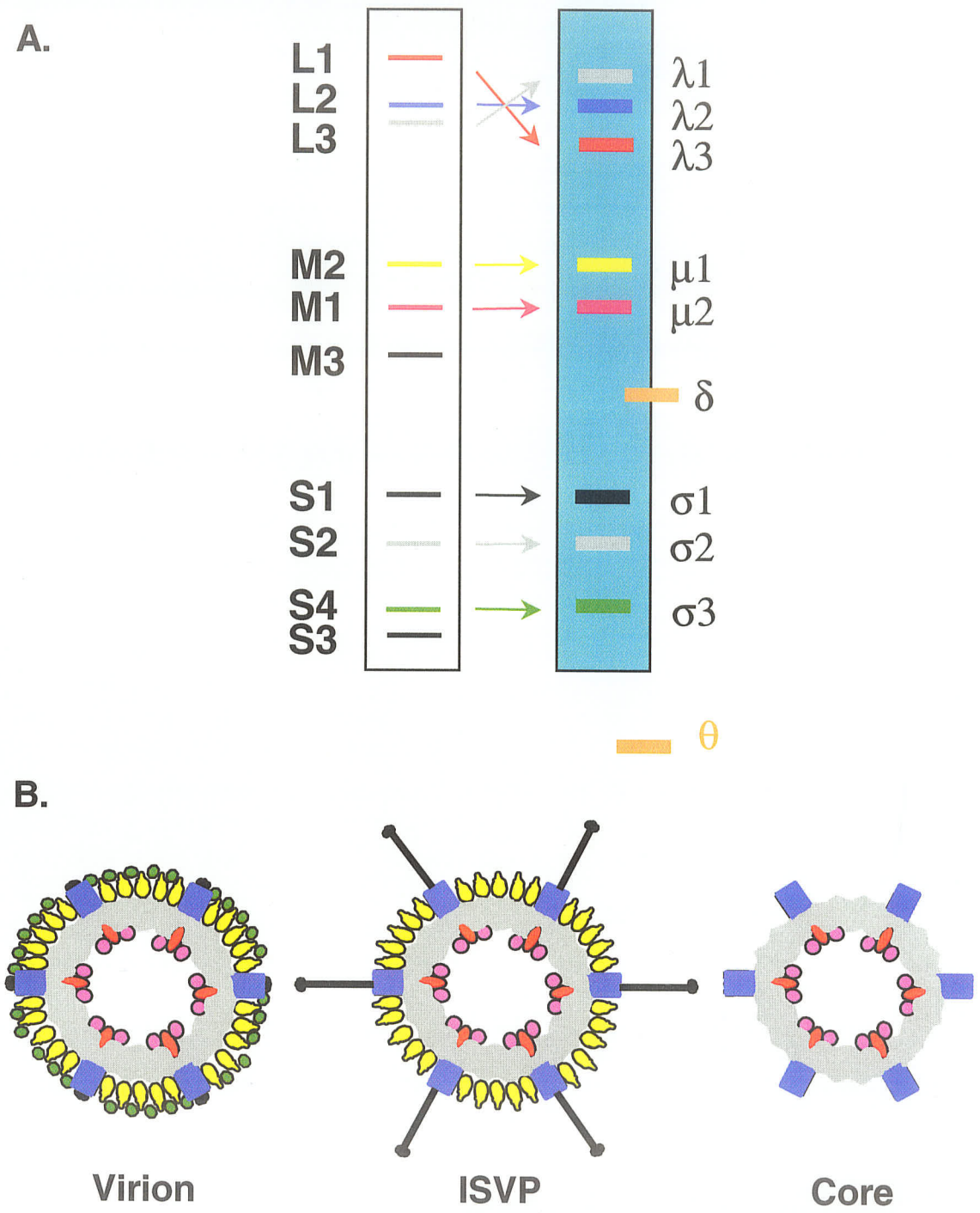

A.
L1
L2
L3
M2
M1
M3
S1
S2
S4
S3
λ1
λ2
λ3
μ1
μ2
δ
σ1
σ2
σ3
θ
B.
Virion
ISVP
Core

Figure 1. Schematic representation of mammalian reovirus electropherotype, protein profile and structure

(A) Electropherotype and protein profile of Type 1 Lang. Migration of gene segments and encoded proteins as seen on SDS-polyacrylamide gel electrophoresis. Genes cluster into large (L), medium (M) and small (S) gene segments. δ and φ are μ1 cleavage products only present in ISVPs. **(B)** Representation of the three reovirus particle forms: whole virions, ISVP and core. Colours indicate location of structural proteins and correlate with proteins indicated in part (A).

Table 3. Reovirus proteins

Gene segment	Protein	Copy Number In[a]			Main functions and properties[b]
		Virion	ISVP	Core	
L1	λ3	13 ± 1	12 ± 1	11 ± 1	RNA-dependent RNA polymerase
L2	λ2	60	60	60	Guanylyltransferase, methyltransferase
L3	λ1	124 ± 3	122 ± 3	123 ± 3	Binds dsRNA, Zn metalloprotein, NTPase?, RNA helicase?, RNA triposphatase
M1	μ2	21 ± 3	17 ± 4	14 ± 3	Putative transcription cofactor, binds RNA, NTPase?
M2	μ1	600	μ1 23 ± 3 δ 575 ± 16	ND	Role in penetration, N-myristoylated, cleaved into fragments, role in transcriptase activation
M3	μNS	0	0	0	Binds core, role in assortment and secondary transcription, binds ssRNA
	μNSC	0	0	0	Unknown
S1	σ1	37 ± 1	23 ± 2	ND	Cell-attachment protein, hemagglutinin, primary serotype determinant
	σ1s	0	0	0	Unknown
S2	σ2	122 ± 12	126 ± 12	117 ± 15	Binds dsRNA
S3	σNS	0	0	0	Binds ssRNA, probable role in RNA assortment or replication
S4	σ3	600	ND	ND	Binds dsRNA, Zn metalloprotein, effects on translation, sensitive to protease degradation

[a] Data on copy number from (Coombs 1998).
[b] Modified from (Nibert and Schiff 2001).
ND – none detected

(Dryden et al. 1993;Reinisch et al. 2000). A folded/retracted arrangement of σ1 monomers (encoded by S1) form trimers associated with λ2 at the surface of each vertex (Strong et al. 1991;Turner et al. 1992;Lee and Leone 1994;Coombs 1998). This protein represents the cell attachment and viral hemagglutinin protein (Masri et al. 1986;Yeung et al. 1987;Banerjea et al. 1988).

Whole virions can be converted by proteolysis *in vitro* and *in vivo* into two distinct subviral particles: ISVPs and cores (Mayor and Jordan 1968;Smith et al. 1969;Shatkin and LaFiandra 1972) with diameters of 80nm and 60nm respectively (Dryden et al. 1993). ISVPs may be formed by intestinal proteases such as pepsin, chymotrypsin or trypsin (Bass et al. 1990;Amerongen et al. 1994) and differ from virions by loss of σ3, cleavage of μ1 into δ and φ and extension of σ1 fibers, 40nm from the particle surface (Furlong et al. 1988;Dryden et al. 1993) possibly to facilitate cell attachment.

Cores are formed from ISVPs by the loss of μ1 cleavage products and σ1 fibers to yield the inner capsid. The inner capsid consists of three major proteins: λ1 (encoded by L3), λ2, and σ2 (encoded by S2) and two minor proteins: μ2 (encoded by M1), and λ3 (encoded by L1). The lattice of the core or inner capsid is thought to be made up of roughly 120 copies of λ1 to form a smooth surface punctated with low lying ridges upon which σ2 (also present in roughly 120 to 150 copies) binds (Coombs 1998;Reinisch et al. 2000). Protein λ2 exists as described above. The minor proteins form a proteinaceous structure internal to the λ1 shell and projects below the shell at each vertex as determined by cryoelectron microscopy and x-ray crystallography (Dryden et al. 1998). This complex is proposed to represent the viral transcriptase complex, which transcribes genomic dsRNA into mRNA. Protein λ3, the RNA-dependent RNA polymerase (RdRp), is believed to be associated with one or two copies of μ2, the alleged polymerase cofactor (Yin et al. 1996;Noble and Nibert 1997). More work is needed to define the structure in detail.

Another type of reovirus particle seen *in vitro* is an "empty virion" with decreased amounts of dsRNA genome (Rhim et al. 1961;Smith et al. 1969). The smaller amount of RNA causes these particles to have a lower buoyant density than whole virions and they

migrate above virions in a cesium chloride gradient; hence, these particles are named "top component." The morphology and protein content of these particles is very similar to whole virions, but negative stains are able to enter the central cavity (White and Zweerink 1976;Wetzel et al. 1997). The derivation of "top component" is unknown, but is unlikely to be a precursor in whole virion assembly (Lai et al. 1973).

1.3. Viral life cycle. For an overview of the reovirus lifecycle, please see Figure 2.

1.3.1. Early Steps: Viral attachment and entry. Reoviruses enter their animal host via respiratory and enteric routes. It is speculated that a region of the σ1 protein may function as a glycosyl hydrolase (mucinase) to facilitate penetration of virions and ISVPs through the mucous layer of intestinal epithelial cells (Bisaillon et al. 1999). Numerous studies have investigated the attachment of reoviruses to erythrocytes and other cells. Studies that have investigated binding of reoviruses within host animals generally indicate defined receptor moieties on the cell surface are responsible for attachment of reovirus particles to cell surface receptors via specific interactions with the cell-attachment protein, σ1 (Lee et al. 1981;Paul and Lee 1987;Choi et al. 1990;Strong et al. 1993). Unfortunately, the nature of these interactions remains incompletely defined. Evidence suggests sialic acid is critically important for infection of some cells (Paul and Lee 1987;Paul et al. 1989). Other data suggests multiple sialylated glycoproteins (Choi 1994), epidermal growth factor receptor (Tang et al. 1993), a 54-kd protein on endothelial cells and undefined moieties on human colonic adenocarcinoma cells (Caco-2) (Ambler and Mackay 1991) and mouse ependymal cells (Nepom et al. 1982) as potential reovirus cellular receptors. More recently, junction adhesion molecule, an integral tight junction protein, has been identified as a reovirus receptor (Barton et al. 2001;Forrest et al. 2003). However, it will be important to determine whether attachment to these potential receptors, alone or in combination, leads to virus uptake and a productive infection. Data also suggests differences in receptors are likely to exist between cell types and between reovirus serotypes or strains. It is unknown at this time whether interactions of cell surface proteins with other reovirus proteins occur. Differences in the attachment process for virions and ISVPs also remain unknown. When reovirus particles attach to a cellular receptor, they undergo a conformational change in viral capsid. The cell-bound virions are more resistant to pepsin digestion than unbound virions (Fernandes et al. 1994). It is

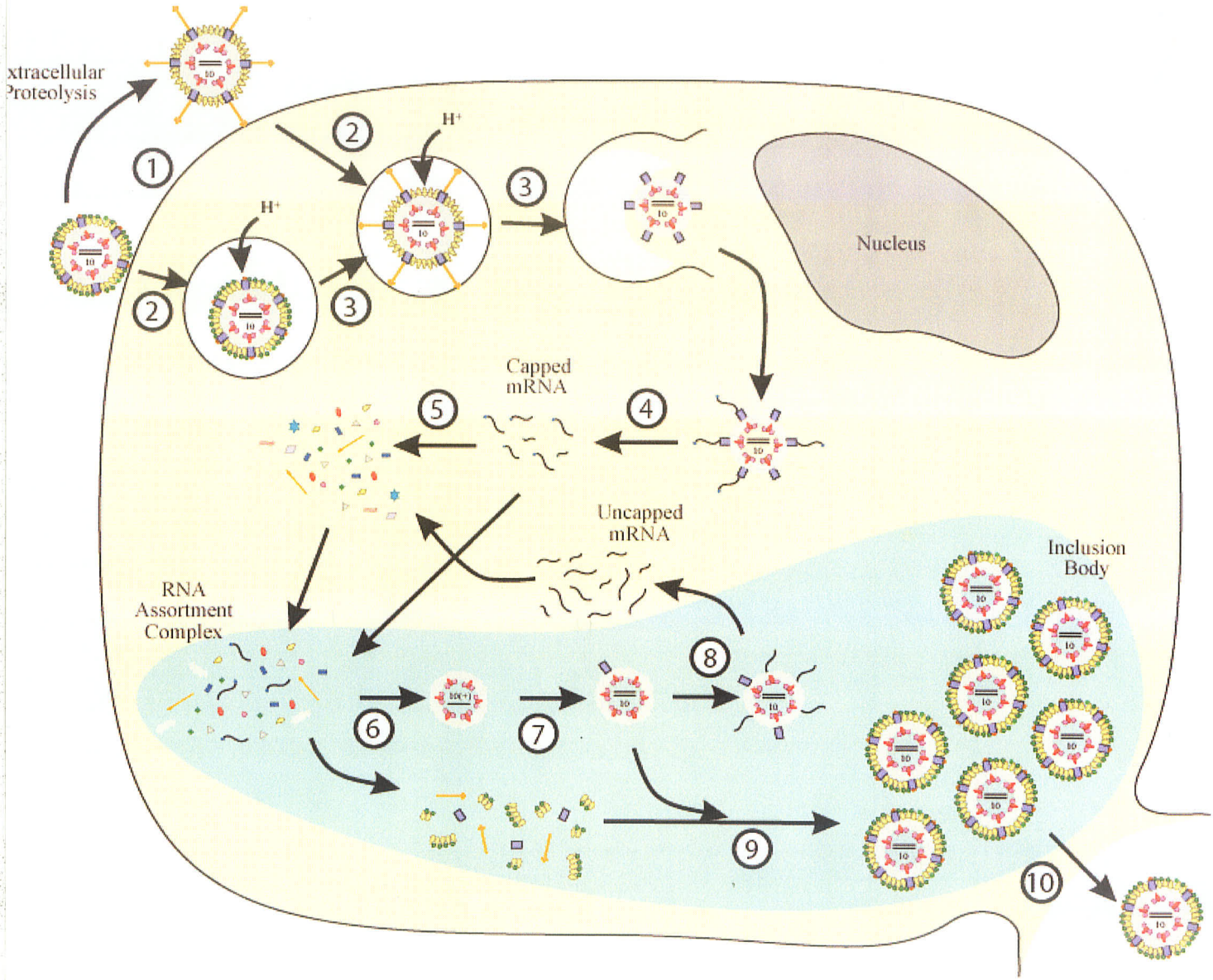

xtracellular
Proteolysis
H+
H+
Nucleus
Capped
mRNA
Uncapped
mRNA
RNA
Assortment
Complex
Inclusion
Body
1
2
3
4
5
6
7
8
9
10

Figure 2. Reovirus life cycle

(1) The first step in reovirus infection is *attachment* of the virion to a cell surface receptor molecule. **(2)** *Entry* of reovirus particles into the cell is by receptor-mediated *endocytosis* where particles are delivered to endosomes and/or lysosomes where **(3)** reovirus outer capsid proteins undergo specific *proteolytic cleavages*. These cleavages are acid-dependent and are essential in the infection process. *Extracellular proteolysis* can generate ISVPs in some situations and these particles may directly cross the cellular membrane into the cytosol. It is unclear how reovirus particles interact with and penetrate lysosomal or plasma membranes, but appears to be dependent on proteolysis as only ISVPs appear to interact with membranes. Once in the cytosol of the cell, the ISVP is likely further processed to the core particle and becomes transcriptionally active. **(4)** *Primary transcription* occurs with the synthesis of ten capped viral mRNAs, which are used for the **(5)** *translation* of viral proteins by cellular protein synthesis machinery. **(6)** The early transcripts also associate with newly made viral proteins to form *replicase complexes*. **(7)** Within these complexes, each of the 10 packaged plus strand RNAs serve as template for minus strand synthesis. **(8)** Viral mRNAs are synthesized by subviral particles during *secondary transcription*. **(9)** These "late" transcripts serve as primary templates for the majority of protein synthesis late in infection. The mechanism of virion *capsid assembly* is poorly defined, but may involve the addition of preformed complexes of outer capsid proteins to replicase complexes. **(10)** Mature virions are likely *released* from infected cells via cell lysis.

thought the altered virion conformation may be necessary for viral entry and subsequent uncoating.

Uptake of reovirus particles into clathrin-coated pits or vesicles at the cell surface occurs via receptor-mediated endocytosis (Borsa et al. 1979;Borsa et al. 1981;Sturzenbecker et al. 1987;Rubin et al. 1992). Proteolysis of viral proteins within hydrolytic compartments of the endocytic pathway (late endosomes or lysosomes) starts 20 to 30 minutes after infection at 37°C (Silverstein et al. 1970;Sturzenbecker et al. 1987). Acidic proteases degrade σ3 protein and convert μ1C protein to its stable cleavage product to generate ISVP-like particles (Chang and Zweerink 1971;Silverstein et al. 1972;Borsa et al. 1981;Sturzenbecker et al. 1987;Nibert and Fields 1992) and appear to be necessary for the next steps in infection (membrane interaction and penetration). Evidence also shows that proteolytic activation of virions can occur outside cells before attachment and endocytosis. Proteolysis to generate ISVPs occurs within the lumen of the small intestine and is probably mediated by pancreatic serine proteases (Bodkin et al. 1989;Bass et al. 1990). These ISVPs can directly cross the cellular membrane and bypass endocytic uptake (Borsa et al. 1979). It remains to be determined whether penetration through the plasma membrane by extracellular generated ISVPs occurs routinely.

The mechanism by which reoviruses cross the membrane barrier remains poorly defined. Unlike enveloped viruses, reovirus cannot use membrane fusion as a general mechanism for this process. Direct interaction of viral components with the lysosomal or cellular plasma membrane is an expected requirement in order to give reovirus particles access to the cytoplasm where substrates for transcription and ribosomes for translation are available. Suggested models propose membrane interaction results in local disruption of the bilayer and allows the virus particle to enter the cytoplasm through a large breach. Perhaps a more precise, pore-like structure is formed by viral proteins within the lipid bilayer such that viral components are able to enter the cytoplasm (Borsa et al. 1974;Borsa et al. 1981;Nibert et al. 1991a;Nibert and Fields 1992;Tosteson et al. 1993). Available data implicate the major outer capsid protein μ1 in mediating direct interactions with membranes. Although not confirmed, it is thought μ1 protein associates with the vacuolar membrane, which results in the release of the reovirus particle into the cytosol (Nibert and Fields 1992;Lucia-Jandris et al. 1993). Work with temperature-

sensitive mutant *ts*A279 provides further evidence μ1 is involved in viral entry. The dominant lesion in the M2 gene segment, which encodes μ1 protein, was associated with defective transmembrane transport of virions (Hazelton and Coombs 1995).

1.3.2. Transcription and replication. A notable feature in the reovirus life cycle is incomplete uncoating of the virus particle during transcription. Core particles in the cytoplasm are transcriptionally active and contain all activities necessary to produce capped methylated mRNAs (Furuichi et al. 1976). Whole virions are unable to make full-length transcripts, but have been reported to synthesize short initiator oligonucleotides (Yamakawa et al. 1982;Farsetta et al. 2000). This suggests transcriptase is constituitively active in virions, but incapable of elongation. Therefore, transcriptase activation in cores must primarily involve a relief in a block to elongation. A conformational change in λ2 protein (Dryden et al. 1993) may be important for transcriptase activation. In cores, λ2 opens a fivefold channel through which substrates or products of transcription may be required to pass. However, even in cores, elongation of initiator oligonucleotides into full-length transcripts only occurs at a low frequency, which suggests the onset of elongation is the rate-limiting step in reovirus transcription (Kapuler 1970;Zarbl et al. 1980a;Yamakawa et al. 1981;Yamakawa et al. 1982). Thus, three phases of transcriptase activity are suggested: (1) repetitive initiation, which yields large amounts of initiator oligonucleotides, (2) onset of elongation and (3) elongation of initiator oligonucleotides to yield smaller amounts of full-length transcripts.

Unlike DNA viruses, reovirus carries its own RNA polymerase as host cells do not encode enzymes for replication of RNA from RNA template. The λ3 core protein combined with μ2 core protein are the RNA-dependent-RNA-polymerase (Drayna and Fields 1982a;Koonin et al. 1989;Morozov 1989;Starnes and Joklik 1993) and putative polymerase cofactor (Wiener et al. 1989;Yin et al. 1996) respectively and are the enzymes responsible for transcription and subsequent replication.

There are two phases of transcription in reovirus replication. *Primary transcription* is mediated by infecting core-like particles and results in the synthesis of 5'-capped, methylated, non-polyadenylated transcripts from each of the ten genomic segments which serve both as mRNA for translation and as templates for minus-strand synthesis. Primary transcripts are detected two hours post infection (hpi) (Zweerink and

Joklik 1970), peak at 6 to 8 hpi and are almost undetectable by 12 hpi. The nascent mRNAs are translated by host cellular ribosomes to generate viral proteins, which associate with primary transcripts to form *replicase complexes*. Each complex packages only one copy of each gene segment into a particle devoid of an outer capsid and the inner core protein λ2 through a poorly understood process (Morgan and Zweerink 1975;Zweerink et al. 1976). Unknown signaling, possibly through conserved regions at the 5' and 3' ends of reovirus plus and minus strands (Antczak et al. 1982), must exist to ensure only viral, not cellular, mRNA is packaged (Zou and Brown 1992a;Chappell et al. 1994). Structural protein σ3 and non-structural proteins σNS and μNS all have the ability to bind ssRNA and may play a role in assortment (Huismans and Joklik 1976;Antczak and Joklik 1992). *Replicase particle* assembly also remains poorly understood. Certain core proteins, such as σ2 and λ1, have been shown to self-associate when co-expressed, whereas another protein, λ2, is formed in large part as a monomer (Mao and Joklik 1991;Xu et al. 1993). It remains unknown if certain protein complexes are required prior to complete core capsid assembly.

Once mRNA transcripts are packaged, each plus-strand serves as template for synthesis of the minus-strand, which remains base-paired to the plus-strand and regenerates genomic dsRNA (Acs et al. 1971;Sakuma and Watanabe 1971;Schonberg et al. 1971;Sakuma and Watanabe 1972;Zweerink 1974). Minus-strand synthesis is initiated at the extreme 3' end of each template, possibly mediated through specific polymerase recognition sequences common to all genes. The viral replicase catalyzes a single round of minus-strand synthesis as the minus-strand remains within the nascent subviral progeny.

Secondary transcription follows regeneration of dsRNA genomic material and is mediated by newly assembled subviral particles. Some studies suggest these particles contain a latent capping enzyme, produce uncapped viral mRNAs (Skup and Millward 1980;Zarbl et al. 1980b) and are proposed to be translated preferentially late in infection. Secondary transcription is the source of 95% of all transcripts formed throughout infection (Ito and Joklik 1972), which serve as template for translation into proteins. Whether they may also serve as template for minus-strand synthesis is still debated (Fields 1971;Schonberg et al. 1971;Morgan and Zweerink 1977).

1.3.3. Late Events: Assembly and release. Virion capsid assembly remains poorly understood. The earliest detectable reovirus particles are ones that contain reovirus mRNAs in complex with σ3 and μNS and σNS (Gomatos et al. 1981;Antczak and Joklik 1992). The next particle observed along the assembly pathway lacks non-structural proteins and includes both inner and outer capsid proteins. These *replicase particles* contain RdRp activity and appear to be engaged in minus-strand synthesis (Morgan and Zweerink 1975). Additional copies of outer capsid proteins may be added to form complete virions (Morgan and Zweerink 1975;Zweerink et al. 1976).

Many reovirus structural proteins exhibit some degree of self assembly in the absence of viral RNA or viral proteins. For example, the major inner core proteins, λ1 and σ2, form particles morphologically similar to spike-less core-like particles when co-expressed in cell culture (Xu et al. 1993). When λ2 and λ3 are also expressed, they form 'spikes' in these core-like particles as expected (Xu et al. 1993). The exact order in which these proteins come together to form nascent infectious particles and the roles of viral RNA and the minor core protein, μ2, are unknown.

Assembly of the outer capsid is also poorly understood. The temperature-sensitive mutant tsG453 fails to assemble outer capsids at nonpermissive temperature, which suggests σ3 plays a role in viral assembly and may be required to interact with μ1 in order to assemble on to core particles (Shing and Coombs 1996). A recently developed system for recoating reovirus core particles with recombinant outer capsid proteins has provided further evidence that preformed complexes of μ1 and σ3 are required for assembly into particles and can occur independently of σ1 (Chandran et al. 1999;Farsetta et al. 2000).

The mechanism by which fully assembled virions are released from infected cells remains unknown. Virion release is thought to follow cell death and the associated breakdown of the plasma membrane. Recent studies indicate apoptosis is an important mechanism of reovirus-induced cell death (Tyler et al. 1996;Connolly and Dermody 2002;Kominsky et al. 2002a;Kominsky et al. 2002b).

1.4. Exploitation of segmented genome.

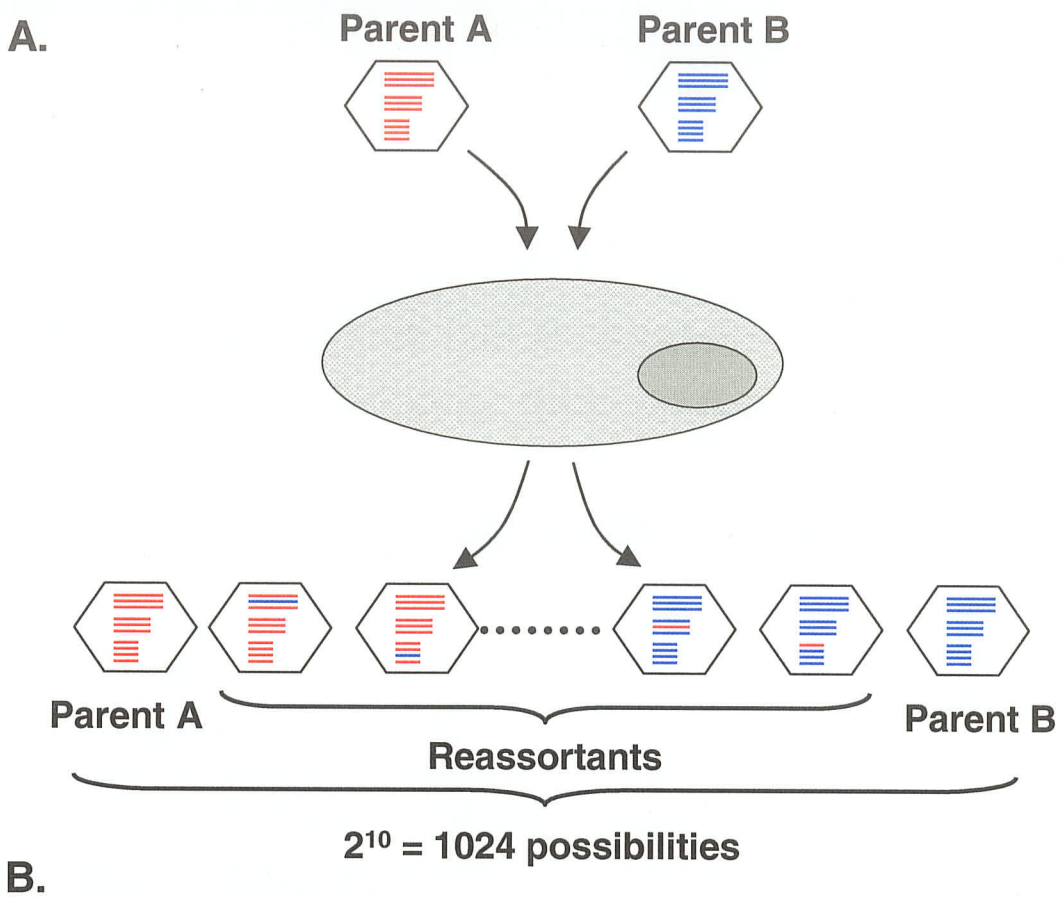
1.4.1. Reassortment. When cells are infected with a mixture of two different reovirus strains, gene segments from each parent are able to mix in a process called *assortment.*

Progeny virions with a mixed set of genes are called *reassortants*. Because homologous gene segments from different reovirus strains are distinguishable by electrophoresis, gel analyses of the parents and each reassortant allow the parental origin of every gene in any given reassortant to be quickly and easily determined. For a schematic example, please see Figure 3A.

If parental viruses are added at a high enough multiplicity of infection to ensure all cells undergo co-infection, then completely random assortment results in 2^{10} or 1024 potential gene combinations, two of which are parental while the remainder are reassortants. This is not the case with mammalian reoviruses. Reassortment frequencies range between 3 – 25% (Fields 1971). The mechanism of the non-random nature of reovirus assortment has yet to be explained. One plausible explanation is two separate areas of replication for each parental virion are established within a cell, which yields inefficient RNA exchange for assortment to occur to a great degree (Joklik and Roner 1995). Another possibility is viral RNA and or protein may interact less efficiently when derived from different parents, which may lead to reassortants with decreased fitness (Roner et al. 1990).

Reassortants result only from mixed infections within species limits and hybrid progeny have been exploited to assign biologic functions to individual genes and their protein products in both mammalian and avian reoviruses and rotaviruses. The segmented nature of these genomes and the ability to exploit this through reassortant analysis has profound implications in the use of reovirus in genetic studies (For a detailed review, see (Ramig and Ward 1991).

1.4.2. Reassortant mapping. As indicated earlier, one unusual feature of mammalian reoviruses is their genomes consists of 10 segments of linear dsRNA, each of which can be resolved by gel electrophoresis to yield a pattern of bands and is known as that virus' "electropherotype." This provides researchers with a tool to characterize viral genes and proteins and help determine their roles in the viral life cycle. Numerous studies have succeeded in attributing phenotypic differences to a single gene segment or to more than one gene segment at a time, which indicates genetic differences in two or more viral components are involved. For example, if T1L virions grew optimally under condition A and T3D virions grew optimally under condition B, then T1L X T3D reassortants can be


A.
Parent A
Parent B
Parent A
Parent B
Reassortants
2^{10} = 1024 possibilities

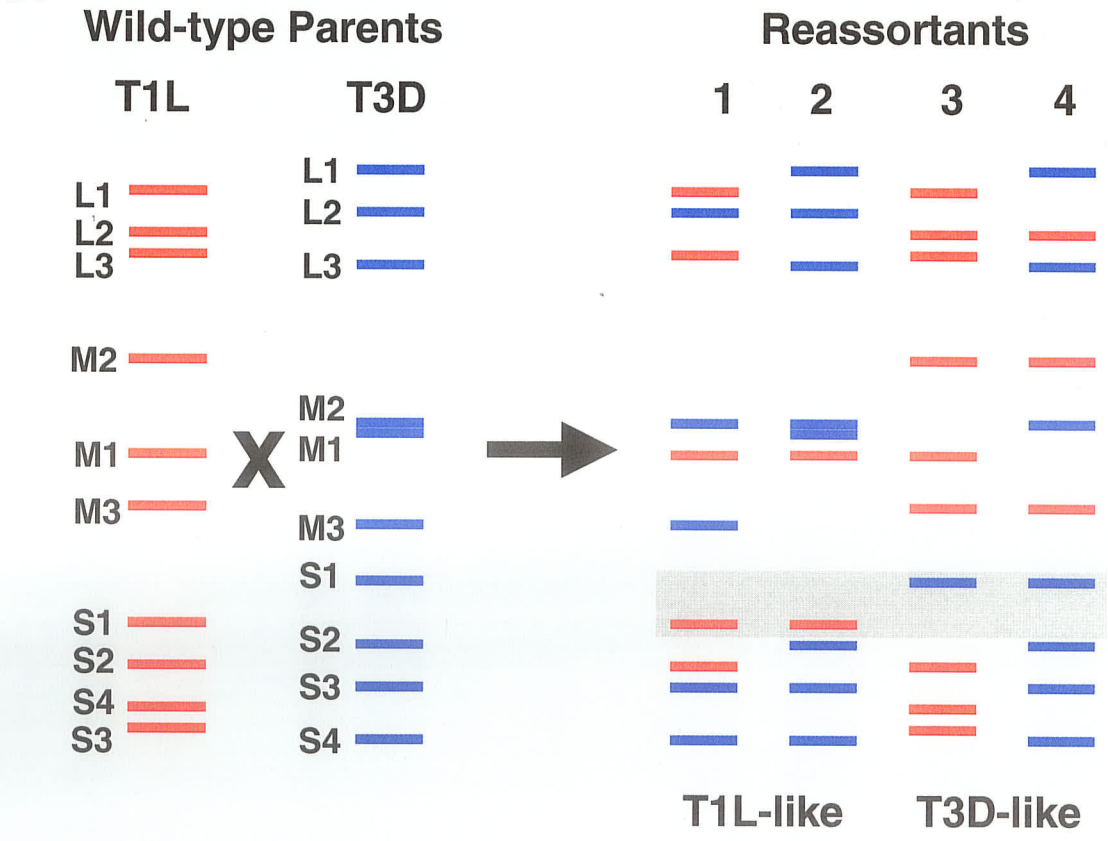

B.
Wild-type Parents
T1L
T3D
Reassortants
1
2
3
4
L1
L2
L3
M2
M1
M3
S1
S2
S4
S3
X
T1L-like
T3D-like

Figure 3. Co-infection and generation of reassortant progeny virions

(A) In a mixed or co-infection, both incoming virions, or parents, undergo transcription and replication. Assortment may occur to generate *reassortants* – progeny virions that contain a mixed set of genes from both parents. If assortment were completely random, 1024 (i.e. 2^n where n = 10 gene segments) possible reassortants could be assembled. Two would be identical to the parental virions while the remainder would be reassortants. **(B)** If a phenotypic difference exists between two different parental strains, reassortants generated from the parents can be used to identify which gene and thus, which protein is responsible for the phenotypic difference. In this example, reassortants were generated from a T1L X T3D co-infection and their electropherotypes resolved by SDS-PAGE. The reassortants were studied to determine whether they behaved more like T1L or T3D under a specific set of growth conditions. One group (1 & 2) behaves like T1L whereas the other group (4 & 5) behaves like T3D. Examination of the electropherotypes of the reassortants show the T1L-like group all derived their S1 gene from T1L whereas the other group which all received their S1 gene from T3D. Since all other genomic segments are randomly distributed, this indicates the phenotypic difference between the two groups is attributed to the M3 gene and is responsible for the observed growth differences between the T1L and T3D parents.

generated and isolated for analysis. Some reassortants will grow better under condition A whereas some will grow better under condition B. Reassortants could then be grouped into two panels of reassortants with respect to their preferred growth conditions. Analysis of their electropherotypes may single out a gene that comes from one parent in one panel and the other parent in the second panel. If all other genes are randomly distributed, then the identified gene is responsible for the phenotypic difference towards growth conditions observed between T1L and T3D (Figure 3B). Use of reassortants to phenotypically identify or "map" a gene is termed *reassortant mapping*.

1.5. Pathogenesis and pathology. Some reovirus strains cause more disease than other strains after inoculation into an experimental host. Newborn mice are the most extensively studied and have provided the most information vis à vis the correlation between pathogenesis and the function of individual viral genes.

In a natural infection, reoviruses behave primarily as respiratory and enteric viruses in mice. After oral inoculation in a newborn mouse, the virion is exposed to luminal serine proteases (Amerongen et al. 1994), which cleave the σ3 protein and convert the virion into a subviral form. Conversion allows the viral particle to adhere to specialized intestinal cells (M cells) overlying the dome of Peyer's patches (reviewed in (Organ and Rubin 1998)). M cells act as a transport system to carry reoviruses across the intestinal mucosa to the surface of mucosal mononuclear cells inside Peyer's patches. A similar sequence of events occurs in the lung, where M cells of the respiratory tract provide a route of entry and spread to regional lymph nodes (Morin et al. 1994).

After primary replication in Peyer's patch, virus spreads from the gastrointestinal tract to extraintestinal organs and the CNS (reviewed in (Tyler 1998)). Reovirus T1L and T3D show marked differences in spread to the CNS after peroral inoculation in newborn mice. T1L can be sequentially detected in Peyer's patches, mesenteric lymph nodes and the spleen. This pattern is consistent with local lymphatic spread (Kauffman et al. 1983). After intramuscular inoculation, the principal route of spread for T1L to the CNS is through the bloodstream (Flamand et al. 1991). In contrast, spread of T3C9 (a type 3 field strain) from the intestinal tract to the CNS is through the myenteric plexus adjacent to Peyer's patches and ultimately through the vagus nerve to brain-stem neurons (Morrison et al. 1991). T3D spreads to the spinal cord after intramuscular inoculation through the

microtubule-associated system of fast axonal transport in nerve tissue (Tyler et al. 1986). Genetic studies indicate the S1 gene segment, which encodes the cell-attachment protein σ1, determines the pattern of spread of reoviruses to the CNS (Kauffman et al. 1983;Tyler et al. 1986).

The importance of the σ1 protein for neurotropism has been extensively studied both *in vitro* and *in vivo*. After intracerebral inoculation into newborn mice T1L infects ependymal cells, which line the ventricles and aqueducts and produces ependymitis (reviewed in (Tyler 1998)). Infected animals frequently develop communicating or non-communicating hydrocephalus due to destruction and sloughing of ependymal cells and subsequent blockage of cerebral spinal fluid (CSF) circulation (Margolis and Kilham 1969;Nielsen and Baringer 1972;Milhorat and Kotzen 1994). Type 3 reovirus strains infect neurons and produce a lethal meningoencephalitis (reviewed in (Tyler 1998)). In the cortex, there is extensive necrosis at times associated with hemorrhage and an inflammatory response (Gonatas et al. 1971;Margolis et al. 1971;Raine and Fields 1973). Reovirus type 3 strains differ in their virulence and peak titre, but not in the basic pattern of CNS injury produced (Hrdy et al. 1982). This indicates the general pattern of CNS tropism is serotype specific. Studies indicate the capacity of reoviruses to infect ependymal cells (T1 pattern) or neurons (T3 pattern) is determined by the S1 gene (reviewed in (Tyler 1998)). This serotype-specific neurotropism is also reflected *in vitro*. T3D, but not T1L, infects neurons in primary explant cultured neural cells derived from fetal rat cortex (Dichter and Weiner 1984). Both strains infect astrocytes and only T1L, not T3D, is able to bind to the surface of human and murine ciliated ependymal cells (Tardieu and Weiner 1982).

The neuropathology of type 2 reovirus strains has not been well characterized. T2J produces encephalitis in suckling mice (Walters et al. 1965), with the majority of injury in the cortex. Unfortunately, the available data are too limited to permit a detailed comparison between T2 strains and other reovirus strains.

In humans, there is generally no pathologic material to evaluate since reoviruses are largely associated with asymptomatic infection. Descriptions of reovirus disease pathology are largely described in experimental animals, in particular neonatal mice. It should be noted reovirus infection is a systemic illness in neonatal mice with evidence of

infection and injury in every major organ system. All three serotypes of reovirus can produce myocardial injury in suckling mice (reviewed in (Sherry 1998)). On gross examination of the heart, small white or yellow patches are seen that coalesce as disease progresses (Sherry et al. 1989). These patches correspond to areas of necrosis and associated calcification. Patchy areas of alveolar hemorrhage, with associated pulmonary edema, have been seen in the lungs after inoculation of newborn mice with strains from any of the three serotypes of reoviruses (Tyler and Fields 1996). Hepatic injury may also occur (reviewed in (Organ and Rubin 1998)). Macroscopic yellow lesions may be visible on the surface of the liver and correspond to regions of hepatic necrosis. A classic presentation of reovirus infection in suckling mice is a syndrome known as oily hair effect (Stanley and Leak 1963;Onodera et al. 1981). Mice appear as if sprayed or dipped in oil. Most of these animals also show reduced growth and small size ('runts') and many animals become jaundiced, likely due to malabsorption produced by concomitant hepatic or biliary disease.

1.6. Clinical features. Reoviruses remain orphan viruses as much today as when Sabin (Sabin 1959) first coined the term over forty years ago. Despite the fact that in early childhood most individuals show serologic evidence of past reovirus infections, it has been difficult to provide convincing evidence to connect reovirus to a specific human disease (Table 4). It is believed most infections are either asymptomatic or remain undistinguished from the many minor upper respiratory and gastrointestinal illnesses seen in infants and toddlers (Tyler and Fields 1996).

1.6.1. Central nervous system disease. One of the hallmarks of reovirus infection in neonatal mice is CNS disease; thus, it is not unexpected there have been isolated reports that describe an association between reovirus and human CNS disease. The best characterized isolate is type 3 reovirus isolated from a 6.5-week old infant with meningitis (Tyler et al. 2004). In this study the S1 gene sequence was determined and the capacity to cause encephalitis in mice was assessed which showed the novel isolate was capable of systemic spread to the CNS after peroral inoculation of newborn mice and provides direct evidence that this isolate can be neurovirulent in humans. Another persuasive case is a report of a previously healthy 3-month-old girl who developed aseptic meningitis (Johansson et al. 1996). The child seroconverted and serotype 1

Table 4. Clinical features of reoviruses

Condition	Reference
Probable associations	
Enteritis in infants and children	(Rosen et al. 1960;Jackson et al. 1961;Jarudi et al. 1973)
Upper respiratory infections	(Rosen et al. 1963)
Possible associations (unproven)	
Exanthema	(Lerner et al. 1962)
Neonatal biliary atresia*	(Bangaru et al. 1980;Morecki et al. 1982;Glaser et al. 1984)
Neonatal hepatitis	(Glaser et al. 1984)
Isolated cases	
Meningoencephalitis	(Krainer and Aronson 1959;Nelson and Tarnowski 1960;Joske et al. 1964;Johansson et al. 1996;Tyler et al. 2004)
Pneumonia	(Tillotson and Lerner 1967)
Myocarditis	(Terheggen et al. 2003)
Keratoconjunctivitis	(Jarudi et al. 1973)

* A recent study provides evidence reoviruses do not play a role in biliary atresia (Saito et al. 2004).

reovirus was isolated after inoculation of CSF onto green monkey kidney cells; therefore it is likely reovirus was responsible for her meningitic symptoms. There are other less well-documented cases, which associate reovirus with human CNS disease. In some cases virus was isolated only from stool and not CSF and seroconversion was not documented (Krainer and Aronson 1959;Nelson and Tarnowski 1960). This raises the possibility that reovirus infection may have been coincidental rather than causal. In some other cases, virus has been isolated from brain tissue (Krainer and Aronson 1959;Joske et al. 1964).

1.7. Diagnosis. Diagnosis of reovirus infection is based on viral isolation from body tissues or fluids, detection of viral antigen and serologic studies (Tyler 2001). Suitable specimens include stool, throat swabs, nasal washings, CSF and urine, although almost all newly discovered isolates come from stool and rarely, respiratory samples. Initial viral isolation is generally on monkey kidney cells (Tyler 2001). Besides characteristic CPE, the presence of viral antigen can be verified by immunofluorescence or immunocytochemistry (Tyler 2001). Immunocytochemical studies can also be used to detect reovirus antigen in tissue samples.

Enzyme-linked immunosorbent assay techniques have been used widely for detection of reovirus antibodies in serum and can easily be applied in clinical settings (Richardson et al. 1988;Selb and Weber 1994). Another diagnostic technique to confirm reovirus infection in human serum is an indirect fluorescence assay (Bangaru et al. 1980;Morecki et al. 1982;Glaser et al. 1984), although its sensitivity and specificity are unknown (Brown 1990). Positive test results can be confirmed by HAI and plaque reduction NT. In addition, with the availability of sequence data for a variety of reovirus genes, a number of molecular techniques have been developed. Dot-blot hybridization, *in situ* hybridization and PCR methods have been used to detect reovirus genomic and mRNA in infected tissues and cells (Parashar et al. 1992;Steele et al. 1995;Tyler et al. 1998). It is likely these techniques will be used more often in the future.

Reovirus is grouped into four serotypes based on HAI and NT tests (Tyler 2001). Determination of reovirus serotype has historically been based on the capacity of a panel of serotype-specific antisera to inhibit hemagglutination (HA) by the test isolate (Rosen 1960). Plaque reduction NT assays with polyclonal antisera can also be used, but are

generally not as reliable as HAI tests (Virgin et al. 1988;Goral et al. 1996). The major-serotype-specific reovirus antigen recognized in HAI and NT assays with type-specific polyclonal antisera is the σ1 protein (Weiner and Fields 1977;Weiner et al. 1978). The availability of type-specific neutralizing monoclonal antibodies directed against the σ1 protein of the prototype virus strains, T1L and T3D, allows a sample to be serotyped based on plaque reduction assays or immunocytochemical reactions (Virgin et al. 1991;Tyler et al. 1993) with greater reliability than with polyclonal antisera (Virgin et al. 1988;Goral et al. 1996).

1.8. Treatment. Reoviruses have not been definitively shown to produce human disease; thus, there has only been limited investigation of antiviral therapy. The administration of antiviral antibodies, virus specific immune cells and protease inhibitors have been tested in experimental animals (Fields 1992). Since ARV is a major cause of economic loss in the poultry industry a variety of vaccines have been developed for veterinary use against ARV with either β-propriolactone or formalin-inactivated viruses and have been shown to induce high-titre antibody responses (Tyler 2001).

1.8.1. Antiviral agents. The activity of a number of antiviral agents against reovirus has been tested in cell culture systems (Table 5). A number of agents have been reported to have *in vitro* activity, but have never been tested *in vivo*. These include neplanocin A and some of its derivatives (De Clercq et al. 1989;Obara et al. 1996;Robins et al. 1998), ribavirin, (Rankin, Jr. et al. 1989;Connolly and Dermody 2002;Labrada et al. 2002), acivicin (Keast and Vasquez 1992), cicloxolone sodium (Dargan et al. 1992) cyclopentenylcytosine (De Clercq et al. 1990;De Clercq 1993) and mycophenolic acid (Gong et al. 1999).

1.8.1.1.Ribavirin. Ribavirin was first described as a broad-spectrum antiviral agent in 1972 (Sidwell et al. 1972). Since then, various analogues have been synthesized (e.g. EICAR, FICAR, tiazofurin, selenazofurin). These derivatives have *in vitro* activity against at least 12 DNA- and 40 RNA-containing viruses including paramyxoviruses, flaviviruses, picornaviruses, orthomyxoviruses, arenaviruses, reoviruses and bunyaviruses and *in vivo* activity against about half as many (For reviews, see (De Clercq 1993;Crotty et al. 2002)). Ribavirin has been approved as an inhaled agent for treatment of severe respiratory syncytial virus infection (Wyde 1998) and orally, in combination

Table 5. Activity of various classes of antiviral agents against reovirus

Class of agent	Activity[a]
Sulfated polysaccharides (e.g. dextran sulphate)	−
SAH hydrolase inhibitors (e.g. neplanocin A)	+
OMP decarboxylase inhibitors (e.g. pyrazofurin)	−
CTP synthetase inhibitors (e.g. cyclopentenylcytosine)	+
IMP dehydrogenase inhibitors (e.g. ribavirin)	+
Nonnucleoside IMP dehydrogenase (e.g. mycophenolic acid)	+
Acyclic guanosine analogues (e.g. ganciclovir)	−
Thymidine analogues (e.g. (E)-5-(2-bromovinyl)-2'-deoxyuridine)	−
Acyclic nucleoside phosphonates (e.g. phosphonoformate)	−
Dideoxynucleoside analogues (e.g. AZT)	−
Specific HIV-1 reverse transcriptase inhibitors (e.g. TIBO)	−
Glutamine analogue (e.g. acivicin)	+
Unknown (e.g. cicloxolone sodium)	+

* modified from (De Clercq 1993;Tyler 2001).

with alpha interferon for the treatment of hepatitis C virus infection (Davis et al. 1998;McHutchison et al. 1998).

There is much debate regarding the mechanism of antiviral activity of ribavirin and its derivatives. Two main mechanisms have been proposed. In the 1970's, inosine monophosphate (IMP) dehydrogenase was recognized as the target for the antiviral action of ribavirin (Streeter et al. 1973). IMP dehydrogenase (IMPDH) catalyzes the rate-limiting step in the *de novo* biosynthesis of purine mononucleotides and is involved in the early steps of GMP synthesis (Figure 4). It is responsible for the conversion of IMP to xanthosine monophosphate, which is further converted to GMP, GDP, dGDP, GTP and dGTP. This can also lead to diminished formation of ATP and dATP because GTP is a cofactor in the conversion of IMP to succinyl AMP by adenylosuccinate sythetase. (For review, see (De Clercq 1993)). IMPDH inhibitors are expected to affect viral RNA and/or DNA synthesis mainly when there is an increased need for synthesis as in the case of virus-infected cells. This mechanism of action for ribavirin is attractive because it potentially explains the broad-spectrum antiviral activity. A recent study of the antiviral activity of ribavirin on the replication of flavi- and paramyxoviruses found a linear correlation over a broad concentration range between ribavirin and GTP pool depletion (Leyssen et al. 2005). This provides compelling evidence that the predominant mechanism of action of ribavirin *in vitro* against these viruses is based on inhibition of cellular IMPDH activity. However, a variety of studies have suggested that inhibition of IMPDH is not sufficient for the antiviral activity and that a second, more potent, mechanism is also present (Crotty et al. 2002).

The inhibition of IMPDH is dependent on the intracellular conversion of ribavirin to its 5'-monophosphate form; however, ribavirin can be also phosphorylated to its 5'-triphosphate. Another proposed mechanism suggests ribavirin acts as a lethal mutagen of viral RNA genomes in its triphosphorylated form. Ribavirin triphosphate accumulates in cells after treatment and has been shown to be incorporated by the poliovirius polymerase as a nucleotide analogue (Crotty et al. 2000;Crotty et al. 2001). Incorporation of ribavirin does not act as a chain terminator, but leads to an accumulation of mutations that decrease the fitness of the virus and ultimately is lethal. This mechanism also provides an explanation to the broad spectrum action of ribavirin against RNA viruses. It is believed

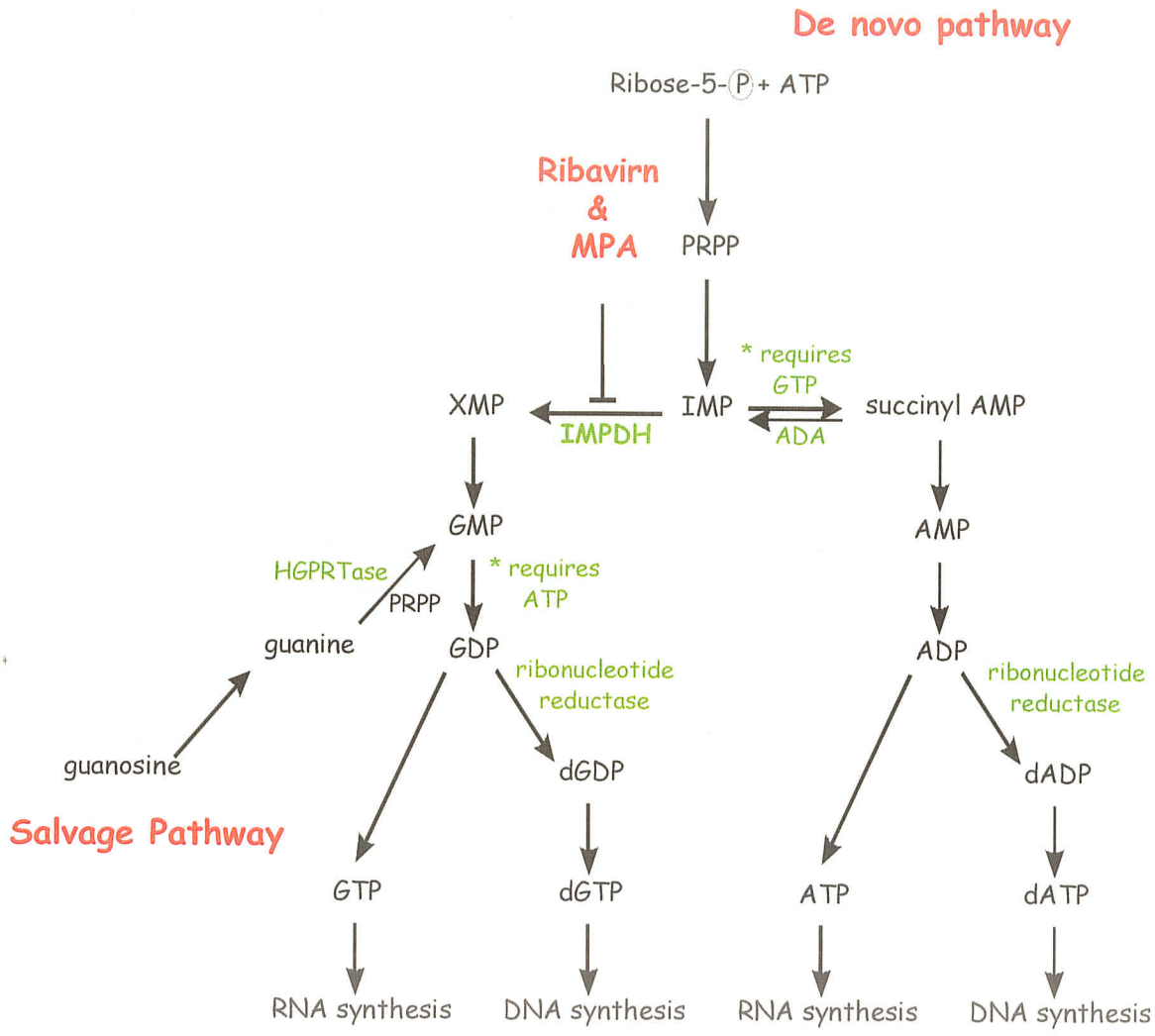

De novo pathway
Ribose-5-P + ATP
Ribavirn
&
MPA
PRPP
* requires
GTP
XMP
IMP
succinyl AMP
IMPDH
ADA
GMP
AMP
HGPRTase
PRPP
* requires
ATP
guanine
GDP
ADP
ribonucleotide
reductase
ribonucleotide
reductase
guanosine
dGDP
dADP
Salvage Pathway
GTP
dGTP
ATP
dATP
RNA synthesis
DNA synthesis
RNA synthesis
DNA synthesis

Figure 4. Pathways of purine biosynthesis

Ribavirin and mycophenolic acid inhibits IMPDH, which directly causes depletion of the guanine nucleotides (GMP, GDP, dGDP, GTP and dGTP) and potentially causes a decrease in succinyl AMP leading to a decrease in adenine nucleotides. Figure compiled from data in references (De Clercq 1993;Lehninger et al. 1993;Allison and Eugui 2000).

the lethal mutagenesis is enhanced by the ability of ribavirin monophosphate to inhibit IMPDH and decrease intracellular GTP pools. The decrease in GTP pools allows for an increase in the frequency with which ribavirin triphosphate is incorporated as a mutagen.

Several other mechanisms of ribavirin action have been proposed. On the surface they do not appear to explain the broad spectrum of antiviral activy of ribavirin, but have yet to be fully explored. It has been observed phosphorylated forms of ribavirin inhibit influenza (Eriksson et al. 1977) and vesicular stomatitis virus RNA polymerases (Fernandez-Larsson et al. 1989) and HIV reverse transcriptase (Fernandez-Larsson and Patterson 1990). It has also been proposed ribavirin can inhibit viral messenger RNA cap formation (Goswami et al. 1979;Scheidel and Stollar 1991). This mechanism is based on observations that ribavirin does not significantly inhibit replication of poliovirus, which lacks a 5' cap (Sidwell et al. 1972).

Ribavirin has been reported to inhibit production of both mammalian and avian reovirus viral ssRNA and dsRNA at concentrations that do not significantly alter the expression of cellular genes or the capacity of mammalian and avian reoviruses to induce apoptosis (Rankin, Jr. et al. 1989;Bodelon et al. 2002;Connolly and Dermody 2002;Labrada et al. 2002).

1.8.1.2.Mycophenolic acid. Mycophenolic acid (MPA) is a non-nucleoside non-competitive, reversible inhibitor of eukaryotic IMPDH (Figure 4; for detailed review, see (Allison and Eugui 2000)). IMPDH inhibition decreases levels of intracellular guanine nucleotide pools required for adequate RNA and DNA synthesis; therefore inhibition of IMPDH with MPA has been shown to have antiproliferative (Carter et al. 1969), immunosuppressive (Mitsui and Suzuki 1969), antimicrobial (Mizuno et al. 1974), antiviral (Williams et al. 1968;Sidwell et al. 1972) and antiparasitic (Berman and Webster 1982;Hupe et al. 1986) effects. Currently, MPA is used clinically to prevent rejection of transplanted kidneys and hearts in combination with steroids and cyclosporine A (Sollinger 1995;Behrend et al. 1997;Kobashigawa et al. 1998). MPA can inhibit the replication of several viruses *in vitro* (Malinoski and Stollar 1981;Ichimura and Levy 1995;Neyts and De Clercq 1998;Neyts et al. 1998;Gong et al. 1999;Margolis et al. 1999;Diamond et al. 2002;Morrey et al. 2002) and potentiates the inhibitory effects of acyclic guanosine analogues (such as acyclovir, penciclovir and ganciclovir) against

herpesviruses (Neyts and De Clercq 1998;Neyts et al. 1998). MPA also potentiates the activity of nucleoside analogues against HIV (Margolis et al. 1999;Heredia et al. 1999;Hossain et al. 2002). This potentiation is thought to occur by an enhancement of antiviral activity caused by depletion of normal dGTP substrate pools, which decreases the competition that nucleoside analogues experience from the normal substrate (dGTP) during the DNA polymerization reaction. Thus, incorporation of the analogue and chain termination is increased (De Clercq et al. 2001;De Clercq 2001a). Despite some progress made in understanding the antiviral properties of MPA, the potential application of its use as a broad spectrum antiviral agent against both positive- and negative-stranded RNA viruses has not been fully realized.

1.9. History of Potential Novel Reovirus Isolate. The focus of this research is on a clinical viral isolate received from Cadham Provincial Laboratory (Winnipeg, Manitoba). The isolate was from an 8-week-old infant girl hospitalized in November 1997 at the Children's Hospital (Winnipeg, Manitoba) for investigation of congenital immune deficiency. She presented with a three-week history of active varicella complicated with *E. coli* sepsis, persistent oral thrush, hypoalbuminemia, intermittent fevers with temperature spikes to 39°C, diarrhea and feeding intolerance. On day 12 of her hospitalization in the Children's Hospital, she developed respiratory distress and irritability and refused to feed. On physical examination, she was found to be hypothermic with a temperature of 34.8°C. Respiratory rate was 50 per minute, heart rate was 140 beats per minute and her blood pressure was 100 over 50. Indrawing and nasal flaring were noted. Course rales were heard throughout her lung fields. She had purulent discharge at the exit site of a previously inserted central line. She also had extensive resolving varicella lesions and was edematous. Complete blood count showed a total white blood cell count of 30,100 cells/mm^3 (N=5,000-15,000 cells/mm^3) with 7,525 mature polymorphonuclear cells, 14,448 band forms, 903 metamyelocyte, 301 myelocyte, 301 promyelocyte, 3,311 lymphocyte and 3,010 monocyte cells. Her hemoglobin had decreased from 106 to 56g/L (N=90-140g/L) and her platelet count had dropped from 903 x 10^9 to 34 x 10^9/L (N=150-500 x 10^9/L). Serum electrolytes, renal and liver function tests were normal. Blood cultures grew *Staphylococcus aureus.* CSF fluid was obtained with difficulty and sent only for bacterial and viral culture. Both reovirus serotype 2

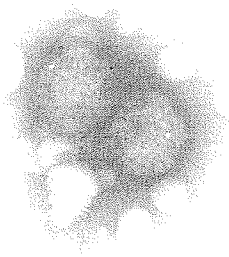
(Figure 5A) and two strains of *Staphylococcus aureus* (one of which was methicillin resistant) were recovered from the CSF sample. A fecal sample and second CSF sample taken at later time points were also culture positive for reovirus, serotype 2. In addition, rotavirus particles were identified in the fecal sample by electron microscopy (EM) (Figure 5B).

The infant was empirically treated with cloxacillin and gentamicin. When antibiotic susceptibilities were reported, cloxacillin was replaced with vancomycin and rifampin. Over the next 24 hours, her respiratory distress worsened and she developed adult respiratory distress syndrome and required intubation and mechanical ventilation for 16 days. She died two months later as a result of sepsis.

Laboratory investigations found the infant to have a congenital immunodeficiency involving profound hypogammaglobulinemia with serum IgG concentrations of 0.55g/L (N=2.61-7.32g/L), IgA values less than 0.07g/L (N=0.06-0.55g/L), IgM values of 0.18g/L (N=0.15-0.64g/L), and IgD less than 7.0mg/L (adult N=0-140mg/L). Her total lymphocyte counts were in the normal range. Repeated studies of T and B cell markers showed there were normal percentages of the major lymphocyte subsets, but the absolute numbers of CD4 cells were reduced for age ranging from 884 to 1,104 cells/mm^3 (N > 1,500 cells/mm^3). Lymphocyte stimulation tests were normal initially, but lymphocytes collected a week before death, when the infant was severely ill, failed to respond. Microscopic examination findings of the lymphatic tissues at autopsy were compatible with the diagnosis of severe combined immune deficiency.

The initial passage of CSF was in primary rhesus monkey kidney cells (RMK), which showed CPE over 48 hours. Standard viral screens employed by Cadham Provincial Laboratory (Winnipeg, Canada) ruled out infection by herpes simplex virus, adenovirus and other enteroviruses. The isolate was determined to be a reovirus serotype 2 based on NT with serotype-specific polyclonal antisera done by the provincial laboratory. This was repeated for all three culture-positive isolates (see above) and all samples were shown to be reovirus serotype 2 (B. Wells, Cadham Provincial Laboratory, personal communication). No reaction was seen with reovirus antisera for serotype 1 or type 3 (B. Wells, Cadham Provincial Laboratory, personal communication). In the past, almost all reovirus clones have been isolated from respiratory or gastrointestinal

A.



B.



0.1 μm

Figure 5. Electron micrograph of reovirus particles isolated from clinical samples

(A) EM of reovirus particles identified in tissue culture fluid from primary RMK cells inoculated with CSF fluid. **(B)** Double-shelled rotavirus particles identified by EM in fecal sample from which reovirus was also recovered by cell culture. EM samples were prepared as described in section 2.4. Bar = 100 nm.

secretions with only a few isolated cases where reovirus was isolated from CNS samples. Since no other known reovirus clones isolated from CSF have been identified as reovirus serotype 2, we hypothesized this viral clone must be unique and tentatively named the clone, Type 2 Winnipeg (T2W). The above case report has been published (Hermann et al. 2004).

1.10. Hypothesis and Objectives of Study. The role of reoviruses in human disease remains uncertain. It is difficult to provide evidence to link reovirus infection to symptomatic human infection, especially CNS infection. Most infections are asymptomatic or produce minor upper respiratory or gastrointestinal symptoms that are indistinguishable from other illnesses seen in infancy or early childhood. There are only a few isolated reports presented in the literature, which associate reovirus with CNS disease (Krainer and Aronson 1959;Joske et al. 1964;Johansson et al. 1996;Tyler et al. 2004). We hypothesize that T2W is a novel reovirus strain associated with CNS symptoms. To test the hypothesis, the objectives of this research work include:

1) grow and purify enough T2W for subsequent experiments;
2) complete molecular and biological characterization of T2W; and
3) examine antiviral agents which may be used for the treatment of a CNS infection with T2W.

Characterization of T2W often required virions in a purified state in the absence of cellular debris. In the past, organic solvent extraction of virions from cellular components with Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane) was used in various viral purification methods (Gomatos and Tamm 1963;Gschwender and Traub 1978;Berman et al. 1981;Richt et al. 1993;Liebermann and Mentel 1994). With its unique physical and chemical properties, Freon is ideal for extracting lipid materials without extracting proteinaceous material such as noneveloped viruses. However, Freon has been implicated in ozone layer depletion and its use has been progressively eliminated and is now prohibited. The unavailability of Freon removes a vital step in virus purification. Therefore, it was necessary to find a substitute solvent with similar physical and chemical properties as Freon. The ability of possible solvent replacements, identified based on their Hansens's solubility parameters, to effectively purify large quantities of virus without affecting virion morphology and infectivity was examined. Vertrel® XF

(1,1,1,2,3,4,4,5,5,5-decafluoropentane) was identified as a suitable solvent for viral purification.

T2W is a clinical isolate obtained from the CSF of an immunocompromised infant and also was identified in the infant's stool. The infant exhibited respiratory, gastrointestinal and CNS symptoms, which may have been due to the reovirus infection. Viral diseases have historically been considered difficult to treat with selective antiviral chemotherapy since it was believed the viral life cycle was too closely related to host cell metabolism and any attempt to suppress viral replication would kill or harm uninfected cells. In the past decade, knowledge of viral replication has allowed for the development of a variety of antiviral agents targeted specifically at the virus' life cycle. Inhibition of reovirus replication has been achieved with a selection of antiviral compounds *in vitro* (De Clercq et al. 1989;Rankin, Jr. et al. 1989;De Clercq et al. 1990;Dargan et al. 1992;Keast and Vasquez 1992;Obara et al. 1996;Robins et al. 1998;Gong et al. 1999;Connolly and Dermody 2002;Labrada et al. 2002;Diamond et al. 2002). This research examined a variety of compounds' ability to attenuate reovirus infection. MPA was the most effective at inhibiting prototype reovirus infection and was also successful at inhibiting T2W replication.

2. *MATERIALS AND METHODS*

2.1. Stock cells and viruses. Spinner-adapted mouse fibroblast L929 cells were grown in suspension culture at a density of 4.5 – 5.0 x 10^5cells/mL in Joklik's modified minimal essential medium (MEM) (Gibco, Grand Island, NY) supplemented with 2.5% fetal calf serum (FCS) (Intergen, Purchase, NY), 2.5% agammaglobulin-neonate bovine viable serum protein (VSP) (Biocell, Carson, CA) and 2mM ℓ-glutamine. T1L, T2J and T3D are laboratory stocks. In 1997, Cadham Provincial Laboratory (Winnipeg, Manitoba) isolated type 2 Winnipeg (T2W) from a clinical sample of CSF from a severe combined immunocompromised infant with respiratory, gastrointestinal and CNS symptoms. Initial inoculation and virus detection was on primary rhesus monkey kidney (RMK) cells. Other reovirus clones used in this study were kindly donated by T. Dermody, Vanderbilt University, and are listed in Table 6. T1L X T3D reassortant viruses were originally isolated as described (Drayna and Fields 1982b;Brown et al. 1983;Coombs et al. 1990) and genotypes confirmed as described (Nibert et al. 1996). Viral infections were carried out either in monolayer (grown in the presence of 5% CO_2 at 37°C) or suspension (grown at 33.5°C) supplemented with 100U/mL of penicillin, 100μg/mL streptomycin sulfate and 100μg/mL amphotericin-B as previously described (Coombs et al. 1994a).

2.2. Virus passaging and plaque assay.

2.2.1. Virus passaging. Wild type reovirus and clones were amplified through two or three passages in mouse fibroblast L929 cells in MEM supplemented as described above. 2 x 10^6 cells were added to T25 Corning flasks (Fisher, Nepean, ON) and incubated overnight or 4 x 10^6 cells were incubated for 1 to 2 hours. Cells were infected when they reached 90% confluency. The media was removed saving some as "pre-adapted" media and cells were infected with 0.5mL of plaque-purified P_0 stock (see section 2.2.3). The virus was allowed to adsorb to the cell monolayer for 1 hour at room temperature with periodic rocking every 10 – 12 minutes. After adsorption, 4.5mL of MEM supplemented as described above and with 25% pre-adapted media was added to the flask and the infected monolayer was incubated at 37°C. Cells were examined daily for CPE. When 80 - 90% CPE was observed, flasks were frozen at -80°C. They were frozen and thawed three times and the P_1 cell lysates were collected. A volume of 0.5mL of P_1 virus was

Table 6. Reovirus clones used for sequence comparisons

Viral Strain	Serotype	Abbreviation	Species	Site	Year
Winnipeg	2	T2W	human	Winnipeg	1997
Lang	1	T1L	human	Ohio	1953
Jones	2	T2J	human	Ohio	1955
Dearing	3	T3D	human	Ohio	1955
Abney	3	T3A	human	Washington, DC	1957
Clone 11	1	T1C11	human	Washington, DC	1957
Clone 62	1	T1C62	human	Washington, DC	1957
Clone N67	1	T1N67	human	Netherlands	1967
Clone N84	1	T1N84	human	Netherlands	1984
Clone N85	1	T1N85	human	Netherlands	1985
Clone N73	1	T1N73	human	Netherlands	1973
Clone N84	1	T1N84	human	Netherlands	1984
SV59	2	SV59	simian	Maryland	1958
Tokyo	2	T2Tok	human	Tokyo	1990
Clone 8	3	T3C8	human	Tahiti	1960
Clone 9	3	T3C9	murine	France	1961
Clone 18	3	T3C18	bovine	Maryland	1961
Clone 31	3	T3C31	bovine	Maryland	1957
Clone 43	3	T3C43	bovine	Maryland	1960
Clone 44	3	T3C44	bovine	Maryland	1960
Clone 45	3	T3C45	bovine	Maryland	1960
Clone 84	3	T3C84	human	Washington, DC	1957
Clone 93	3	T3C93	human	Washington, DC	1955
Clone N83	3	T3CN83	human	Netherlands	1983
Feline	3	T3Fel	feline	California	1968
Colorado	3	T3Col	human	Colorado	1996

used to infect T75 flasks in a similar manner to generate a larger volume of P_2 cell lysate. On day 2 of the infection, $2/3^{rds}$ of the media was removed from the T75 and replaced with fresh supplemented MEM. If further passage of virus (P_3) was required, cell monolayers in T75 flasks were infected at a multiplicity of infection (MOI) between 0.1 and 5 plaque forming units (PFU) per cell. Cells were harvested as described for P_1 cell lysate. The dsRNA genomes were extracted from the cytoplasm of the cells and the presence of virus confirmed by SDS-PAGE as described below (section 2.5).

2.2.2. Plaque Assay. 1 x 10^6 L929 cells were plated onto Corning Costar 6-well cluster dishes (Fisher, Nepean, ON) and incubated overnight at 37°C with 5% CO_2 to form monolayers. Serial 1:10 dilutions of virus were made 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). On day 1, cell monolayers were infected in duplicate with 100µL of each dilution and virus was allowed to adsorb for 1 hour with periodic rocking every 10 – 12 minutes. After adsorption, each well was overlaid with 3mL of a 50:50 ratio of 2% agar and 2x Medium 199 (M199) supplemented with 2.5% FCS, 2.5% VSP and a final concentration of 2mM ℓ-glutamine, 100U/mL of penicillin, 100µg/mL streptomycin sulfate and 100µg/mL amphotericin-B. The infections were incubated at 37°C. On day 4 (3 days pi) cells were fed with 2mL fresh agar/M199 (supplemented as above). Cells were stained on day 7 with a 0.04% neutral red solution (neutral red in a 1:1 ratio of 2x PBS to 2% agar). Viral plaques were counted 18 – 24 hours later and titres calculated.

2.2.3. Plaque Purification. To replenish viral stocks, viral plaques were isolated and picked. Samples were infected, overlaid and incubated as described earlier (see section 2.2.2). Plaques were stained with 0.04% neutral red, incubated for approximately 18 hours and picked ($P_{0,\#1}$) with a sterile pasteur pipette. The viral/agar plugs were re-suspended in 0.5mL MEM supplemented with antibiotics and placed at 4°C for 24 hours to allow viral diffusion into the media. $P_{0,\#1}$ stock was used as the inoculum for an additional round of plaque purification to yield $P_{0,\#2}$ stock. The double plaque purified stock ($P_{0,\#2}$) was amplified twice (P_1, P_2) and harvested by freeze/thawing three times. To ensure picked plaques were identical to the initial clinical isolate, growth characteristics and sequence data were compared.

2.3. Purification of reovirus particles.

2.3.1. Virus amplification. To purify large amounts of virus particles, batch infections of $\geq$ 500mL were set up. For a 1 L batch infection, 6.5 x 10^8 cells were centrifuged at 700rpm for 10 minutes in an IEC refrigerated floor centrifuge. The cell pellet was re-suspended in fresh MEM supplemented as above for virus infections to a final concentration of 1 – 2 x 10^7cells/mL, which included the addition of viral cell lysate. Virus from a P_2 or P_3 stock was added at a MOI of 3 – 5 PFU/cell. The virus was allowed to adsorb for 1 hour at room temperature with periodic swirling every 10 – 12 minutes, then the infected cells were diluted to 7 x 10^5 cells/mL with 75% fresh MEM and 25% pre-adapted media. The suspension of infected cells was incubated at 33.5°C for 65 hours for most clones. Some slower growing clones required longer incubation times. In these cases, the infected cells were fed fresh media at 48 hours. Cells were centrifuged at 700rpm for 10 minutes in an IEC floor centrifuge. Media was decanted and cells re-suspended in 75% fresh MEM supplemented as above and 25% pre-adapted media. Cells were harvested when there was 60% CPE determined by Trypan Blue exclusion using a hemocytomer. After incubation, cells were centrifuged at 3,500rpm for 20 minutes in a fixed-angle rotor (JA-10) in a Beckman RC centrifuge (Beckman, Mississauga, ON). Cells were re-suspended in 12mL homogenization buffer (HO Buffer) (10mM Tris, pH 7.4, 250mM NaCl, 10mM β-mercaptoethanol) and transferred to a 30mL COREX tube and total volume was recorded. This mixture could be used immediately for viral purification or frozen at -80°C for later use.

2.3.2. Virus purification. Cells in HO Buffer were thawed if frozen and kept on ice throughout. Samples were sonicated for 10 seconds to disrupt cell membranes and any cell clumps formed during freezing. Cell membranes were further disrupted with the addition of 1/50th the sample volume of 10% desoxycholate (DOC). Samples were briefly vortexed then incubated on ice for 30 minutes. Virus particles were obtained by organic solvent extraction with Freon 113 (Freon) (1,1,2-trichloro-1,2,2-trifluoroethane) (Caledon Laboratories, Ltd., Georgetown, ON) or Vertrel® XF (Vertrel) (1,1,1,2,3,4,4,5,5,5-decafluoropentane) (DuPont Chemicals, Wilmington, DW). A volume of solvent equal to 2/5th of the sample volume was added to the cell suspension and emulsified by sonication for 30 seconds or more at 35% duty cycle with sonicator probe (Vibra Cell, Sonics & Materials Inc., Danbury, CN). A second aliquot of 2/5th volume of the same solvent was

added and sample re-emulsified before centrifugation at 9,000rpm for 10 minutes in a fixed-angle rotor (JA-20) in a Beckman RC centrifuge. Two phases were present after centrifugation. The top aqueous layer was collected with a plastic pipette and transferred to a fresh 30mL COREX tube. The bottom organic layer was discarded. A second extraction was completed by addition of $9/10^{th}$ volume of solvent to the sample and re-emulsified and re-centrifuged as above. If the collected aqueous phase remained hazy a third solvent extraction was completed. The resultant aqueous phase was layered onto a pre-formed 1.2 – 1.4g/mL cesium chloride gradient made in a SW-28 tube (Beckman Instruments Inc., Palo Alto, CA). Samples were ultracentrifuged for a minimum of 5 hours in a Beckman SW-28 rotor at 25,000rpm. Purified virus particles formed a band within the gradient that was collected through a puncture in the bottom of the tube. The density was checked by refractometer (Bausch & Lomb, USA) then loaded into dialysis tubing for extensive dialysis against dialysis buffer (D-buffer) (150mM NaCl, 15mM $MgCl_2$, 10mM Tris, pH 7.4) to remove cesium chloride. Virus was collected in appropriate storage vessels and stored at 4°C or frozen with 25% glycerol or 10% dimethylsulfoxide (DMSO). Before addition of glycerol or DMSO, the concentration of purified virus particles was measured by UV spectroscopy where 1 OD_{260} = 2.1 x 10^{12} particles/mL (Smith et al. 1969).

2.4. Electron microscopy of reovirus particles. For investigation by EM, gradient purified virus particles were prepared by standard drop method described elsewhere (Hammond et al. 1981;Hazelton and Coombs 1995). Briefly, a drop of untreated virions was placed on clean parafilm. In some cases, virions were diluted 100-fold with D-buffer and 0.1% glutaraldehyde was added to stabilize viral structures. Samples were mounted on a 400-mesh formvar-coated electron microscopy copper grid (3.05 mm) by placing the carbon side down onto the surface of a sample drop for 30 seconds. Excess sample was wicked away by touching a clean piece of filter paper to the edge of the grid. The sample was then negatively stained with 1.2mM phosphotungstic acid, pH 7.0 for 30 seconds and excess stain was wicked away. Samples were viewed with a Philips model 201 transmission electron microscope at magnifications of 100,000x or 200,000x. Electron micrographs were photographed onto Kodak 5303 direct positive film and printed onto Kodak Polycontrast III paper.

2.5. SDS-PAGE. All samples to be resolved by SDS-PAGE contained 1x electrophoresis sample buffer (ESB) (240mM Tris-HCl, pH 6.8, 1.5% dithriothreitol, 1% SDS). For RNA analysis, 10% SDS slab gels (16 x 16 x 0.15 cm) were poured and allowed to polymerize for 1 hour. 5 – 15% gradient SDS gels (16 x 16 x 0.1 cm) were poured and allowed to polymerize overnight for protein analysis. RNA and protein samples were heated to 65° and 95°C respectively for 3 – 5 minutes before equal numbers of virus particles (1 x 10^{11} particles for RNA analysis, 7 x 10^{11} particles for protein analysis) were loaded. Resolution was at 18mA and 250V for 45 hours for separation of RNA gene segments or at 5mA and 200V overnight for separation of structural proteins. Gels were stained with ethidium bromide (3μg/mL) and RNA gene segments visualized with UV irradiation by a Gel Doc 2000 (BioRad) apparatus. Protein gels were fixed in an 30% isoproponal / 10% acetic acid solution and stained with Coomassie Brilliant Blue R250 (0.5% Coomassie Brilliant Blue solution). Gels were dried between layers of cellophane for preservation.

2.6. Determination of serotype. Serotype was determined by Cadhman Provincial Laboratory using NT. Antisera to serotype 1, 2 and 3 reovirus was inactivated at 56°C for 30 minutes after dilution to 20U. Serial 1:10 dilutions of virus were made in gel saline and 200μL of antiserum was added to 200μL of the diluted virus. Hank's balanced salt solution with 2% FCS was used as a virus control. The virus-antiserum solution was mixed by hand for 5 – 10 seconds then incubated at 37°C for 1 hour with periodic mixing. RMK cells were inoculated with 200μL of the virus-antiserum mixture and incubated at 37°C. The cultures were examined every day for the presence of CPE. Identification of serotype was made by neutralization of specific antisera with a minimum of a 2+ CPE difference (i.e. minimum CPE difference of 50%) between the virus control tube and the neutralized tube.

2.7. Determination of T2W S gene sequences.

2.7.1. Virus template preparation. Virus template was obtained from purified T2W virions. In a 17 x 100 mm "snap-cap" polypropylene tube, 2 – 6 x 10^{13} virions were diluted with 150μL 1M Tris, pH 8.0, 15μL 200mM EDTA, pH 8.0 and 300μL 10% SDS and D-buffer to a total volume of 2mL. The mixture was vortexed then incubated at 42°C for 15 minutes. The genomic dsRNA was isolated by two rounds of phenol/chloroform

extraction followed by one round of chloroform only extraction. Briefly, 60μL of 5M NaCl and 3mL of phenol/chloroform (1:1 v/v ratio) was added to the diluted virion mixture. The sample was vortexed to mix thoroughly, reheated to 42°C for 5 minutes, followed by centrifugation at 6,000rpm, 15 – 20°C for 5 minutes (Beckman JA-20). The aqueous phase was collected, transferred to a sterile "snap-cap" tube and phenol/chloroform extraction was repeated. The aqueous phase from this second extraction was collected and extracted a third time with 3mL of chloroform only. The aqueous phase was precipitated overnight at -20°C in a silanized COREX tube by addition of 0.1 volume of 3M sodium acetate and 2.5 volumes of ice-cold ethanol. The precipitated dsRNA was pelleted at 9,000rpm, 5°C for 30 minutes (Beckman JA-20). The gelatinous pellet was washed with 70% ethanol and re-suspended in 100μL of 90% DMSO / 10% 10mM Tris, pH 6.8. For nested-PCR reactions, virus stock (i.e. sample received from Cadham Provincial Laboratory) was used as starting material and volumes were scaled to micro levels, i.e. 250μL of diluted stock versus 2mL of a diluted purified virion mixture.

2.7.2. Primer design for S gene segments. The nucleotide sequences for the prototype viruses and many other reovirus clones are known for the four S gene segments and are available in Genbank. The S gene sequences for T1L were used to design terminal primers for T2W (Figures 6 – 9). The terminal upper strand primer for each S gene segment is complementary to bases that were 5' or contained the start codon. The terminal lower strand primer for each S gene segment are situated beyond the stop codon. Internal primers were designed as sequence was determined and were used to 'walk' along gene segments. Primers were designed with PrimerSelect (DNASTAR Inc., Madison, WI) and ordered in a desalted form from GIBCO BRL (Life Technologies). Primers were resuspended in DEPC-treated water and their concentration determined by UV spectroscopy.

2.7.3. Reverse transcriptase polymerase chain reaction (RT-PCR). dsRNA template was converted to ssDNA by reverse transcriptase (RT). A 3μL volume of purified dsRNA template (prepared as described in section 2.7.1) was heated for 45 minutes at 50°C to denature the strands. Samples were immediately transferred to an ice/water bath and "snap-cooled" by the addition of 31.5μL of ice-cold DEPC-treated ddH_2O (double-

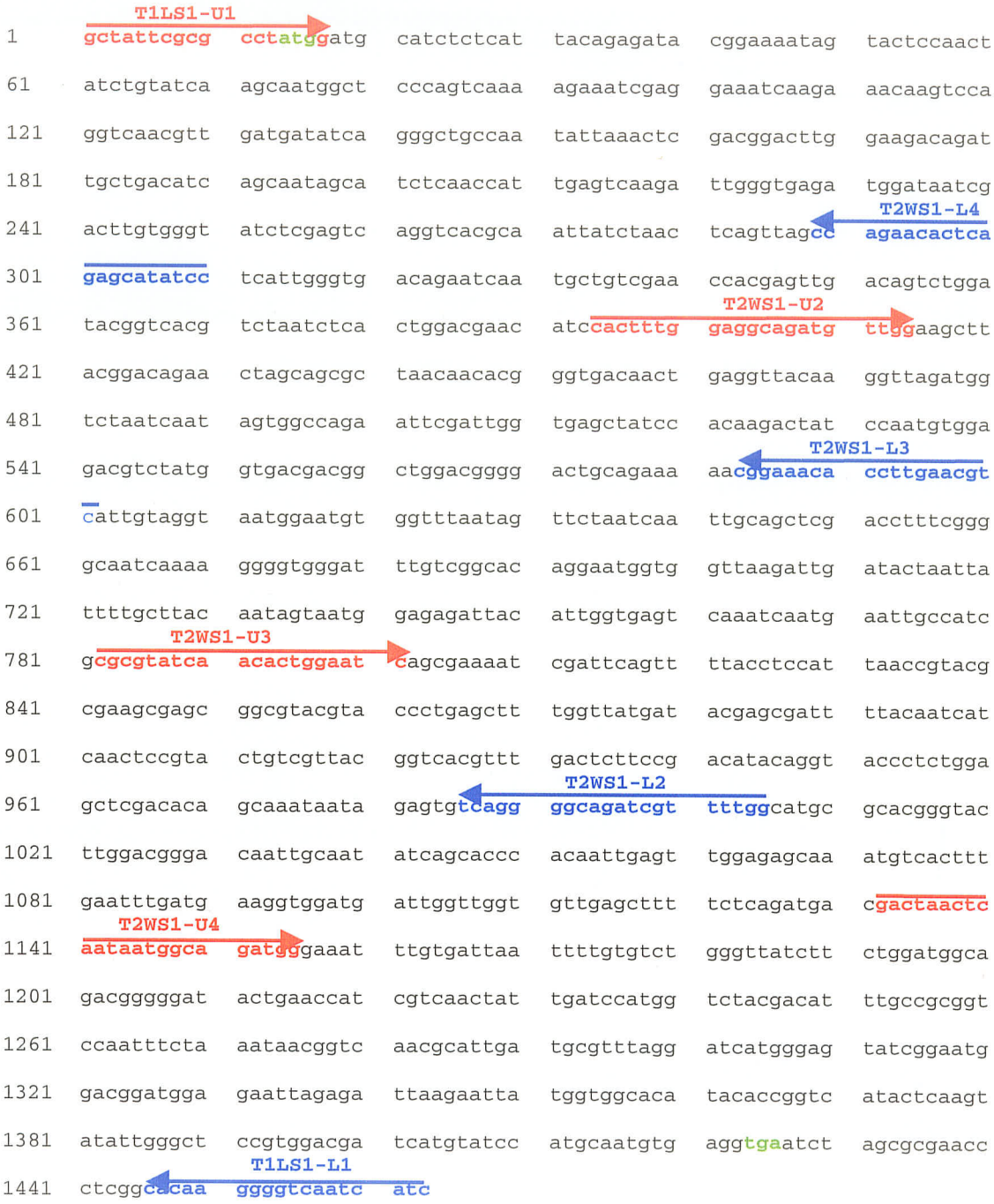

T1LS1-U1
T2WS1-L4
T2WS1-U2
T2WS1-L3
T2WS1-U3
T2WS1-L2
T2WS1-U4
T1LS1-L1

Figure 6. T1L S1 cDNA sequence and designed primers

The plus strand of the double-stranded cDNA sequence is shown (1,463 nucleotides). Primers were designed to sequence the entire ORF of the T1L S1 gene segment in both directions (Genbank Accession # M35963). Red arrows represent primers whose sequences are identical to the bases shown and read in the forward direction. Blue arrows represent primers whose sequences are complementary to the bases shown and read in the opposite direction. The start and stop codons are highlighted in green.

T1LS2-U1

```
   1 gctattcgct ggtcagttat ggctcgcgct gcgttcctat tcaagactgt tggatttggt
  61 ggtctgcaaa atgtgccaat taatgatgag ttatcgtcac atctacttcg agccggtaat
 121 tcgccatggc agctgactca gttcttagat tggataagtc ttggaagagg attagctaca
 181 tcagctcttg ttccaaccgc tggttcaaga tattatcaga tgagttgttt attgagtggt
 241 accctccaga ttccatttcg tcctaatcat cgatggggag atattaggtt tctgcgtcta
 301 gtgtggtcag ctcctacgct tgacgggttg gttgttgccc caccgcaggt cttagctcag
 361 ccagcgttac aggcgcaggc agatcgagtg tatgattgtg atgattaccc attcttagca
 421 cgtgacccga gatttaagca tcgagtgtat caacaattga gcgccgtaac tctgcttaac
 481 ttgacggggt ttggtccaat ttcctatgtt cgagtagacg aggatatgtg gagtggggat
 541 gtgaaccagc ttctcatgaa ctacttcggg catacgtttg cggaaattgc gtatacatta
 601 tgtcaagctt cagccaatag accttgggag catgatggta cgtatgcgag gatgactcaa
 661 attatactgt ccttattttg gttatcatat gtcggtgtaa ttcatcagca gaacacgtac
 721 cggacgttct atttccaatg caatcggcgc ggtgatgctg ctgaagtatg gattctttcc
 781 tgctcattaa accactccgc ccagattaga ccgggtaatc gcagtctatt cgtcatgcca
 841 acaagtccag attggaatat ggacgtcaat ttgattttaa gttcgacgtt gacagggtgc
 901 ttgtgttcgg gctctcagtt accgcttatt gacaataact cagtgcctgc ggtttcgcgt
 961 aacatccatg gttggactgg cagagctggc aaccagctgc atggtttcca agtgcgacga
1021 atggtgactg aattctgtga cagattaaga cgcgatggag ttatgactca agctcagcaa
1081 aatcaaattg aagcgttggc agatcaaact caacagttta agagggataa acttgaggcg
1141 tgggctaggg aagatgatca gtataatcag gctaatccga attctacgat gttccgtacg
1201 aagccattta cgaatgcgca atggggacga ggaaataccg gagcgactag tgccgcaatt
1261 gcagcccttа tctaatcgtc ttggagtgag gggatccccc cacacccctc acgactgacc
1321 acacattcat c
```

T1LS2-L1

Figure 7. T1L S2 cDNA sequence and designed primers

The plus strand of the double-stranded cDNA sequence of T1L is shown (1,331 nucleotides). The initial primer pair was designed to sequence the entire ORF of the T1L S2 gene segment in both directions (Genbank Accession # L19774). No inner primers were designed as attempts to amplify and sequence the S2 gene segment were unsuccessful despite changes in primer design and PCR conditions (see section 3.2.3.2). Red arrows represent primers whose sequences are identical to the bases shown and read in the forward direction. Blue arrows represent primers whose sequences are complementary to the bases shown and read in the opposite direction. The start and stop codons are highlighted in green.


T1LS3-U1
T2WS3-U2
T2WS3-L3
T1LS3-L2
T2WS3-U3
T1LS3-L1

Figure 8. T2W S3 cDNA sequence and designed primers

The plus strand of the double-stranded cDNA sequence is shown (1,152 nucleotides). Primers were designed to sequence the entire ORF of the T2W S3 gene segment in both directions. Outer primers were designed based on T1L S3 sequence data (GenBank Accession # M18389). Inner primers were designed as T2W S3 sequence data was available. Red arrows represent primers whose sequences are identical to the bases shown and read in the forward direction. Blue arrows represent primers whose sequences are complementary to the bases shown and read in the opposite direction. The start and stop codons are highlighted in green.


T1LS4-U1
T1LS4-U1B
T1LS4-L3
T1LS4-U2
T1LS4-L2B
T1LS4-L2
T1LS4-U2B
T1LS4-U3
T1LS4-L1B
T1LS4-L1
1 gctatttttg cctcttccca aacgttgtcg caatggaggt gtgcttgccc aacggtcatc
61 agatcgtgga cttgattaac aacgcttttg aaggtcgtgt atcaatctac agcgcgcaag
121 agggatggga caaaacaatc tcagcacagc cagatatgat ggtatgtggt ggtgccgtcg
181 tttgcatgca ttgtctaggt gttgttggat ctctgcaacg caagctgaag catttgcctc
241 accatagatg taatcaacag attcgtcatc aggattacgt cgatgtacag ttcgcagatc
301 gtgttactgc tcactggaag cggggtatgc tgtcctttgt tgcgcagatg cacgcgatga
361 tgaatgacgt atcaccagag gatctagacc gtgtgcgtac tgagggaggt tcactagtgg
421 agttaaactg gcttcaggtt gatccaaatt caatgtttag atcaatacac tcaagttgga
481 cagatcctct gcaggtagtg gatgatcttg acactaagct ggatcaatac tggacggccc
541 tgaatctgat gattgattca tccgacttgg tgcccaactt catgatgaga gacccatcac
601 atgcattcaa tggtgtgaga ctggagggag acgcccgcca aactcaattc tctaggactt
661 tcgattcgag atcgagttta gaatggggtg tgatggttta cgattactct gagttagagc
721 atgatccatc gaagggccgt gcttacagga aggaattggt gacgccagca cgagacttcg
781 gtcattttgg attatcccac tactctaggg cgactacccc aatccttgga aagatgccag
841 ctgtattctc gggaatgttg actgggaatt gtaaaatgta tccattcatc aaaggaacgg
901 ctaagttaaa gacagtgcgc aagctagtgg attcagtcaa tcatgcgtgg ggcgtcgaga
961 agatcagata tgcgcttgga ccaggtggca tgacgggatg gtacaacagg actatgcagc
1021 aggcccccat tgtactaact cccgctgctc tcacaatgtt ctcagacacc accaagttcg
1081 gggatttgga ttatccggtg atgattggcg atccgatgat tcttggctaa acacccccat
1141 cttcacagcg ccgggcctga ccaacctggt gtgacgtggg acaggctcca ttcatc

Figure 9. T1L S4 cDNA sequence and designed primers

The plus strand of the double-stranded cDNA sequence is shown (1,196 nucleotides). Primers were designed to sequence the entire ORF of the T1L S4 gene segment in both directions (Genbank Accession # X61586). Red arrows represent primers whose sequences are identical to the bases shown and read in the forward direction. Blue arrows represent primers whose sequences are complementary to the bases shown and read in the opposite direction. The start and stop codons are highlighted in green.

distilled water). A "master mix" was prepared which contained 16U (0.4μL of 40U/μL) of RNAse inhibitor (Boehringer Manheim), 0.1μM (0.5μL of 10μM) each of the appropriate upstream and downstream primer (dependant on gene segment to be amplified), 0.5mM each of dATP, dCTP, dGTP, dTTP (2.5μL of dNTP mix with 10mM of each nucleotide), 10μL of 5x 1st Strand Buffer (250mM Tris-HCl, pH 8.3, 375mM KCl, 15mM $MgCl_2$), 0.1mg/mL (0.5μL of 10mg/mL) BSA (bovine serum albumin) (Sigma Aldrich), 1μM (0.5μL of 10μM) DTT and 80U (0.4μL of 200U/μL) Superscript II reverse transcriptase enzyme (Gibco-BRL) for each sample. A 15.3μL aliquot of master mix was added to each sample and the final volume brought up to 50μL with ddH_2O and then incubated at 42°C for 1.5 – 2 hours. Samples were used for amplification immediately as ssDNA is relatively unstable.

2.7.4. Amplification of S genes and purification of cDNA. The cDNA produced by RT as described above was amplified with the Expand High Fidelity PCR System with Expand HF Buffer (Roche Applied Science). To amplify one strand, 16μL of cDNA was diluted to a final volume of 100μL with 69.25μL ddH_2O, 0.1 μM (1 μL of 100 μM) of each terminal primer, 0.2mM each of dATP, dCTP, dGTP, dTTP (2μL of dNTP mix with 10mM of each nucleotide), 1x Expand HF Buffer, 1.5mM $MgCl_2$ (10μL of 10x concentration of Expand HF Buffer with 15mM $MgCl_2$) and 2.6U Expand High Fidelity PCR System enzyme mixture. The cycling reaction was performed on a GeneAmp PCR system 9700 (PE Applied Biosystems; Foster City, CA) for 5 cycles (94°C for 1 minute to denature cDNA, 56°C for 1 minute to allow primers to anneal and 68°C for 2 minutes for elongation) followed by 30 cycles (92°C for 1 minute to denature, 56°C for 1 minute to anneal and 68°C for 2 minutes to elongate) with a final elongation step at 68°C for 10 minutes. Samples were then cooled to 4°C and could be stored indefinitely.

To confirm the amplified cDNA product, a small aliquot of product was diluted in DNA ESB and run on a 0.9% horizontal agarose gel at 100V for ~1.5 hours in 0.5x TBE buffer (4.5mM Tris, 4.5mM boric acid, 1mM EDTA, pH 8.0) which contained 0.1% ethidium bromide to allow for DNA visualization under UV irradiation. A 1 kilobase DNA ladder (Boehringer Mannheim) or purified prototype virions were used as markers. 1,463 base, 1,152 base and 1,143 base products were observed which corresponded to S1,

S3 and S4 gene products respectively and indicated successful amplification. The S2 gene segment was not successfully amplified despite several attempts with four different primer sets. Next, a large volume of cDNA product was run on a larger 0.9% agarose gel as described above. The resultant band was excised from the gel with a sterile razor blade on top of a UV light box. The QIAquick™ gel extraction kit (Qiagen, Germany) was used according to manufacturer's instructions to separate cDNA from the agarose mixture. The quantity of purified product was determined by visual comparison to a control marker with predetermined amounts on an agarose gel as described above.

2.7.5. Cycle sequencing. The ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit 2.0 (Perkin Elmer Applied Biosystems; Foster City, CA) was used for cycle sequencing. Separate sequencing reactions were set up for all primers for both T2W and T1L (used as a control): 5pmol (0.35μM) primer, 20-50ng cDNA template, 6μL BigDye™ Terminator Ready Reaction Mix and deionized water to a final volume of 15μL. Cycle sequencing was performed on a GeneAmp 9700 system for 40 cycles of 96°C for 30 seconds, 52°C for 30 seconds and 60° for 2 minutes then cooled to 4°C. The products were purified by sodium acetate/ethanol precipitation, dried on a thermoblock at 90°C for 1 minute and resuspended in template suppression reagent (ABI PRISM®, Perkin Elmer) for ~ 1 hour (minimum 20 minutes). Samples were then heated to 95°C for 2 minutes to denature the strands and immediatedly "snap-cooled" in an ice-water bath. The ABI PRISM® 310 Genetic Analyser (PE Applied Biosystems; Foster City, CA) was used to determine the sequence for each reaction. Chromas version 1.45 (McCarthy 1998) was used to manually analyze sequences to ensure accuracy. SeqMan (DNASTAR Inc., Madison, WI) was used to construct the entire gene sequence for S1, S3 and S4 and MegAlign (DNASTAR Inc., Madison, WI) was used for sequence comparison between T2W and other known reovirus clones and construction of phylogenetic trees.

2.8. Antiviral treatment. Subconfluent L929 cell monolayers in 24-well plates were infected with prototype viral strains at an MOI of 0.5 PFU/cell. Virus was allowed to adsorb to the cell monolayer for 1 hour at room temperature with periodic rocking every 10 – 12 minutes. After adsorption, MEM supplemented as described in section 2.1, with 25% pre-adapted media and the appropriate concentration of antiviral, was added. The infected monolayer was incubated at 37°C for 72 hours. Virus was harvested and samples

were frozen at -80°C and thawed twice. A 0.5mL aliquot of gel saline or media was added and samples were sonicated for 10 - 15 seconds at 35% duty cycle with the sonicator probe (Vibra Cell) (Sonics & Materials Inc., Danbury, CN). This was followed by a plaque assay (section 2.2.2) to determine viral titre. Antiviral agents investigated in this manner included: amantadine HCl (Sigma Aldrich), azidothymidine (AZT) (Sigma Aldrich), phosphonoformate (foscarnet) (Sigma Aldrich), rifampin (Sigma Aldrich) and acyclovir (Sigma Aldrich). High concentration stock solutions of the antivirals were made by dissolving the compound in water, except rifampin, which was dissolved in DMSO, and stored at -20°C until use. Working solutions of rifampin kept the concentration of DMSO below 2% in order to minimize the toxicity of DMSO.

2.8.1. Ribavirin. Initial experiments with ribavirin (Sigma Aldrich) were completed as described above (section 2.8). In a second experiment, subconfluent L929 cell monolayers were either not treated or pre-treated with ribavirin at various concentrations for 3 hours before infection with T1L or T3D at an MOI between 1 and 3 PFU/cell. Ribavirin at the appropriate concentration was added to the cells after virus adsorption and virus was harvested between 0 and 72 hours. Viral titre was determined by plaque assay (section 2.2.2). In another experiment, cells were infected with T1L or T3D at an MOI of 1 PFU/cell in the presence of 100μM ribavirin. At 48 hpi, an aliquot of cell lysate was taken and used to infect a second set of L929 cells also in the presence of 100μM ribavirin. Virus was harvested between 0 and 48 hours and titre determined. For all experiments, a 0.49mg/mL (2mM) stock solution of ribavirin was made and stored at -20°C until use.

2.8.2. Mycophenolic Acid. Subconfluent monolayers of L929 cells were pre-treated with between 0ng/mL and 100μg/mL MPA (Sigma Aldrich) for 45 – 60 minutes or MPA was added at the appropriate time pi. Cells were infected with T1L, T3D, T2W or a variety of reassortant viruses at an MOI of 0.12, 1.2 or 12 PFU/cell and virus was allowed to adsorb for 1 hour at room temperature with periodic rocking. After adsorption, MEM supplemented as described in section 2.1 with 25% pre-adapted media was added and the infected monolayer was incubated at 37°C. The appropriate concentration of MPA was added at pre-determined times between 0 and 24 hpi. Virus was harvested between 0 and 72 hpi and titre determined as described above (section 2.2.2). In some

experiments, 50μg/mL of exogenous guanosine (Sigma Aldrich) was added to the media. A 3mg/mL stock solution of MPA in ethanol was made and stored at -20°C.

2.9. Cell Toxicity. L929 cell monolayers were evaluated microscopically for the presence of CPE due to the antiviral agents. The cells were then harvested, counted in a hemocytometer and cell viability was determined by cell doubling times and Trypan Blue exclusion. Cytotoxic effects of MPA were also determined with the Cell Proliferation Reagent WST-1 (Roche Applied Science). Briefly, 5 x 10^3 L929 cells were added to each well of a 96-well plate and incubated with various concentrations of MPA at 37°C. After 24 hours, 10μL of WST-1 reagent was added and the cells were incubated for an additional 4 hours. After incubation cells were thoroughly shaken for 1 minute on a shaker and the absorbance of the sample was determined at 440nm with a Spectromax Plus microplate reader (Molecular Devices).

2.10. Identification of total viral dsRNA production.

2.10.1. Infection and $^{32}PO_4^{3-}$ labelling of viral dsRNA. Subconfluent L929 monolayers in P60 dishes were infected with viral clones at an MOI of 10 PFU/cell. Virions were labelled by the addition of ^{32}P-orthophosphate (Perkin Elmer Applied Biosystems) after adsorption of virus to a final concentration of >20mCi/mL. Infections were incubated at 37°C and harvested at 24 or 72 hpi. Labelled dsRNA was quantified with a Geiger counter.

2.10.2. Cytoplasmic extraction of radiolabelled viral dsRNA. Immediately after incubation, infections were placed on ice and cell monolayers were "scraped" from the plate to harvest cells. Cells were pelleted by centrifugation at 1,100rpm (IEC floor centrifuge). Cell pellets were resuspended in 500μL NP-40 buffer (140mM NaCl, 1.5mM $MgCl_2$, 10mM Tris, pH 7.4) with 0.5% NP-40 detergent, vortexed and incubated on ice with periodic mixing for 30 minutes in order to lyse cell membranes. Cellular nuclei and organelle were pelleted by centrifugation at 3,000rpm in the Biofuge (VWR Scientific, Toronto, ON) for 10 minutes and supernatants were collected and transferred to a sterile microfuge tube. A pre-mixed solution was prepared (400μL 1M Tris, pH 7.8, 40μL 200mM EDTA pH 8.0, 800μL 10% SDS) and 95μL was added to each sample. Crude cell extracts were incubated with 50μg/mL proteinase K (Sigma Aldrich) at 37°C for 30 minutes to aid in protein removal. Samples were then heated for 15 minutes at 42°C and

viral dsRNA genomes were purified by phenol/chloroform extraction (addition of 14μL 5 M NaCl followed by 600μL phenol/chloroform in a 1:1 ratio). Samples were mixed thoroughly and reheated to 42°C for 30 seconds followed by centrifugation at 12,500rpm for 5 minutes. The top aqueous phase was transferred to a fresh sterile microfuge tube and placed on ice. RNA was precipitated at -20°C overnight by the addition of 0.1 volumes of 3M sodium acetate and 2.5 volumes of ice-cold ethanol. Precipitated dsRNA was pelleted at 12,500 rpm for 30 minutes in a Biofuge at 4°C. Viral dsRNA pellets were lyophilized (SC100 speed-vac, Savant, Holbrook, NY) for 30 minutes and resuspended in 30 μL of 1x ESB. Samples were stored at -20°C if not used immediately.

2.10.3. Labelled viral dsRNA resolved by SDS-PAGE. Viral dsRNA was resolved either on a 10% SDS-PAGE gel as described earlier (section 2.5) or run on a 0.9% horizontal agarose gel at 100V for ~1.5 hours in 0.5x TBE buffer which contained 0.1% ethidium bromide to allow for RNA visualization under UV irradiation. T3D purified virion was used as a marker. The gel was dried onto filter paper at 80°C (Slab Gel Dryer, SGD4050, Savant) for 2 hours for the acrylamide gel or at room temperature for 1 hour followed by 2 hours at 80°C for the agarose gel, then autoradiographed by exposure to Kodak X-AR sheet film (Kodak, Rochester, NY) at -80°C.

2.11. Identification of total viral protein production

2.11.1. Infection and ^{35}S labelling of viral proteins. Prototype virus and viral clones were used to infect subconfluent L929 monolayers in P60 dishes at an MOI of 10 PFU/cell. Virions were labelled by the addition of ^{35}S-protein labelling mix (Perkin Elmer Applied Biosystems) after adsorption of virus to a final concentration of >20mCi/mL. Infections were incubated at 37°C and harvested 24 or 72 hpi. Labelled proteins were quantified by scintillation counts.

2.11.2. Immunoprecipitation of radiolabelled viral proteins. Immediately after incubation, infections were placed on ice and cell monolayers were "scraped" from the plate to harvest cells. Cells were pelleted by centrifugation at 1,100rpm (IEC floor centrifuge) and resuspended in 400μL immunoprecipitation buffer (50mM Tris pH 8.0, 100mM NaCl) with 0.5% NP-40 detergent and protease inhibitors (50μg/mL PMSF, 1μg/mL TLCK, 1μg/mL leupeptin, 1μg/mL pepstatin A, 0.1U/mL aprotinin). Samples were vortexed and incubated on ice with periodic mixing for 30 minutes in order to lyse

cell membranes. Cellular nuclei and organelles were pelleted by centrifugation at 5,000rpm in the Biofuge (VWR Scientific, Toronto, ON) for 15 minutes and supernatants were collected and transferred to a sterile microfuge tube. Cell extracts were rocked overnight with anti-T3D reovirus-conjugated protein A-Sepharose beads at 4°C. The samples were washed 5 times with wash buffer (50mM Tris 8.0, 100mM NaCl, 1% NP-40, 1% DOC) with a final centrifugation at 10,000rpm for 1 minute in the Biofuge at 4°C. The protein pellet was resuspended in 50μL 1x ESB and samples were stored at -20°C if not used immediately.

2.11.3. Labelled viral proteins resolved by SDS-PAGE. Viral proteins were resolved on a 5-15% SDS-PAGE gel as described earlier (section 2.5). Protein gels were fixed in an 30% isopropanol / 10% acetic acid solution and impregnated with Enlightning (Perkin Elmer Applied Biosystems) for 1 hour. The gel was dried onto filter paper at 80°C (Slab Gel Dryer, SGD4050, Savant) for 2 hours, then fluorographed by exposure to Kodak X-AR sheet film (Kodak, Rochester, NY) at -80°C.

2.12. Statistical analyses. The student's t-test was used to test for the significance between two groups (SISA 2003). The Wilcoxon rank sum test was performed on the ranks of both raw and averaged values to determine the relative contribution of any single gene in reassortant mapping experiments (SISA 2003). Chi-square and multiple linear regression analyses were carried out by Mrs. Mary Cheang (Department of Community Health, University of Manitoba, Canada). A minimum of two trials were carried out for all experiments and the standard deviation or standard error of the mean were calculated.

3. *RESULTS*

T2W is a clinical sample isolated from the CSF of a severe combined immunocompromised infant with respiratory, gastrointestinal and CNS symptoms. Most reovirus strains have been isolated from respiratory or gastrointestinal secretions, so it is unusual to isolate reovirus from the CNS. There are only a few isolated reports presented in the literature, which associate reovirus with CNS disease. T2W provides evidence reovirus can infect the CNS and examination of this isolate may provide a better understanding of the requirements for reovirus CNS infection. Since reovirus infections are generally asymptomatic, there has been little evaluation of agents that could be used both clinically and in the laboratory to inhibit reovirus infection. The second half of this research examined various compounds for their ability to inhibit reovirus replication of the prototype reoviruses, T1L and T3D. Ribavirin and MPA, both IMPDH inhibitors, were investigated in greater detail and MPA was also shown to inhibit T2W replication. This suggests MPA, or its prodrug mycophenolate mofetil, could possibly be used to treat an infant infected with T2W.

3.1. A comparative analysis of Freon substitutes in the purification of reovirus virions. Characterization of T2W often required virions in a purified state in the absence of cellular debris. In the past, Freon was used in our laboratory for organic solvent extraction of virions from cellular components. However, Freon has been implicated in ozone layer depletion and its use is now prohibited. Therefore, it was necessary to find a substitute solvent that could effectively purify large quantities of virus without affecting virion morphology and infectivity before lab stores of Freon were exhausted.

3.1.1. Comparison of organic solvents. Organic solvents are characterized by Hansen's three solubility parameters (D – dispersion energy, P – polar energy and H – hydrogen bonding energy) (Hansen and Beerbower 1971;Goldschmidt 1993). To find a replacement for Freon in viral purification methods, solvents with similar properties to Freon were considered (see Table 7). Alternate halogenated hydrocarbons with similar Hansen solubility parameters were not chosen because of other unsuitable properties. For example, carbon tetrachloride has high toxicity and 1,2-dichloro-1,1,2,2,-tetrafluoroethane has a boiling point of 4°C which means it is a gas at room temperature; thus both are unsuitable for viral purification methods. Other hydrocarbon solvents have

Table 7. Hansen's solubility parameters of various solvents

Solvent	Solubility Parameters			MP[b] (°C)	BP[b] (°C)	Density[c] (g/mL)
	D[a]	P[a]	H[a]			
Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane)	14.7	1.6	0.0	-36	48	1.56
Carbon tetrachloride	17.8	0.0	0.6	-23	77	1.59
1,2-dichloro-1,1,2,2-tetrafluoroethane	12.6	1.8	0.0	-94	4	>1[d]
Hexane	14.9	0.0	0.0	-56	128	0.66
Isopentane	13.7	0.0	0.0	-56	30	0.62
1-chlorobutane	16.2	5.5	2.0	-123	78	0.89
Toluene	18.0	1.4	2.0	-95	110	0.87
Chloroform	17.8	3.1	5.7	-64	62	1.48
Isobutanol	15.1	5.7	15.9	-108	108	0.81
n-butanol	16.0	5.7	15.8	-90	118	0.81
Vertrel® XF (1,1,1,2,3,4,4,5,5,5-decafluoropentane)	—[e]	—	—	-80	55	1.58
Hexane/1-chlorobutane (7:3)	15.3	1.7	0.6	—	—	0.73[f]
Isopentane/1-chlorobutane (7:3)	14.5	1.7	0.6	—	—	0.70[f]
Isopentane/1-chlorobutane (1:1)	15.0	2.8	1.0	—	—	0.76[f]
Chloroform/isobutanol (1:1)	16.5	4.4	10.8	—	—	1.15[f]
Chloroform/*n*-butanol (1:1)	16.9	4.4	10.8	—	—	1.15[f]

[a] Hansen's solubility parameters where D is dispersion energy, P is polar enery and H is hydrogen bonding energy
[b] MP, melting point; BP, boiling point (The Merck Index, 1983)
[c] Density of liquid form at 24°C unless otherwise indicated by '[d]' (1977)
[d] Density of gaseous form at room temperature (1977)
[e] Unknown
[f] Calculated and not empirically determined because of differential evaporation rates

some parameters similar to Freon, but are quite different in other parameters. Combinations of solvents in various ratios were used to yield solubility parameters similar to Freon and were used for further study (see Table 7). The resultant solubility parameters of the mixtures are theoretical values calculated manually, not empirically because of different evaporation rates. Initially hexane/1-chlorobutane (7:3 v/v mixture), isopentane/1-cholorbutane (7:3), toluene and Vertrel® XF (Vertrel) were tested as replacement solvents. Vertrel (1,1,1,2,3,4,4,5,5,5-decafluoropentane) is a Freon substitute developed by DuPont which is not implicated in ozone depletion (DuPont 2001). Solvents were also examined either alone or in mixture ratios (other than those listed), to test whether the proportions of each solvent in the mixture affected the results.

3.1.2. Viral purification with Freon substitutes Proposed solvent replacements were tested using basic viral purification methods with the prototype reovirus, T3D, as previously described for Freon (Smith et al. 1969). Briefly, T3D infected L929 mouse fibroblast cells were harvested, lysed with DOC and divided into equal aliquots. Each aliquot was emulsified with equal volumes of the various solvents to be tested and emulsified samples were centrifuged to separate the aqueous and organic phases. The location of the aqueous phase depended on the density of the organic solvent. Freon, which is denser than water, formed a thick solid phase at the bottom of the tube which allowed for easy removal of the top aqueous phase. Vertrel, chloroform and chloroform/isobutanol (1:1) were the only substitute solvents tested with densities greater than the aqueous phase. The other solvents tested formed a semi-solid layer above the aqueous phase with the exception of toluene, which formed a slurry-like layer. The semi-solid solvent layer was punctured with a pipette in order to collect the aqueous phase. This was a concern, since debris from the solvent phase could potentially contaminate the aqueous phase in the course of collection. After initial extraction, emulsification with test solvents and centrifugation was repeated on the aqueous phase. Twice-extracted aqueous phases were collected and layered onto 1.2 – 1.4g/mL cesium chloride gradients and virus banded by ultracentrifugation at 25,000rpm for a minimum of 5 hours.

Gradient purification of Freon-extracted reovirus-infected cells resulted in two bands that could be collected and corresponded to virus and top component (empty virus particles devoid of genome) (Smith et al. 1969), indicated as V and T respectively in

Figure 10. All solvents tested, except chloroform/isobutanol (1:1), produced two bands in approximately the same regions of the gradient. Toluene-extracted samples consistently produced larger proportions of top component than virus. No visible virus band was observed when reovirus-infected cells were treated with chloroform/isobutanol (1:1) whereas treatment with chloroform/*n*-butanol (1:1) resulted in only a very faint virus band. Density measurements by refractive index of bands collected from solvents tested, including Freon, differed in density by no more than 0.003g/mL and top component by no more than 0.002g/mL. This suggests viral particles are not significantly different from Freon purified particles.

3.1.3. Electron microscopic analysis of purified virions. Dialyzed gradient purified virus and top component band material was examined by negative stain transmission EM (Hammond et al. 1981) to determine whether viral morphology was affected by the solvent used for virus extraction. No difference was observed in complete virion morphology for most solvents tested (Figure 11). Greater than 95% of particles from virus band material were complete virions for all solvents tested with the exception of chloroform/isobutanol (1:1) and chloroform/*n*-butanol (1:1) which produced disrupted virions. Freon and Vertrel treatments yielded equivalent apparent concentrations of virus and much higher concentrations of virus than for the other solvents used. Also, Freon and Vertrel treatments contained little non-virion debris whereas the other treatments contained comparatively large amounts (data not shown).

Top component fractions were composed of mostly genome deficient virions and other shell structures with less than 10% of particles as complete virions (Figure 12). Similar to virus fractions, treatment with Freon and Vertrel yielded equivalent amounts of particles with little or no cellular debris while treatment with the other solvents yielded reduced quantity of top component particles with relatively large amounts of non-particulate structures. Samples from other bands seen higher in some gradients (e.g. hexane/1-chlorobutane (7:3)) were comprised mostly of debris with minimal viral structures identified (data not shown).

These experiments were repeated with T1L, another prototype reovirus strain. Results were similar to those described for T3D; however, a faint virus band was seen for

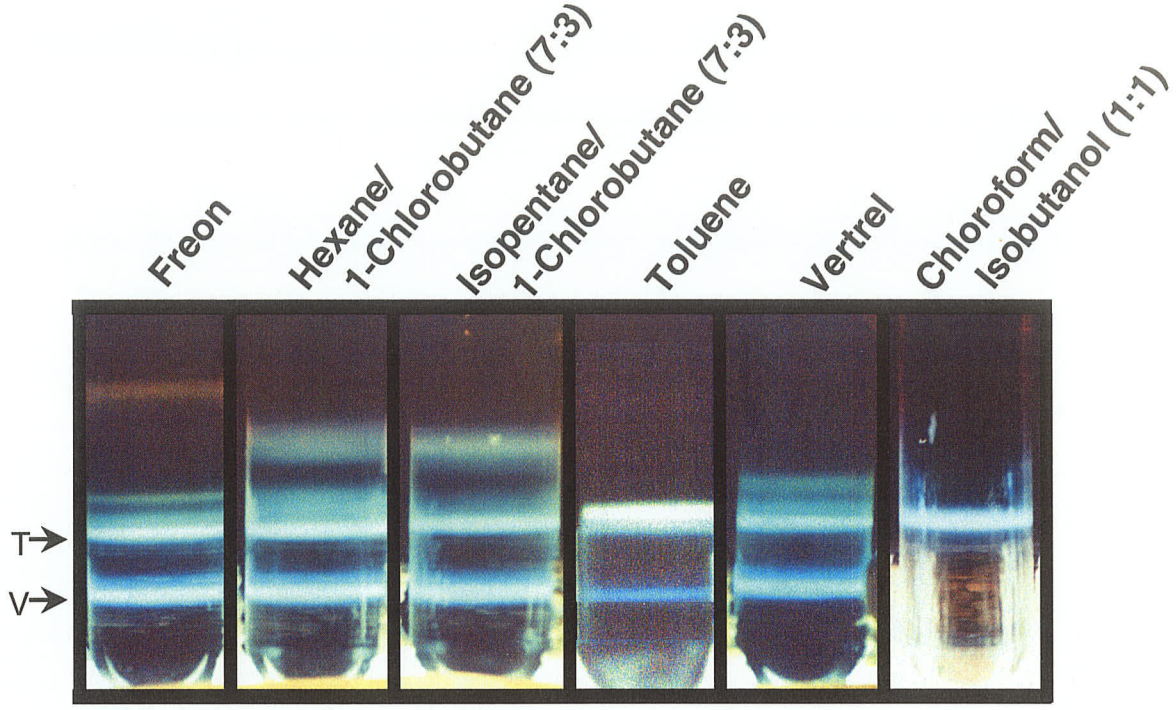

Freon
Hexane/
1-Chlorobutane (7:3)
Isopentane/
1-Chlorobutane (7:3)
Toluene
Vertrel
Chloroform/
Isobutanol (1:1)
T
V

Figure 10. Buoyant density cesium chloride gradients of reovirus-infected cell lysates extracted with the indicated solvents

Twice-extracted aqueous phases were layered onto 1.2 – 1.4 g/mL cesium chloride gradients, centrifuged in a Beckman SW28 rotor at 25,000rpm for a minimum of 5 hours and photographed. The results are representative of three trials. **V** = virions; **T** = top component.

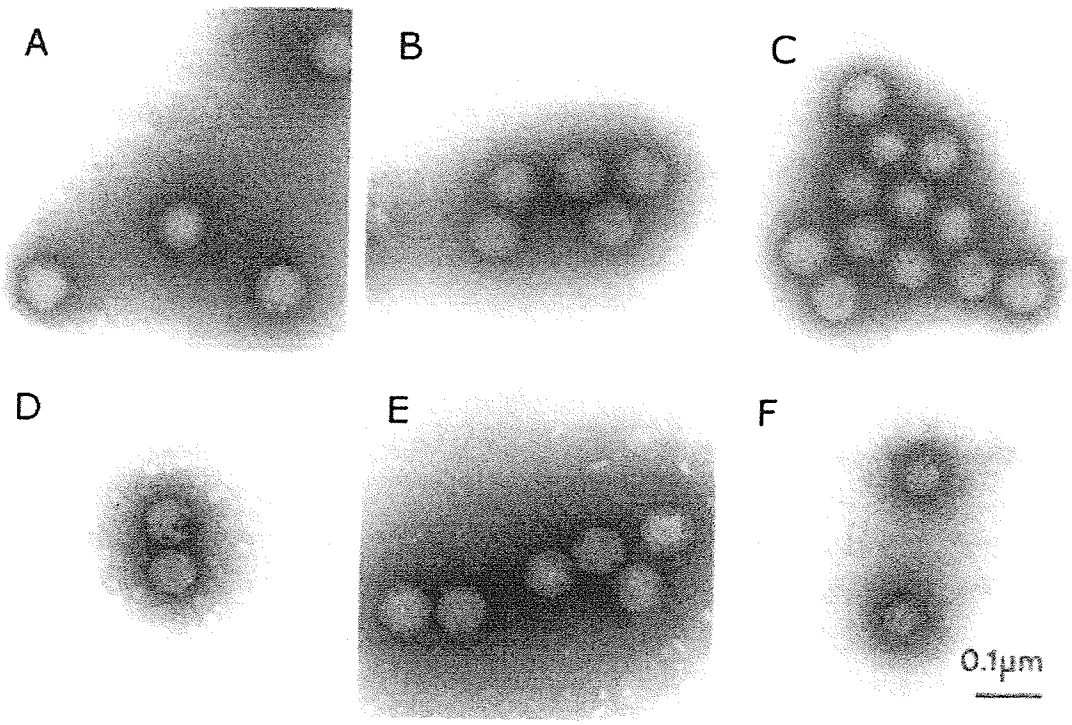

A
B
C
D
E
F
0.1μm

Figure 11. Electron micrographs of gradient purified virion particles

Virus bands such as those depicted in Figure 10 were harvested and dialysed against D-buffer. Aliquots were diluted 100-fold with D-buffer and 0.1% glutaraldehyde was added to stabilize viral structures. Samples were mounted on 400-mesh formvar-coated copper grids by drop method, negatively stained with 1.2mM phosphotungstic acid, pH 7.0 and examined in a Philips model 201 transmission electron microscope as previously described (Hazelton and Coombs 1995). **(A)** Freon; **(B)** Hexane/1-chlorobutane (7:3); **(C)** isopentane/1-chlorobutane (7:3); **(D)** Toluene; **(E)** Vertrel; **(F)** Chloroform/isobutanol (1:1). Bar = 100nm

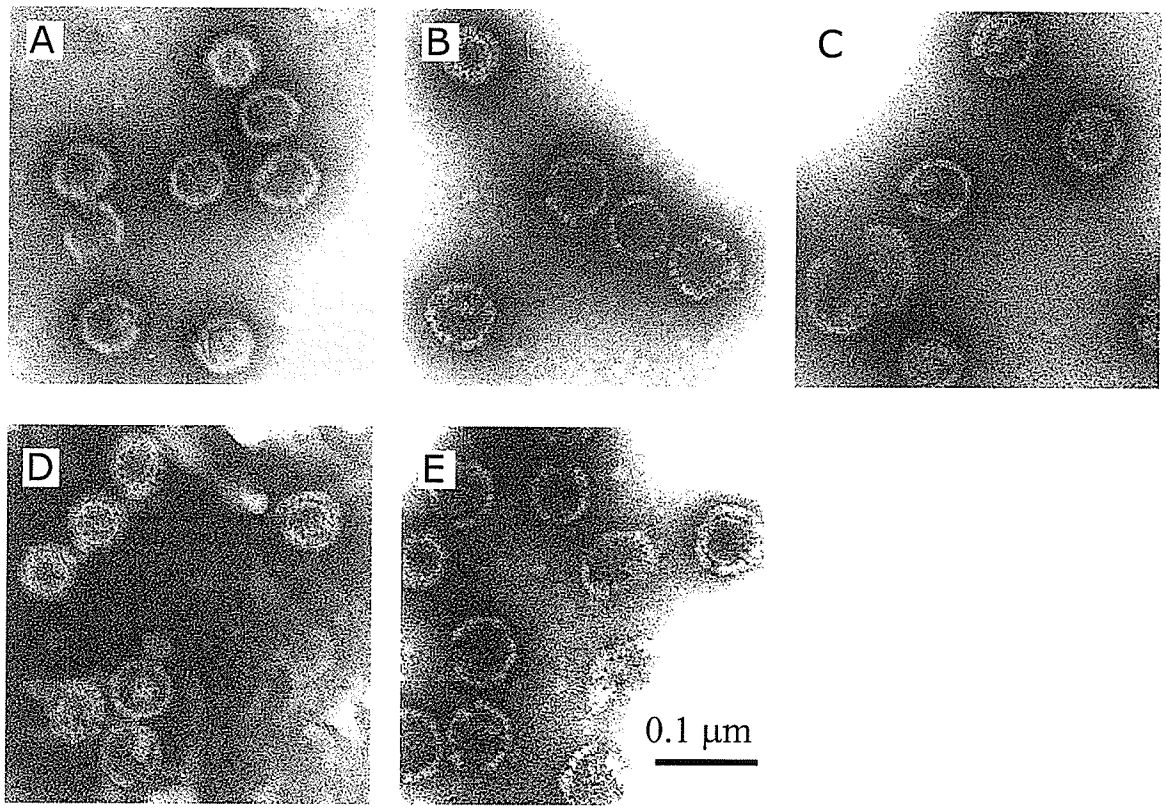

A
B
C
D
E
0.1 μm

Figure 12. Electron micrographs of gradient purified top component particles

Top component bands such as those depicted in Figure 10 were harvested and dialysed against D-buffer. Aliquots were diluted 100-fold with D-buffer and 0.1% glutaraldehyde was added to stabilize viral structures. Samples were mounted on 400-mesh formvar-coated copper grids by drop method, negatively stained with 1.2mM phosphotungstic acid, pH 7.0 and examined in a Philips model 201 transmission electron microscope as previously described (Hazelton and Coombs 1995). **A.** Freon; **B.** Hexane/1-chlorobutane (7:3); **C.** isopentane/1-chlorobutane (7:3); **D.** Toluene; **E.** Vertrel. Bar = 0.1μm.

chloroform/isobutanol (7:3). Morphological examination of this virus band showed few virus particles and those that were observed appeared to have disrupted outer capsids.

3.1.4. SDS-PAGE analysis of purified virions. The integrity of virus samples after treatment with the various solvents was confirmed by examination of equivalent numbers of purified virions suspended in 1x ESB by SDS-PAGE. Resolution of genomic material from each of the treated samples revealed an equivalent amount of each of the 10 viral gene segments (Figure 13A) which indicates none of the solvents had differential effects on the presence of the genome.

Viral proteins were resolved in a 5 – 15% gradient SDS-PAGE and Coomassie stained (Figure 13B). The migration pattern of proteins was not altered for any solvent and there was no apparent difference in the quantities of each protein. This supports the morphological evidence that virions were structurally unaffected by test solvents when compared to Freon-treated samples. The hexane/1-chlorobutane (7:3) treated samples contained some low molecular weight contaminants which indicates the purification was not as complete as with the other treatments.

3.1.5. Functional analysis of virions purified with Freon substitutes. Specific infectivity of each virion band is a more sensitive assay of the structural and functional integrity of each purified virus sample. Viral particle concentrations were determined by UV spectrophotometric analysis and virus titres were determined by plaque assay on sub-confluent L929 mouse fibroblast cells as described in the Materials and Methods section 2.2.2 (Smith et al. 1969;Coombs et al. 1994a). The total number of virus particles purified with each solvent was determined and compared to the number of virus particles purified with Freon (Table 8). Most extraction treatments recovered fewer virus particles from an equivalent number of infected cells. Only extraction with Vertrel yielded as many or more virus particles than extraction with Freon. Relative infectivity ratios were determined by taking the ratio of particles/mL to PFU/cell (Table 8). Only Vertrel and the isopentane/1-chlorobutane (7:3) mixture had comparable ratios to Freon. Thus, treatment with other solvents yielded both decreased amounts of virus and less infectious virus preparations. Similar results to those described above for T3D were also obtained when T1L was tested (data not shown). The above results have been published (Mendez et al. 2000).

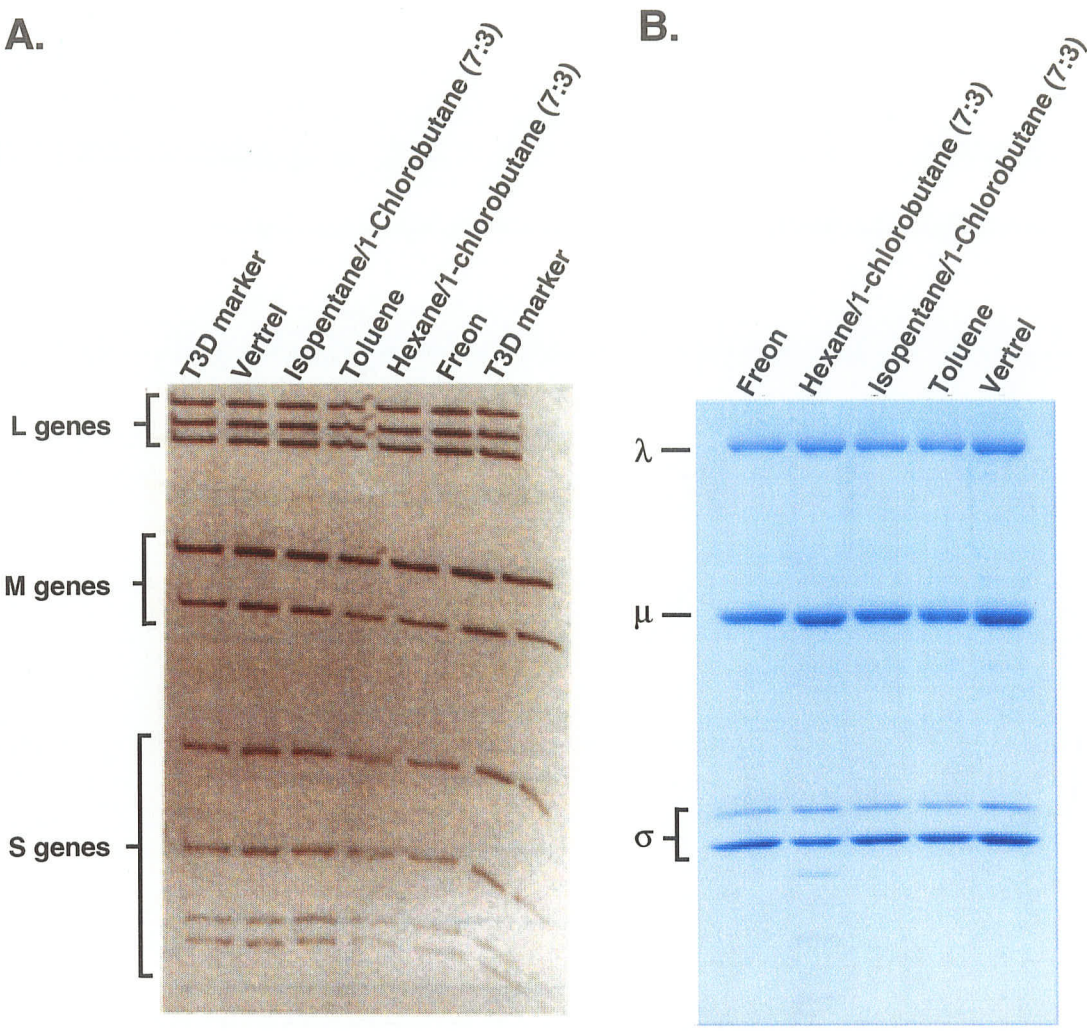

A.
T3D marker
Vertrel
Isopentane/1-Chlorobutane (7:3)
Toluene
Hexane/1-chlorobutane (7:3)
Freon
T3D marker
L genes
M genes
S genes
B.
Freon
Hexane/1-chlorobutane (7:3)
Isopentane/1-Chlorobutane (7:3)
Toluene
Vertrel
λ
μ
σ

Figure 13. Electropherotype and protein profile of virions purified with indicated solvent

(A) Equal amounts of virus harvested from gradients similar to those depicted in Figure 10 were heated to 65°C for 5 minutes in 1 x ESB and resolved in a 10% SDS-PAGE at 18mA for 45 hours. The gel was treated with 0.1% ethidium bromide and visualized under UV light with a Gel Doc 2000 (BioRad) apparatus. Solvent treatments are indicated above each lane. T3D marker is T3D virions previously purified with Freon. Locations of specific classes of reovirus genes are indicated on the left. **(B)** Equal amounts of virus were heated to 95°C for 5 minutes in 1 x ESB and resolved in a 5 - 15% SDS-PAGE at 5mA overnight. The gel was fixed and stained with Coomassie Blue. Solvent treatments are indicated above each lane. Locations of specific classes of reovirus proteins are indicated on the left.

Table 8. Summary of reovirus yield and specific infectivity for various solvents

Solvent	Total virus particles recovered ($x10^{13}$)[a] ± SEM[b]	Total infectivity recovered ($x10^{11}$ PFU)[c] ± SEM	Specific Infectivity[d] ± SEM	Relative Infectivity[e] ± SEM
Freon	2.97 ± 0.71	1.84 ± 0.59	176 ± 29	1.00
Hexane/1-chlorobutane (7:3)	1.70 ± 0.54	0.67 ± 0..54	367 ± 158	1.82 ± 0.43
Isopentane/1-chlorobutane (7:3)	2.44 ± 0.45	1.20 ± 0.28	197 ± 64	1.24 ± 0.52
Toluene	1.37 ± 0.78	0.41 ± 0.01	341 ± 197	2.19 ± 1.58
Vertrel	4.07 ± 0.75	1.97 ± 0.17	204 ± 21	1.19 ± 0.12

* Values represent the averages of at least three experiments.

[a] In each experiment 7.0 x 10^8 cells were infected with reovirus T3D, harvested and extracted with indicated solvent. Value represents the average of at least three experiments.

[b] SEM: standard error of the mean represents the average of the absolute deviation of data points from their mean.

[c] Total amount of infectious particles recovered from indicated solvent.

[d] Determined by dividing the total number of virus particles recovered by the total infectivity for the indicated solvent.

[e] Determined by dividing the specific infectivity of the indicated solvent by the specific infectivity of virus recovered after Freon extraction.

3.2. Characterization of a viral clinical isolate. After a Freon substitute for viral purification was determined, work with T2W was begun. The isolate came from an 8-week-old infant girl with respiratory, gastrointestinal and CNS symptoms hospitalized in November 1997 at the Children's Hospital (Winnipeg, Manitoba) for investigation of congenital immune deficiency. A reovirus serotype 2 strain and two strains of *Staphylococcus aureus* (one of which was methicillin resistant) were recovered from a CSF sample. A fecal sample and second CSF sample taken at later time points were also culture positive for reovirus serotype 2. RMK cells inoculated with CSF, examined by negative stain transmission EM revealed reovirus-like particles (Figure 5A). In addition, rotavirus particles were identified in the fecal sample by EM (Figure 5B). Unfortunately, CSF material was not available for direct EM investigation; however, dialyzed gradient purified virus material examined by EM also showed whole virion, ISVP and top component particles typical of reovirus (Figure 14).

Initial isolation of virus was after inoculation of primary RMK cells with an aliquot of CSF from the infant. Over the next 48 hours, CPE was seen. Standard viral screens employed by Cadham Provincial Laboratory (Winnipeg, Canada) ruled out infection by herpes simplex virus, adenovirus and other enteroviruses. The serotype was determined to be reovirus serotype 2 based on NT studies with serotype-specific polyclonal antisera done by the provincial laboratory. This was repeated for all three culture positive isolates (i.e. two CSF and one stool sample) and all samples were shown to be reovirus serotype 2. No reaction was seen with polyclonal reovirus antisera for serotype 1 or 3 (B. Wells, Cadham Provincial Laboratory, personal communication). Since reovirus is not often isolated from CNS samples and previously reported examples have been either type 1 or type 3, we hypothesized this isolate was a novel reovirus clone and was potentially neurotropic. The sample was tentatively named Type 2 Winnipeg (T2W).

3.2.1. Growth characteristics. We received a small aliquot of an RMK cell lysate (< 1mL) from Cadham Provincial Laboratory as starting material for these experiments. The first step required to characterize this isolate, was to amplify sufficient virus stocks for subsequent experiments. This required initial investigations to determine appropriate growth conditions for T2W.

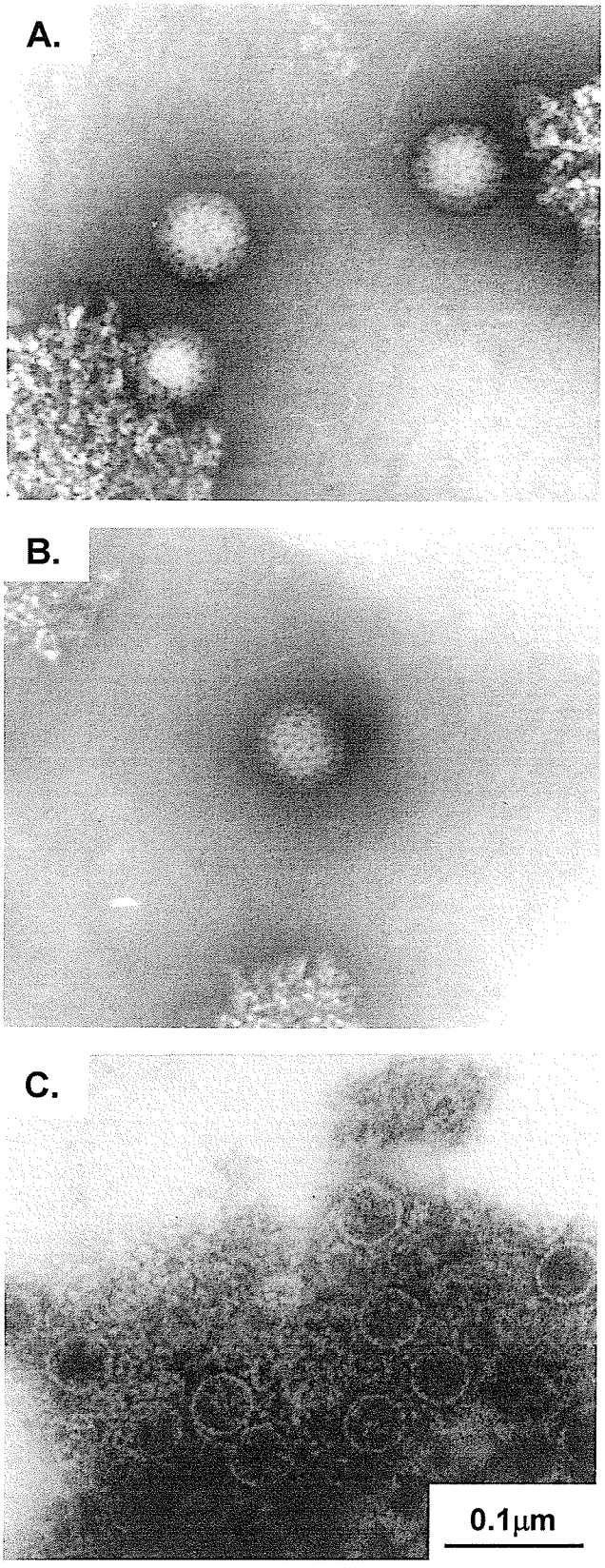

A.
B.
C.
0.1μm

Figure 14. Electron micrograph of gradient purified T2W virion particles

Bands harvested from cesium chloride gradients were harvested and dialysed against D-buffer. Aliquots were diluted 100-fold with D-buffer and 0.1% glutaraldehyde was added to stabilize structures. Samples were mounted on 400-mesh formvar-coated copper grids by drop method, negatively stained with 1.2mM phosphotungstic acid, pH 7.0 and examined in a Philips model 201 transmission electron microscope as previously described (Hazelton and Coombs 1995). **(A)** Whole virion **(B)** ISVP and **(C)** top component particles were all visualized.

A preliminary experiment looked at the ability of reovirus to grow in different cell lines. L929, Vero (African green monkey kidney cells) and primary RMK cells were each inoculated with T2W at various MOIs. Cell monolayers were examined daily and when CPE was >90% virus was harvested and viral titre determined. Results indicated T2W grew equally well in L929 and Vero cells with titres of cell lysates between 10^6 and 10^7 PFU/mL. Viral titres were 10-fold less in RMK cells (data not shown). For further virus amplification, L929 cells were used because stock L929 cells are maintained in the laboratory. As well, L929 cells can be grown in suspension, which allows for amplification of large quantities of cells more easily as compared to primary cells (RMK) or cells grown in monolayer (Vero).

Experiments with T2W were limited by the small amounts of stock sample received from Cadham Provincial Laboratory (Winnipeg, Manitoba), therefore growth of T2W was further characterized in L929 cells with time course studies to help establish ideal amplification conditions. L929 cells were infected with T2W at MOIs of 0.05, 0.5 and 5 PFU/cell. Virus was harvested at different time points over 14 days and viral titre determined. There were no significant differences in viral growth patterns when inoculated with an MOI of 0.5 or 5 PFU/cell and near maximal levels of virus production was seen after 5 days (Figure 15A). Virus production was significantly slower when an MOI of 0.05 PFU/cell was used. However, maximum viral titres of 10^7 were obtained for each MOI examined at 14 days (Figure 15A).

In an attempt to produce high-titre virus stocks, in some experiments α-chymotrypsin was added to the cell culture medium to generate ISVP-like particles from crude viral stocks. Virion uncoating is an essential early event in the reovirus lifecycle. Evidence shows the requirement for capsid proteolysis restricts reovirus cellular host range and that the addition of a protease to the culture medium can enhance virus replication (Golden et al. 2002). Subconfluent L929 cells were infected at an MOI of 0.1 PFU/cell for 1 hour and overlaid with serum-free MEM supplemented with concentrations of α-chymotrypsin between 0 and 25μg/mL. Virus was harvested between 1 and 3 days post-infection (pi) and virus titre determined. Viral titres > 10^8 were obtained with the addition of 1 and 2.5 μg/mL of α-chymotrypsin when virus was harvested 2 or 3 days pi (Figure 15B). Harvesting virus at later time points (i.e. 4 and 5

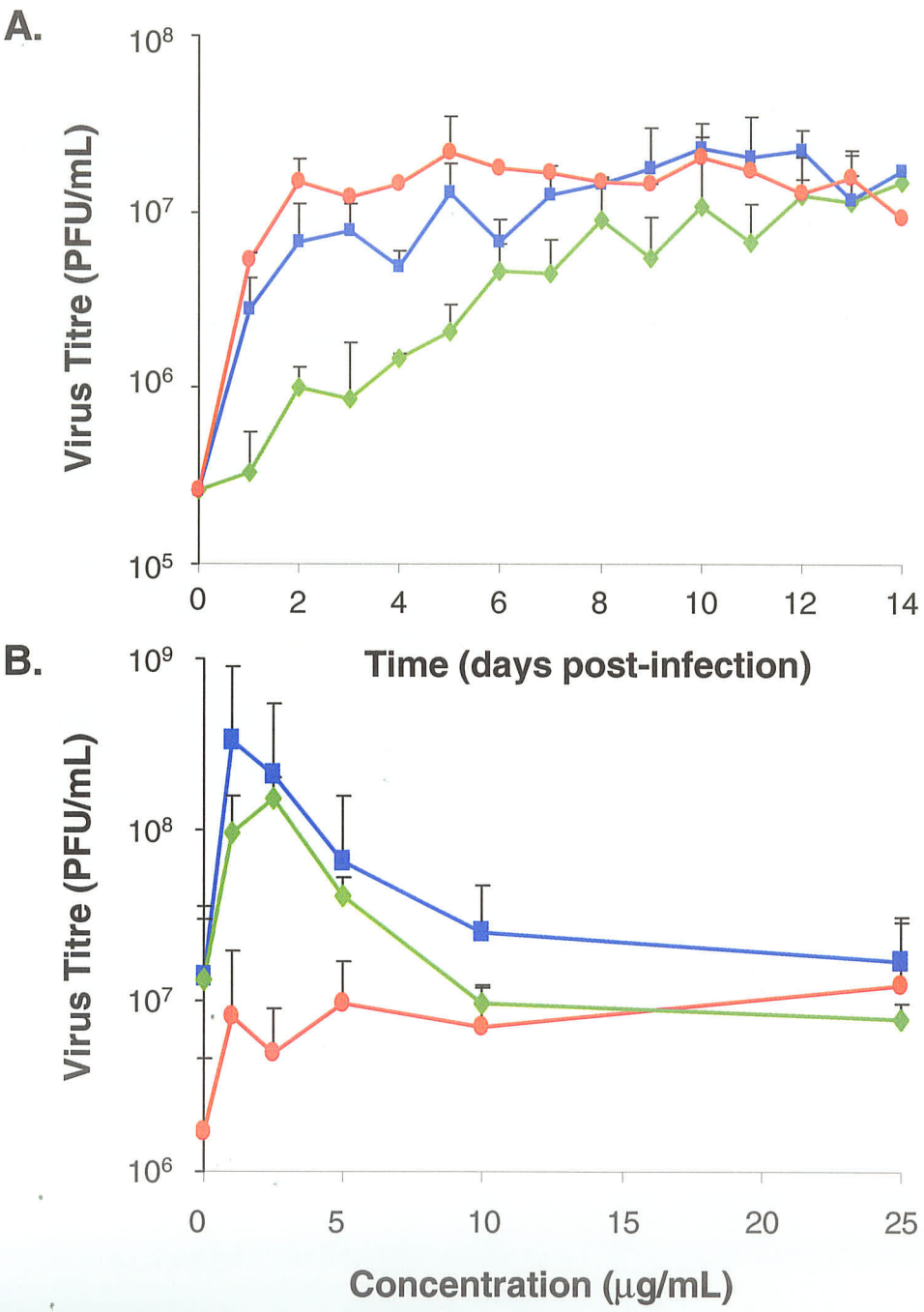

A.
Virus Titre (PFU/mL)
10^8
10^7
10^6
10^5
0
2
4
6
8
10
12
14
Time (days post-infection)
B.
Virus Titre (PFU/mL)
10^9
10^8
10^7
10^6
0
5
10
15
20
25
Concentration (μg/mL)

Figure 15. Growth of T2W in L929 cells

(A) Subconfluent L929 cells were infected with T2W at an MOI of 0.05 (◆), 0.5 (■) or 5 (●) PFU/cell. Cells were overlaid with fresh MEM and incubated at 37°C. Virus was harvested between 0 and 14 days post-infection and viral titre was determined. The data represents the average of a minimum of three experiments (n=3) and the error bars represent one standard deviation. **(B)** Dose response of α-chymotrypsin on T2W replication. Subconfluent L929 cells infected with T2W at an MOI = 0.1 PFU/cell for 1 hour and overlaid with serum-free MEM supplemented with 0 to 25 μg/mL of α-chymotrypsin. Virus was harvested at 1 (●), 2 (■) or 3 (◆) days post-infection and viral titre was determined. The data represents the average of a minimum of three experiments (n=3) and the error bars represent one standard deviation.

days pi) did not increase titre further (data not shown). This represents a 10-fold increase in titre over the untreated control. Higher concentrations of α-chymoptrypsin resulted in lower titres, due to decreased cell viability as previously reported (Golden et al. 2002). It is also possible the decrease in viral titres is due to an adverse effect on the reovirus particles themselves, e.g. at higher α-chymotrypsin concentrations, further proteolysis occurs and the virion may become unstable. This method of amplication, i.e. virus grown in media supplemented with 2.5 μg/mL α-chymotrypsin and harvested at 2 days, was used to amplify high-titre T2W stocks for batch infections and viral purification.

3.2.2. Protein and RNA Profiles. To further characterize this isolate, batch infections of L929 cells in suspension were employed to amplify virus as described in section 2.3.1. Viral purification was by standard methods of solvent extraction with Freon or Vertrel followed by concentration by cesium chloride gradient ultracentrifugation (Mendez et al. 2000).

Viral proteins were resolved in a 5 – 15% gradient SDS-PAGE and Coomassie stained. The migration pattern of proteins in the T2W sample was typical of reovirus, i.e. with separation into groups of λ, μ and σ proteins with no gross differences from T1L or T3D (Figure 16A). RNA was extracted and examined in a 10% SDS-PAGE and ethidium bromide stained as described in section 2.5. A sample of T2W's RNA was run along with the RNA from the reovirus prototypes and a number of other reovirus strains (Figure 16B). The electrophoretic gel pattern revealed 10 gene segments indicative of reovirus and was different from all the reovirus strains examined. This evidence further supports T2W is a novel reovirus clone.

3.2.3. Analysis of the S gene sequences of T2W. To further analyze this clinical isolate, the S gene segments of T2W were sequenced and compared to genes from other reovirus clones listed in GenBank for identity and phylogenetic analysis. The S gene segments were chosen because they are small and well characterized and sequence data for a large number of clones is available for analysis. Appropriate gene segments from T1L were used as controls. The sequences obtained for T1L were identical to previously published sequence data. To confirm T2W the sequence had not been altered in amplified samples (i.e. higher passage virus), nested PCR amplification and sequencing were completed on the original sample received from Cadham Provincial Laboratory.

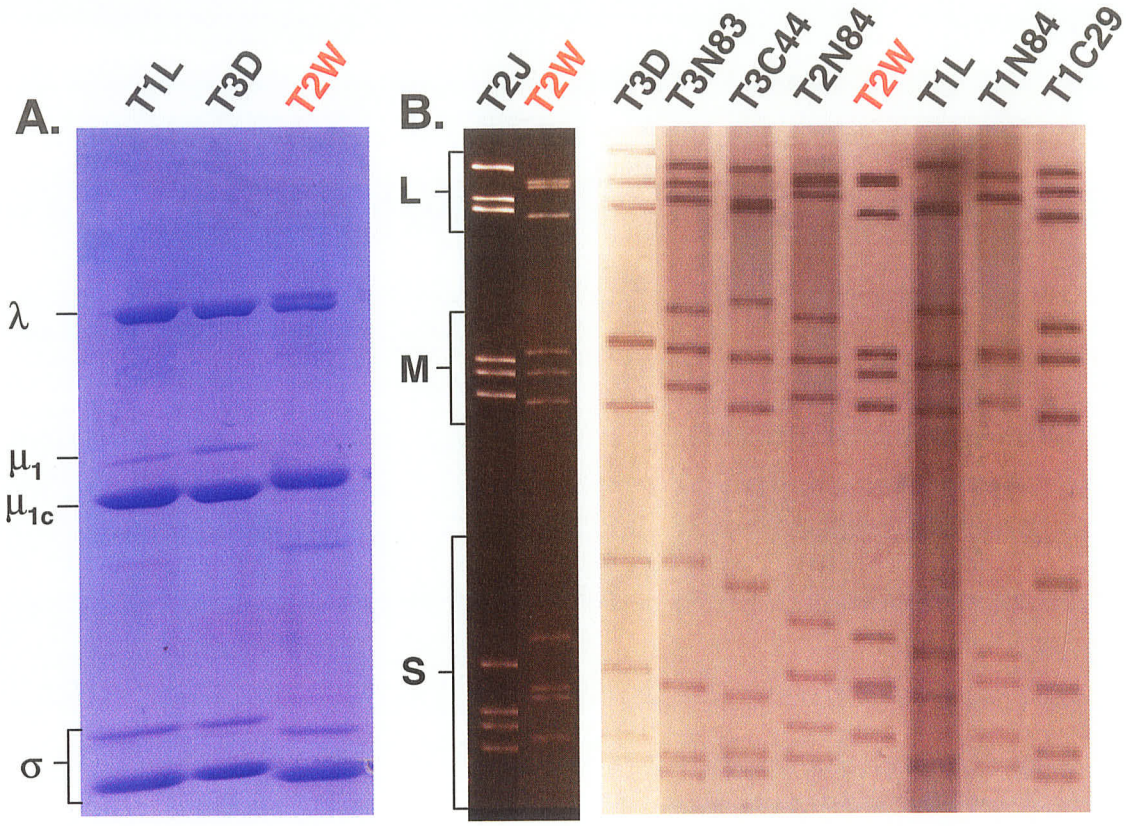

A.
T1L
T3D
T2W
λ
μ1
μ1c
σ
B.
T2J
T2W
L
M
S
T3D
T3N83
T3C44
T2N84
T2W
T1L
T1N84
T1C29

Figure 16. Protein and RNA profile of gradient purified T2W virions

(A) Equal amounts of purified virus were heated to 95°C for 5 minutes in 1 x ESB and resolved in a 5 - 15% SDS-PAGE at 5mA overnight. The gel was fixed and stained with Coomassie Blue. Virus names are indicated above each lane. Locations of specific classes of reovirus proteins are indicated on the left. **(B)** Equal amounts of purified virus were heated to 65°C for 5 minutes in 1 x ESB and resolved in a 10% SDS-PAGE at 18mA for 45 hours. The gel was treated with 0.1% ethidium bromide and visualized under UV light with a Gel Doc 2000 (BioRad) apparatus. Reovirus clones are indicated above each lane. Locations of specific classes of reovirus genes are indicated on the left.

3.2.3.1.Comparison of S1 nucleotide and σ1 amino acid sequences. The T2W S1 gene segment was PCR-amplified and the resulting cDNA was purified and sequenced. These contiguous sequences (contigs) were then assembled into one single, non-redundant sequence and compared to reovirus sequences reported in GenBank. By this method, the sequence of the entire open reading frame (ORF) was read in both directions a minimum of two times. The complete T2W S1 ORF nucleotide and deduced σ1 aa sequences are shown in Figure 17.

The T2W S1 ORF is 1,416 nucleotides in length, which is 3 bases longer than the T1L S1 ORF. This encodes a 471 aa and 119 aa σ1 and σ1s protein. In order to evaluate the degree of sequence identity, the S1 ORF nucleotide sequence and deduced amino acid sequences of σ1 and σ1s were examined. Pairwise nucleotide identity between T2W and most clones inspected ranged between 23.2 and 47.8%; however, the T2W S1 ORF showed 99.4% similarity with the T1L S1 ORF (Figure 18). The sequence variability in the 5'- and 3'- non-coding regions was not evaluated because the PCR products were prepared using primers which represented the T1L gene sequence and 'fixed' these nucleotide bases. Compared to the T1L S1 ORF sequence, the T2W S1 ORF was found to have 6 nucleotide base changes: a transition at base number 18 where a thymidine was replaced with a cytosine, an insertion of 3 nucleotide bases from base number 19 to 21, a transition at base number 843 where a cytosine was replaced with a thymidine and a transversion at base number 1229 where a guanosine was replaced with a thymidine (Table 9).

The nucleotide base changes at base number 18 and 843 represent silent mutations with no change in the deduced aa sequence for the T2W σ1 protein. However, the insertion of 3 nucleotides lead to the insertion of a lysine molecule at the 7^{th} aa position. This insertion occurs in the tail (T(i) morphological region) of the σ1 protein which is hydrophobic and forms a short α-helical coiled coil. The nucleotide change at base number 1229 also lead to an aa change from a tryptophan to a leucine at aa 410 when compared to T1L (GenBank Accession # M35963) (Nibert et al. 1990). This change is located in the head region of the σ1 protein. It is interesting to note, this aa change is also present in T2J and in another T1L S1 sequence listed in Genbank (GenBank Accession # M32860; (Duncan et al. 1990)).

A.

```
T2W   atggatg-ca tctctcatca agacagagat acggaaaata gtact-ccaa ctatc-tgta     57
T1L   atggatg-ca tctctcatt- --acagagat acggaaaata gtact-ccaa ctatc-tgta     54
T2J   ----atgtcg gatctagtgc agctcataag aagggagatc ttactgttaa ctgg--g-aa     53
T3D   atggatcctc gcctacgtga ag-aagtagt acggctgata atcgcattaa cgagtgataa     59

T2W   tcaagcaatg --gctc---- --ccagtcaa aagaaatcga ggaaatcaag aaacaagtcc    109
T1L   tcaagcaatg --gctc---- --ccagtcaa aagaaatcga ggaaatcaag aaacaagtcc    106
T2J   tggagaatca --gccaa--- --ctcgaaac acgagatcga ggaaattaag aaacaaatta    106
T3D   tggagcatca ctgtcaaaag ggcttgaatc aag-ggtctc ggcgctcgag aagacgtctc    118

T2W   aggtcaacgt tgatgatatc agggctgcca atattaaact cgacggactt ggaagacaga    169
T1L   aggtcaacgt tgatgatatc agggctgcca atattaaact cgacggactt ggaagacaga    166
T2J   aagacatctc tgctgatgtc aacaggatca gtaacatcgt tgattcaatc caaggacaac    166
T3D   aaatacactc tgatactatc ctccggatca cccagggact cgatgatgca aacaaacgaa    178

T2W   ttgctgacat cagcaata-g catctcaacc attgagtcaa gattgggtga gatggataat    228
T1L   ttgctgacat cagcaata-g catctcaacc attgagtcaa gattgggtga gatggataat    225
T2J   tgggtgg-at tatctgtacg cgtgtcagcc attgaatcgg gagttagtga gaacggcaat    225
T3D   tcatcgctct tgagcaaagt cgggatgact -tggttgcat cagtcagtga tgctcaactt    237

T2W   cgacttgtgg gtatctcgag tcaggtcacg caattatcta actcagttag ccagaacact    288
T1L   cgacttgtgg gtatctcgag tcaggtcacg caattatcta actcagttag ccagaacact    285
T2J   cgaattgata gactcgagcg agatgtctcc ggcatatcgg ctagcgttag cggaatcgat    285
T3D   gcaatctcca gattggaaag ctctatcgga gccctccaaa cagttgtcaa tggacttgat    297

T2W   cagagcatat cctcattggg tgacagaatc aatgctgtcg aaccacgagt tgacagtctg    348
T1L   cagagcatat cctcattggg tgacagaatc aatgctgtcg aaccacgagt tgacagtctg    345
T2J   tcgcgtttat ccgagctggg tgaccgagtc aatgttgcag aacagcgaat tggccagttg    345
T3D   tcgagtgtta cccagttggg tgctcgagtg ggacaacttg agacaggact tgcagagcta    357

T2W   gatac-ggtc acgtctaatc tcactggacg aacatccact ttggaggcag atgttggaag    407
T1L   gatac-ggtc acgtctaatc tcactggacg aacatccact ttggaggcag atgttggaag    404
T2J   gatac-agtc acggataatc tccttgagcg agcatcaaga ctggaaactg aagtatcagc    404
T3D   cgcgttgatc acgac-aatc tcgttgcgag agtggatact gcagaacgta acat-tggat    415

T2W   c-ttacggac agaactagca gcgctaacaa cacgggtgac aactgaggtt acaaggttag    466
T1L   c-ttacggac agaactagca gcgctaacaa cacgggtgac aactgaggtt acaaggttag    463
T2J   cattactaat -gaccttgga tcattgaata cgagggtgac gactgaa--- ----------    450
T3D   cattgaccac tgagctatca actctgacgt tacgagtaac atccata--- ----------    462

T2W   atggtctaat caatagtggc cagaattcga ttggtgagct atccacaaga ctatc-caat    525
T1L   atggtctaat caatagtggc cagaattcga ttggtgagct atccacaaga ctatc-caat    522
T2J   -----ttgaa cgatgtccgc caaact---a ttgctgcgat agacacgcgt ctcac-gaca    501
T3D   ------caag cggatttcg- -aatctagga taaccacgtt agagcgcacg gcggtcacta    514

T2W   gtggaga--c gtctatggtg acgacggctg gacggggact gcagaaaaac ggaaacacct    583
T1L   gtggaga--c gtctatggtg acgacggctg gacggggact gcagaaaaac ggaaacacct    580
T2J   ctggaga--c cgatgccgtg acgtcggttg gtcaagggct tcagaagact gggaactcga    559
T3D   gcgcgggagc tcccctctca atccgtaata accgtatgac -catgggatt aaatgatgga    573

T2W   tgaacgtcat tgtaggtaat ggaatgtggt ttaatagttc taatcaattg cagctcgacc    643
T1L   tgaacgtcat tgtaggtaat ggaatgtggt ttaatagttc taatcaattg cagctcgacc    640
T2J   ttaaggttat tgtgggtacg gggatgtggt tcgaccgcaa taatgttctg cagttattct    619
T3D   ctcacgttgt ca-gggaata atctcgccat ccgattgcca ggaaatacgg ----------    622

T2W   tttcggggca atcaaaaggg gtgggatttg tcggcacagg aatggtggtt aagattgata    703
T1L   tttcggggca atcaaaaggg gtgggatttg tcggcacagg aatggtggtt aagattgata    700
T2J   tatcgaacca gcagaaaggg ttgggattca tagacaatgg aatggtagtg aaaatagata    679
T3D   -gtctgaata ttcaaaatgg -tggacttca gtttcgattt aatactgatc a------att    674

T2W   ctaattattt tgcttacaat agtaatggag agattacatt ggtgagtcaa atcaatgaat    763
T1L   ctaattattt tgcttacaat agtaatggag agattacatt ggtgagtcaa atcaatgaat    760
T2J   cccagtattt cagcttcgat agcaatggca acataactct gaacaacaac ataagtggtc    739
T3D   ccagatagtt -aataataac ttgactctca agacgactgt gtttgattct atcaac----    729
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T2W   tgccatcgcg cgtatcaaca ctggaatcag cgaaaatcga ttcagtttta cctccattaa    823
T1L   tgccatcgcg cgtatcaaca ctggaatcag cgaaaatcga ttcagtttta cctccattaa    820
T2J   tgccggcgcg aacaggttcc ctcgaggcat ctcgtatcga tgtggtagcg ccaccgcttg    799
T3D   -----tcaag gataggcgca actgagcaaa gttacgtggc gtcggcagtg actcccttga    784

T2W   ccgtacgcga agcgagcggt gtacgtaccc tgagctttgg ttatgatacg agcgatttta    883
T1L   ccgtacgcga agcgagcggc gtacgtaccc tgagctttgg ttatgatacg agcgatttta    880
T2J   tgatacagtc tactggtagc actcggctac tgcgtctcat gtacgaggct gtggacttcg    859
T3D   gattaaac-- ----agtagc acgaaggtgc tggatat--g ctaatagaca gttcaacact    836

T2W   caatcatcaa ctccgtactg tcgttacggt cacgtttgac tcttccgaca tacaggtacc    943
T1L   caatcatcaa ctccgtactg tcgttacggt cacgtttgac tcttccgaca tacaggtacc    940
T2J   tggttactaa caacgttctc acactgagaa atcgatcggt cacgccaaca ttcaagtttc    919
T3D   tga-aattaa ttctagtgga cagctaactg ttagatcgac atccccgaat ttgaggtatc    895

T2W   ctctggagct cgacacagca aataatagag tgcaggtggc agatcgtttt ggcatgcgca   1003
T1L   ctctggagct cgacacagca aataatagag tgcaggtggc agatcgtttt ggcatgcgca   1000
T2J   ctctggagtt gaatagtgct gataactcag tgagcattca tagaaattac cgcattagac    979
T3D   cgatagctga tgttagcggc ggtatcggaa tgagtcc--- ---aaattat aggtttaggc    949

T2W   cgggtacttg gacgggacaa ttgcaatatc agcacccaca attgagttgg agagcaaatg   1063
T1L   cgggtacttg gacgggacaa ttgcaatatc agcacccaca attgagttgg agagcaaatg   1060
T2J   ttgggcaatg gtcaggtcaa ttggaatatc acacgccgag tttgcgttgg aatgctcccg   1039
T3D   agagcatgtg gataggaatt gtctcctatt ctggtagtgg gctgaattgg agggtacagg   1009

T2W   tcactttgaa tttgatgaag gtggatgatt ggttggtgtt gagcttttct cagatgacga   1123
T1L   tcactttgaa tttgatgaag gtggatgatt ggttggtgtt gagcttttct cagatgacga   1120
T2J   tcacggttaa tttgatgcga gtagacgatt ggctcatttt gagttttact cggttttcga   1099
T3D   tgaactccga catttttatt gtagatgatt acatacatat atgtctt--c cagcttttga   1067

T2W   ctaact--ca ataatggcag atgggaaatt tgtgattaat tttgtgtctg ggttatcttc   1181
T1L   ctaact--ca ataatggcag atgggaaatt tgtgattaat tttgtgtctg ggttatcttc   1178
T2J   cgagcgg-cg atcttagcgt caggaaagtt tgtattgaac ttcgtaactg gtttgtctcc   1158
T3D   cggtttctct atagctgacg gtggagatct atcgttgaac tttgttaccg gattgttacc   1127

T2W   tggatggcag acgggggata ctgaaccatc gtcaactatt gatc------ cattgtctac   1235
T1L   tggatggcag acgggggata ctgaaccatc gtcaactatt gatc------ catggtctac   1232
T2J   agggtgggcg actgggagta ccgagccctc gacaactact aa-------- ----------   1200
T3D   accgttactt acaggagaca ctgagcccgc ttttcataat gacgtggtca catatggagc   1187

T2W   gacatttgcc gcg-gtccaa tttctaaata acggtcaacg cattgatgcg tttaggatca   1294
T1L   gacatttgcc gcg-gtccaa tttctaaata acggtcaacg cattgatgcg tttaggatca   1291
T2J   ---------- ---------- ---------- ---------- ---------- ----------   1200
T3D   acagactgta gctatagggt tgtcgtcg-g gtggtgcgcc tcagtatatg agtaagaatc   1246

T2W   tgggagtatc ggaatggacg gatggagaat tagagattaa gaattatggt ggcacataca   1354
T1L   tgggagtatc ggaatggacg gatggagaat tagagattaa gaattatggt ggcacataca   1351
T2J   ---------- ---------- ---------- ---------- ---------- ----------   1200
T3D   tgtgggtgga gcagtggcag gatggagtac ttcggttacg tgttgagggg ggtggctcaa   1306

T2W   ccggtcatac tcaagtatat tgggctccgt ggacgatcat gtatccatgc aatgtgaggt   1414
T1L   ccggtcatac tcaagtatat tgggctccgt ggacgatcat gtatccatgc aatgtgaggt   1411
T2J   ---------- ---------- ---------- ---------- ---------- ----------   1200
T3D   ttacgcactc aaacagtaag tggcctgcca tgaccgtttc gtacccgcgt agtttcacgt   1366

T2W   ga                                                                 1416
T1L   ga                                                                 1413
T2J   --                                                                 1200
T3D   ga                                                                 1368
```

B.

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T2W   MDASLIKTEI RKIVLQLSVS SNGSQSK--- -EIEEIKKQV QVNVDDIRAA NIKLDGLGRQ     56
T1L   MDASLI-TEI RKIVLQLSVS SNGSQSK--- -EIEEIKKQV QVNVDDIRAA NIKLDGLGRQ     55
T2J   --MSDLVQLI RREILLLTGN GESANSKH-- -EIEEIKKQI KDISADVNRI SNIVDSIQGQ     55
T3D   -MDPRLREEV VRLIIALTSD NGASLSKGLE SRVSALEKTS QIHSDTILRI TQGLDDANKR     59

T2W   IADISNSIST IESRLGEMDN RLVGISSQVT QLSNSVSQNT QSISSLGDRI NAVEPRVDSL    116
T1L   IADISNSIST IESRLGEMDN RLVGISSQVT QLSNSVSQNT QSISSLGDRI NAVEPRVDSL    115
T2J   LGGLSVRVSA IESGVSENGN RIDRLERDVS GISASVSGID SRLSELGDRV NVAEQRIGQL    115
T3D   IIALEQSRDD LVASVSDAQL AISRLESSIG ALQTVVNGLD SSVTQLGARV GQLETGLAEL    119

T2W   DTVTSNLTGR TSTLEADVGS LRTELAALTT RVTTEVTRLD GLINSGQNSI GELSTRLSNV    176
T1L   DTVTSNLTGR TSTLEADVGS LRTELAALTT RVTTEVTRLD GLINSGQNSI GELSTRLSNV    175
T2J   DTVTDNLLER ASRLETEVSA ITNDLGSLNT RVTTELNDVR -------QTI AAIDTRLTTL    168
T3D   RVDHDNLVAR VDTAERNIGS LTTELSTLTL RVT------- -------SIQ ADFESRITTL    165

T2W   ETSMVTTAGR GLQKNGNTLN VIVGNGMWFN SSNQLQLDLS GQSKGVGFVG TGMVVKIDTN    236
T1L   ETSMVTTAGR GLQKNGNTLN VIVGNGMWFN SSNQLQLDLS GQSKGVGFVG TGMVVKIDTN    235
T2J   ETDAVTSVGQ GLQKTGNSIK VIVGTGMWFD RNNVLQLFVS NQQKGLGFID NGMVVKIDTQ    228
T3D   ERTAVTSAGA PLSIRNNRMT MGLNDGLTLS GNN-LAIRLP G-NTGLNIQN GGLQFRFNTD    223

T2W   YFAYNSNGEI TLVSQINELP SRVSTLESAK IDSVLPPLTV REASGVRTLS FGYDTSDFTI    296
T1L   YFAYNSNGEI TLVSQINELP SRVSTLESAK IDSVLPPLTV REASGVRTLS FGYDTSDFTI    295
T2J   YFSFDSNGNI TLNNNISGLP ARTGSLEASR IDVVAPPLVI QSTGSTRLLR LMYEAVDFVV    288
T3D   QFQIVNNNLT LKTTVFDSIN SRIGATEQSY VASAVTPLRL NSS--TKVLD MLIDSSTLEI    281

T2W   INSVLSLRSR LTLPTYRYPL ELDTANNRVQ VADRFGMRTG TWTGQLQYQH PQLSWRANVT    356
T1L   INSVLSLRSR LTLPTYRYPL ELDTANNRVQ VADRFGMRTG TWTGQLQYQH PQLSWRANVT    355
T2J   TNNVLTLRNR SVTPTFKFPL ELNSADNSVS IHRNYRIRLG QWSGQLEYHT PSLRWNAPVT    348
T3D   N-SSGQLTVR STSPNLRYPI ADVSGG--IG MSPNYRFRQS MWIGIVSYSG SGLNWRVQVN    338

T2W   LNLMKVDDWL VLSFSQMTTN SIMADGKFVI NFVSGLSSGW QTGDTEPSST IDPLST--TF    414
T1L   LNLMKVDDWL VLSFSQMTTN SIMADGKFVI NFVSGLSSGW QTGDTEPSST IDPWST--TF    416
T2J   VNLMRVDDWL ILSFTRFSTS GILASGKFVL NFVTGLSPGW ATGSTEPSTT TNPLST--TF    406
T3D   SDIFIVDDYI HICLPAFDGF SIADGGDLSL NFVTGLLPPL LTGDTEPAFH NDVVTYGAQT    398

T2W   AAVQFLNNGQ RIDAFRIMGV SEWTDGELEI KNYGGTYTGH TQVYWAPWTI MYPCNVR       471
T1L   AAVQFLNNGQ RIDAFRIMGV SEWTDGELEI KNYGGTYTGH TQVYWAPWTI MYPCNVR       470
T2J   AAIQFINGSS RVDAFRILGV AEWNAGELEI TNYGGTYTAH TNVDWAPMTI MYPCLG-       462
T3D   VAIGLSSGGA PQYMSKNLWV EQWQDGVLRL RVEGGGSITH SNSKWPAMTV SYPRSFT       455
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Figure 17. T2W S1 nucleotide and deduced σ1 amino acid sequence alignments

(A) The S1 nucleotide sequences included in the alignment are T2W (determined in this study), Lang (Genbank Accession # M35963), Jones (Genbank Accession # M35964) and Dearing (Genbank Accession # M32094). **(B)** The σ1 amino acid sequences were deduced from the S1 nucleotide sequences. The single-letter amino acid code is utilized. Differences in nucleotide or amino acid sequence between T2W and T1L are highlighted in red.

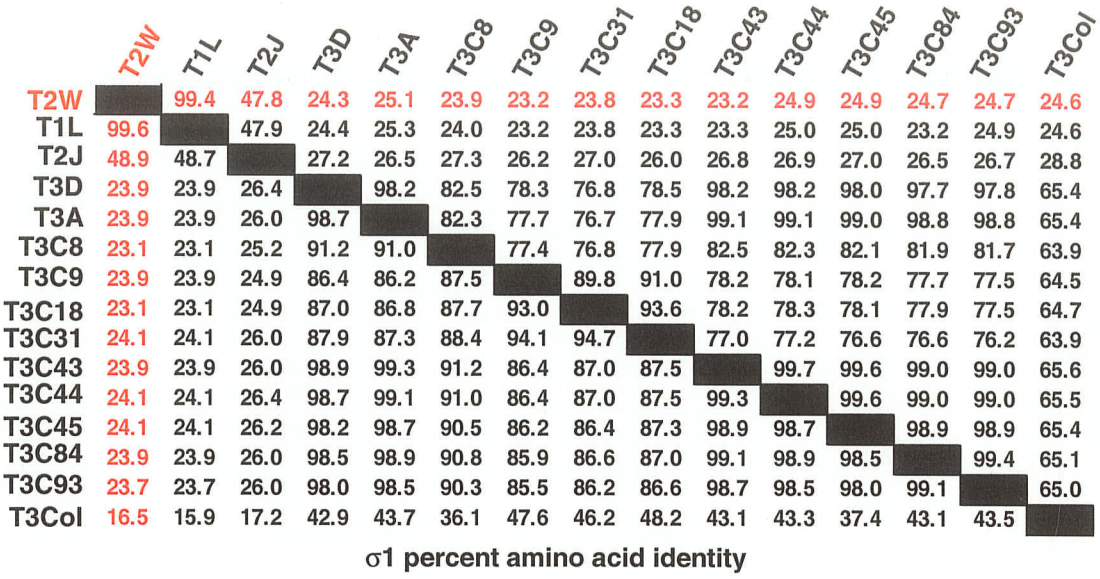

T2W T1L T2J T3D T3A T3C8 T3C9 T3C31 T3C18 T3C43 T3C44 T3C45 T3C84 T3C93 T3Col
T2W 99.4 47.8 24.3 25.1 23.9 23.2 23.8 23.3 23.2 24.9 24.9 24.7 24.7 24.6
T1L 99.6 47.9 24.4 25.3 24.0 23.2 23.8 23.3 23.3 25.0 25.0 23.2 24.9 24.6
T2J 48.9 48.7 27.2 26.5 27.3 26.2 27.0 26.0 26.8 26.9 27.0 26.5 26.7 28.8
T3D 23.9 23.9 26.4 98.2 82.5 78.3 76.8 78.5 98.2 98.2 98.0 97.7 97.8 65.4
T3A 23.9 23.9 26.0 98.7 82.3 77.7 76.7 77.9 99.1 99.1 99.0 98.8 98.8 65.4
T3C8 23.1 23.1 25.2 91.2 91.0 77.4 76.8 77.9 82.5 82.3 82.1 81.9 81.7 63.9
T3C9 23.9 23.9 24.9 86.4 86.2 87.5 89.8 91.0 78.2 78.1 78.2 77.7 77.5 64.5
T3C18 23.1 23.1 24.9 87.0 86.8 87.7 93.0 93.6 78.2 78.3 78.1 77.9 77.5 64.7
T3C31 24.1 24.1 26.0 87.9 87.3 88.4 94.1 94.7 77.0 77.2 76.6 76.6 76.2 63.9
T3C43 23.9 23.9 26.0 98.9 99.3 91.2 86.4 87.0 87.5 99.7 99.6 99.0 99.0 65.6
T3C44 24.1 24.1 26.4 98.7 99.1 91.0 86.4 87.0 87.5 99.3 99.6 99.0 99.0 65.5
T3C45 24.1 24.1 26.2 98.2 98.7 90.5 86.2 86.4 87.3 98.9 98.7 98.9 98.9 65.4
T3C84 23.9 23.9 26.0 98.5 98.9 90.8 85.9 86.6 87.0 99.1 98.9 98.5 99.4 65.1
T3C93 23.7 23.7 26.0 98.0 98.5 90.3 85.5 86.2 86.6 98.7 98.5 98.0 99.1 65.0
T3Col 16.5 15.9 17.2 42.9 43.7 36.1 47.6 46.2 48.2 43.1 43.3 37.4 43.1 43.5
σ1 percent amino acid identity
S1 percent nucleotide identity

Figure 18. T2W S1 nucleotide and σ1 amino acid identity

S1 nucleotide sequence data are presented in the top right triangle and σ1 amino acid sequence data are presented in the bottom left triangle. Sequence identity was determined using MegAlign software based on Clustal method with weighted residue weight table. Clone abbreviations are indicated in Table 6.

Table 9. Details of changes in T2W S1 ORF compared to T1L S1 ORF

Base Number	aa[a] number	Type of Change	Codon change	aa[a] change
18	6	transition	ATT to ATC	no change
19-21	7	insertion	AAG	K[b]
843	281	transition	GGC to GGT	no change
1229	410	transversion	TGG to TTG	W[b] to L[b]

* T1L sequence was obtained from GenBank (Accession # M35963)

[a] aa = amino acid

[b] K = lysine; W = tryptophan; L = leucine

Hydropathy is defined as a measure of attraction to water where positive attraction is defined as hydrophilic and negative being hydrophobic. All amino acids are assigned a relative hydropathy index value based on their water-vapour transfer free energies and polarity. T2W σ1 protein was compared to T1L σ1 protein using the BioEdit Sequence Alignment Editor software (Kyte and Doolittle 1982;Hall 1999). This program uses a variety of hydropathy analyses based on Kyte and Doolittle (1982). No gross changes in hydrophobicity or hydrophilicity were seen when T2W and T1L σ1 proteins were compared.

The nucleotide base changes seen in T2W S1 ORF did not alter the deduced aa sequence of the σ1s protein which was identical to the equivalent T1L protein (data not shown).

3.2.3.2.Comparison of S2 nucleotide sequence. Attempts to amplify and sequence the T2W S2 gene segment were not successful. Initial attempts were made using primers based on T1L (Figure 7). Subsequent attempts involved changes in primer design (e.g. degenerate primers or primers based on areas of sequence homology between known sequences), temperature gradient PCR varying the annealing temperature and changes in Mg^{2+} concentration. Studies indicate the S2 gene and σ2 protein are highly conserved among reovirus strains (Chappell et al. 1994). This suggests the inability to amplify the S2 segment is likely because the T2W S2 gene segment is significantly different from previously reported clones.

3.2.3.3.Comparisons of S3 nucleotide and σNS amino acid sequences. Sequence of the T2W S3 gene segment was read in both directions of the 1,152 base amplification product and compared to sequences reported in GenBank. The entire ORF was sequenced in both directions a minimum of two times. The complete T2W S3 ORF nucleotide and deduced σNS aa sequences are shown in Figure 19. The ORF of the S3 gene of T2W is 1,101 nucleotides in length, which is identical to T1L, T2J and T3D and encodes a 366 aa σNS protein. In pairwise comparisons of nucleotide sequence variability, identity of nucleotide positions ranged between 68.1 and 73.6% (Figure 20A) and was most like the Type 1 Netherlands 1985 clone and least similar to T2J. In pairwise comparisons of deduced aa sequence of the σNS protein, alignment variability with known clones ranged between 10.1 to 13.7% (Figure 20A). Thirteen cysteine residues, previously identified in

A.

```
T2W   atggcttctt cactcagagc agcgatctcc aagattaaac gtgatgatct tggtcagcaa     60
T1L   atggcttcct cactcagggc tgcgatctct aagatcaaga gagatgatgt tggtcagcaa
T2J   atggcttcct cactcagagc tgcgatctca aagatcaagc gcgatgatgt cggtcagcaa
T3D   atggcttcct cactcagagc tgcgatctcc aagatcaaga gggatgacgt cggtcagcaa

T2W   gtatgtccca gctatgtcat gctgagatca tctgtgacaa ccaaagttgt tcgtaacgtg    120
T1L   gtttgtccaa attatgtcat gcttagatca tcggtcacaa cgaaggtggt acgaaacgtt
T2J   gtatgcccta actatgtcat gttgaggtct tctgtgaata ccaaagtggt gagaaatgtg
T3D   gtttgtccta attatgtcat gctgcggtcc tctgtcacaa caaaggtggt acgaaatgtg

T2W   gttgagtacc aaattaggac tggtggtttc ttttcttgtc tgtcaatgtt gcgtccgctc    180
T1L   gttgagtatc aaatccgtac aggtggattc ttttcgtgcc tagctatgtt gagaccgctc
T2J   gttgactacc agattaaaac tggcggcttc ttctcctgta tcgcaatgct gcgtcctttg
T3D   gttgagtatc aaattcgtac gggcggattc ttttcgtgct tagctatgct aaggccactc

T2W   cagtatgcca agcgtgagcg gttgttaggc cagcgcaatt tggaacgcat atcctctaga    240
T1L   cagtatgcta aacgtgagcg tctgcttgga caaaggaatc tggaacgcat atcgactagg
T2J   caatatgcca agcgtgaaag attgctgggt caacgcaacc tagaacgtat cgctgcccga
T3D   cagtacgcta agcgtgagcg tttgcttggt cagaggaatc tggaacgtat atcgactagg

T2W   gatattcttc agactcgtga tctgcattcc ttgtgtatgc ctactcctga tgctcctatg    300
T1L   gacattcttc agacacgcga tttgcactca ttgtgcatgc caactcctga cgcgccaatg
T2J   gacgtgctgc aaaccaggga tctacactca ctatgcatgc ccacgcctga tgctccgatg
T3D   gatatccttc agactcgtga tttacactca ctatgtatgc caactcctga tgcgccaatg

T2W   tcaaactatc aggcatcgac tatgagagag ctcatatgta gttattttaa gacagatcat    360
T1L   tccaatcatc aggcagccac catgagagag ctgatctgca gctacttcaa ggtcgaccac
T2J   actaattacc aggcttccac catgagagaa ttagtttgtg accacttcaa ggttgatcac
T3D   tctaatcatc aagcatccac catgagagag ctgatttgca gttacttcaa ggtcgatcat

T2W   gctgatggtc tgagatatgt accaatggat gatcgatatt cgccgtcatc gctggctagg    420
T1L   actgatgggt tgaaatatat acccatggat gagagatatt ctccatcatc gcttgccaga
T2J   gtggatggac tacgctatgt ccccatggat gacaggtact ccccctcttc cttagctcga
T3D   gcggatgggt tgaaatatat acccatggat gagagatact ctccgtcatc acttgccaga

T2W   cttttttacta tgggaatggc tggacttcac attactacag acccttcata taagagagtg    480
T1L   ttgttcacta tgggtatggc tggactacac attaccactg agccttccta caaacgtgtg
T2J   ctgtttacaa tgggaatggc tggtctccac atcacgactg agcctgcgta taagcgagtc
T3D   ttgtttacca tgggcatggc tgggctgcac attaccactg agccatctta taagcgtgtt

T2W   ccgataatgc atttggcggc agaccttgat tgcatgacat tggccttgcc ttacatgatc    540
T1L   cccatcatgc acttggcagc agatttggac tgcatgacgt tagctttacc ctacatgatt
T2J   ccaattatgc accttgctgc tgatcttgac tgcatgacat ttgcacttcc atacatgatc
T3D   ccgattatgc acttagctgc ggacttggac tgtatgacgc tggctctacc ttacatgatt

T2W   actctcgatg gtgacacagt tgttcctgtc gctcctgtgg cttctgctga gcagttgttg    600
T1L   acacttgatg gtgacacagt ggtacctgtc gccccaacgc tttctgcaga acagcttttg
T2J   actgttgatg gtgatactgt agtacctgtg gctccaactt taccagctga acgccttttg
T3D   acgcttgatg gtgatactgt ggttcctgtc gctccaacac tgtcagcgga acagcttctg

T2W   gatgatggct tcaagggctt ggcttgtatg gacatttcat acggttgtga ggtcgacgcc    660
T1L   gatgatggac ttaagggatt agcatgcatg gatatctcat acggatgtga ggtggacgct
T2J   gatgacggtt ttaaaggata tggttgttta gacatctcat atggatgtga ggttgatgcc
T3D   gacgacggac tcaaaggatt agcatgcatg gatatctcct atggatgtga ggtggacgcg

T2W   aataatcggt cagctggaga tcagagtatg gactcttcgc gttgtatcaa cgagctgcac    720
T1L   agcaaccgat cagctggtga tcagagcatg gattcttcac gatgcatcaa tgagttatat
T2J   aacaaccggt cagcgggaga tcaaagtatg gactcatcca gatgcattaa tgagctgtat
T3D   aatagccggc cggctggtga tcagagtatg gactcttcac gctgcatcaa cgagttgtat

T2W   tgtgatgata cggctgaggc gatatgcata ctaaagacat gtctgatact taactgcatg    780
T1L   tgcgaggaaa cggcagaagc tatctgtgta ctcaaaacat gtcttgtgct gaactgtatg
T2J   accgctgaaa cagctgaggc catttgtata cttaaaactt gtttgatcct gaattgtatg
T3D   tgcgaggaga cagcagaagc catctgtgtg cttaagacat gccttgtgtt aaattgcatg
```

```
T2W   cagtttaagt tagagatgga cgacctggca cataacagcg tagagctgga taaagtgcag    840
T1L   caattcaaac ttgagatgga tgatttagca cacaacgcta ctgagctgga caagatacag
T2J   cagtttaagt tggagatgga cgacttggca cacaatggat tcgaattaga taaggtacag
T3D   cagtttaaac ttgagatgga tgacctagca cataacgctg ctgagctgga caagatacag

T2W   atgatgattc cgttcagtga gcgtatcttt aggatggcct ccgcgttcgc cacaatcgat    900
T1L   atgatgatac cttttagtga acgtgttttc agaatggctt cttcatttgc taccattgat
T2J   atgatgatac cattcagcga gagagtgttt cggatggctt ccgcatttgc taccatagat
T3D   atgatgatac ccttcagtga gcgtgttttt aggatggcct cgtcctttgc gactattgat

T2W   gcgcagtgct tccgtttctg tgtcatgatg aaagacaaga atttgaaagt tgatatgcga    960
T1L   gcccagtgtt tcaggttttg tgtgatgatg aaggataaga atttgaagat agacatgcgt
T2J   gttcagtgtt tcagattttg tcttttgatg aaggataaga acctcaaaat tgatatgagg
T3D   gcccagtgtt ttaggttttg cgtgatgatg aaggataaaa atctgaaaat agatatgcgt

T2W   gagactatga ggacatggac gaaagccagc tccgatgacg ctattgttat ctcatcattg   1020
T1L   gaaacgatga gactttggac tcgatcggcg ttggacgatt cagtggttac gtcatctttg
T2J   gagaccatgc gcttgtggac gcgcgctggc tcggacgacg ccatctctac atcttccctg
T3D   gaaacgacga gactgtggac tcgttcagca tcagatgatt ctgtggccac gtcatcttta

T2W   actgtcaccc tggatagggg tagatgggtt gcttctgatg caaatgacac tcgactgctc   1080
T1L   agtatttcgc tggatcgagg tcgatgggta gcagctgatg ctactgatgc tagattgctg
T2J   actatttcac tggaccgtgg acggtgggtg gcgatggata tgaacgaggt gcgtctctta
T3D   agtatttcct tggaccgggg tcgatgggtg gcggctgacg ccagtgatgc tagactgctg

T2W   gtattcccta ttcgtgtgta a                                            1101
T1L   gtgtttccaa ttcgcgtgta a
T2J   gtgttccctg ctcgtgtgta a
T3D   gttttccga ttcgcgtgta a
```

B.

```
T2W   MASSLRAAIS KIKRDDLGQQ VCPSYVMLRS SVTTKVVRNV VEYQIRTGGF FSCLSMLRPL     60
T1L   MASSLRAAIS KIKRDDVGQQ VCPNYVMLRS SVTTKVVRNV VEYQIRTGGF FSCLAMLRPL
T2J   MASSLRAAIS KIKRDDVGQQ VCPNYVMLRS SVNTKVVRNV VDYQIKTGGF FSCIAMLRPL
T3D   MASSLRAAIS KIKRDDVGQQ VCPNYVMLRS SVTTKVVRNV VEYQIRTGGF FSCLAMLRPL

T2W   QYAKRERLLG QRNLERISSR DILQTRDLHS LCMPTPDAPM SNYQASTMRE LICSYFKTDH    120
T1L   QYAKRERLLG QRNLERISTR DILQTRDLHS LCMPTPDAPM SNHQAATMRE LICSYFKVDH
T2J   QYAKRERLLG QRNLERIAAR DVLQTRDLHS LCMPTPDAPM TNYQASTMRE LVCDHFKVDH
T3D   QYAKRERLLG QRNLERISTR DILQTRDLHS LCMPTPDAPM SNHQASTMRE LICSYFKVDH

T2W   ADGLRYVPMD DRYSPSSLAR LFTMGMAGLH ITTDPSYKRV PIMHLAADLD CMTLALPYMI    180
T1L   TDGLKYIPMD ERYSPSSLAR LFTMGMAGLH ITTEPSYKRV PIMHLAADLD CMTLALPYMI
T2J   VDGLRYVPMD DRYSPSSLAR LFTMGMAGLH ITTEPAYKRV PIMHLAADLD CMTFALPYMI
T3D   ADGLKYIPMD ERYSPSSLAR LFTMGMAGLH ITTEPSYKRV PIMHLAADLD CMTLALPYMI

T2W   TLDGDTVVPV APVASAEQLL DDGFKGLACM DISYGCEVDA NNRSAGDQSM DSSRCINELH    240
T1L   TLDGDTVVPV APTLSAEQLL DDGLKGLACM DISYGCEVDA SNRSAGDQSM DSSRCINELY
T2J   TVDGDTVVPV APTLPAERLL DDGFKGYGCL DISYGCEVDA NNRSAGDQSM DSSRCINELY
T3D   TLDGDTVVPV APTLSAEQLL DDGLKGLACM DISYGCEVDA NSRPAGDQSM DSSRCINELY

T2W   CDDTAEAICI LKTCLILNCM QFKLEMDDLA HNSVELDKVQ MMIPFSERIF RMASAFATID    300
T1L   CEETAEAICV LKTCLVLNCM QFKLEMDDLA HNATELDKIQ MMIPFSERVF RMASSFATID
T2J   TAETAEAICI LKTCLILNCM QFKLEMDDLA HNGFELDKVQ MMIPFSERVF RMASAFATID
T3D   CEETAEAICV LKTCLVLNCM QFKLEMDDLA HNAAELDKIQ MMIPFSERVF RMASSFATID

T2W   AQCFRFCVMM KDKNLKVDMR ETMRTWTKAS SDDAIVISSL TVTLDRGRWV ASDANDTRLL    360
T1L   AQCFRFCVMM KDKNLKIDMR ETMRLWTRSA LDDSVVTSSL SISLDRGRWV AADATDARLL
T2J   VQCFRFCLLM KDKNLKIDMR ETMRLWTRAG SDDAISTSSL TISLDRGRWV AMDMNEVRLL
T3D   AQCFRFCVMM KDKNLKIDMR ETTRLWTRSA SDDSVATSSL SISLDRGRWV AADASDARLL

T2W   VFPIRV                                                            366
T1L   VFPIRV
T2J   VFPARV
T3D   VFPIRV
```

Figure 19. Alignment of S3 ORF and the predicted σNS amino acid sequences

(A) The S3 ORF nucleotide sequences included in the alignment are T2W (determined in this study), Lang (Genbank Accession # M18389), Jones (Genbank Accession # M18390) and Dearing (Genbank Accession # X01627). **(B)** The σNS amino acid sequences were deduced from the S3 nucleotide sequences. The single-letter amino acid code is utilized. Nonconserved nucleotides and amino acids are highlighted in red.

A.

	T2W	T1L	T2J	T3D	T3A	T1C11	T1C62	T1N67	T1N84	T1N85	SV59	T2N73	T2N84	T2Tok	T3C8	T3C9	T3C18	T3C31	T3C84	T3N83	T3Fel
T2W		72.4	68.1	71.7	71.9	72.8	72.8	73.0	73.0	73.6	71.7	72.7	72.5	73.4	72.6	73.3	72.7	71.9	71.8	72.1	71.8
T1L	88.8		66.8	84.7	83.5	96.9	97.5	85.3	90.3	90.5	94.6	90.6	90.4	89.9	84.1	85.6	97.4	96.4	83.7	85.1	96.8
T2J	86.3	86.1		68.2	68.3	67.1	67.1	67.9	69.1	68.9	67.3	69.0	68.2	68.9	68.4	67.7	67.2	66.9	68.3	67.8	67.4
T3D	88.8	97.3	86.1		98.5	83.7	84.0	91.7	84.3	83.9	85.0	84.7	83.7	83.7	87.3	91.7	84.3	84.7	98.9	91.3	84.8
T3A	88.3	96.2	85.5	98.9		82.4	82.7	90.8	83.6	83.4	83.8	83.9	83.0	83.6	86.2	90.7	82.9	83.1	98.9	90.3	83.5
T1C11	88.5	97.5	85.8	96.7	95.6		98.6	84.0	90.0	90.3	94.2	90.3	89.9	89.6	83.8	83.9	98.7	96.4	82.5	84.2	96.5
T1C62	89.1	98.1	86.3	97.3	96.2	98.9		84.3	90.5	90.6	94.8	90.8	90.4	90.0	84.3	84.4	99.0	96.8	82.9	84.3	96.9
T1N67	89.6	97.5	86.1	98.1	97.0	96.5	97.0		83.7	83.0	85.6	83.7	83.3	83.0	87.3	98.5	84.2	84.8	91.1	97.9	85.0
T1N84	89.3	97.5	85.8	97.5	96.5	97.9	98.6	98.1		97.2	90.2	98.5	97.7	96.9	83.7	84.1	90.3	90.0	83.7	83.8	90.5
T1N85	89.3	97.8	86.3	97.5	97.0	98.1	98.6	97.3	98.6		90.1	97.6	97.2	98.8	83.7	83.7	90.6	90.3	83.6	83.7	90.6
SV59	89.1	97.0	86.1	96.7	95.6	97.3	97.8	96.5	97.8	98.1		91.0	90.7	97.3	84.6	85.5	94.6	94.3	84.0	83.8	90.8
T2N73	88.8	97.8	85.8	97.5	96.5	98.1	98.6	97.3	98.6	98.9	98.1		98.2	96.6	84.2	84.1	90.6	90.6	84.1	83.3	90.5
T2N84	88.8	97.0	85.3	96.7	95.6	97.3	97.8	97.0	98.4	98.1	97.3	98.1		89.2	84.0	83.7	90.2	90.4	83.3	85.5	94.8
T2Tok	88.8	97.0	85.5	96.7	96.2	97.3	97.8	96.7	98.1	99.2	97.3	98.1	97.3		83.6	83.5	89.9	89.6	83.7	83.7	90.0
T3C8	88.8	96.2	85.3	97.0	95.9	96.5	97.0	96.7	97.3	97.8	96.5	97.3	96.5	97.3		87.4	84.7	85.5	86.6	84.2	97.0
T3C9	89.9	98.1	86.3	98.1	97.0	96.5	97.0	99.5	98.1	97.3	96.5	97.3	97.0	96.7	96.7		84.3	84.7	91.3	84.8	97.4
T3C18	89.1	98.1	86.3	97.3	96.2	98.9	99.5	97.0	98.4	98.6	97.8	98.6	97.8	97.8	97.0	97.0		97.2	83.2	90.6	83.8
T3C31	88.5	98.1	85.8	97.3	96.2	98.4	98.9	97.0	98.4	98.6	97.8	98.6	97.8	97.8	97.0	97.0	98.9		83.6	87.6	84.8
T3C84	89.1	97.0	86.3	99.7	99.2	96.5	97.0	97.9	97.3	97.8	96.5	97.3	96.5	97.0	96.7	97.8	97.0	97.0		97.8	85.3
T3N83	89.6	97.5	86.1	98.1	97.0	96.5	97.0	99.5	98.1	97.3	96.5	97.3	97.0	96.7	96.7	99.5	97.0	97.0	97.8		85.2
T3Fel	88.8	98.1	86.1	97.5	96.5	98.4	98.9	97.3	98.4	98.6	97.8	98.6	97.8	97.8	97.3	97.3	98.9	98.9	97.3	97.3	

σNS percent amino acid identity

S3 percent nucleotide identity

B.

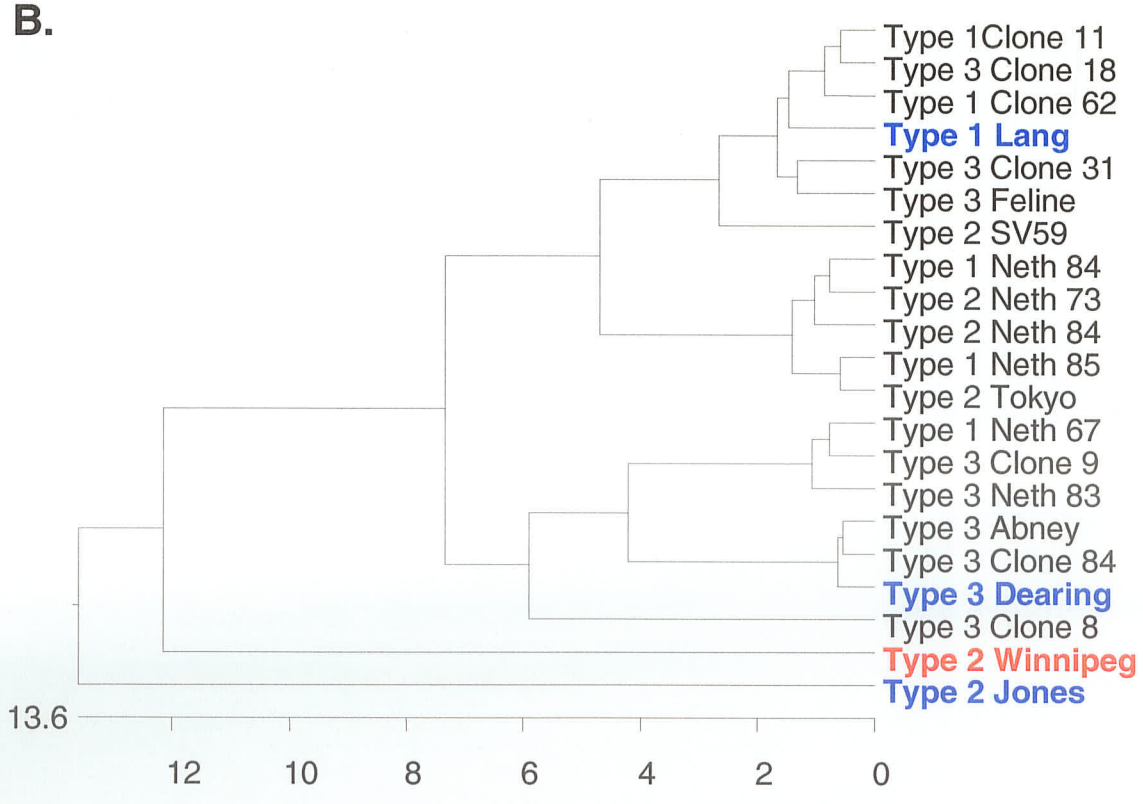


Figure 20. T2W S3 nucleotide and σNS amino acid identity and phylogeny of reovirus S3 gene segment

(A) S3 nucleotide sequence data are presented in the top right triangle and σNS amino acid sequence data are presented in the bottom left triangle. Sequence identity was determined using MegAlign software based on Clustal method. Clone abbreviations are indicated in Table 6. **(B)** Phylogenetic tree for reovirus S3 gene segment constructed by MegAlign using Clustal method with weighted residue weight table.

the deduced aa sequence of T1L and conserved in field isolates, were also conserved in T2W. The aa substitution of methionine to threonine at position 260, seen in the deduced aa sequence of *ts*E320, was also not seen (Wiener and Joklik 1987).

To define the evolutionary relationship of the T2W S3 gene segment, a phylogenetic tree was produced using variation in the S3 ORF nucleotide sequences of T2W, 4 prototype and 16 field-isolate strains. The phylogenetic tree generated by MegAlign using Clustal method with weighted residue table is shown in Figure 20B. The resulting minimum-length phylogenetic tree delineates the most probable evolutionary relatedness of the T2W S3 ORF. Like T2J, T2W formed its own lineage, which indicates T2W S3 gene segment is more distantly related to other known reovirus clones.

3.2.3.4.Comparison of the S4 nucleotide sequence. The T2W S4 gene segment was PCR-amplified and the resulting cDNA was purified and sequenced as described in section 2.7. After compilation of the contigs, it was seen the T2W S4 ORF sequence was identical to T1L. This was unexpected since the mobility of gene segments between T1L and T2W are different when compared by electrophoresis (Figure 16B). However, results were confirmed after the experiment was repeated on numerous occasions with extra care taken to avoid cross-contamination. Also, a 509 nucleotide amplification product from a nested PCR experiment completed on the original sample received from Cadham Provincial Laboratory was identical to the T1L sequence.

Serologic, EM and molecular studies indicate T2W is a novel reovirus clone isolated from an immunocompromised child. The persistent reovirus infection may have contributed to her respiratory, gastrointestinal and CNS symptoms. Since reovirus infections are not generally associated with human disease, there has not been much research into the treatment of reoviruses infections. In the next phase of this study, we wanted to find an antiviral compound that could inhibit reovirus and T2W replication, that could have been used to treat this infant. Due to small quantities of T2W viral stocks, potential antiviral agents were initially screened with prototype reoviruses, T1L and T3D. Inhibition of T2W was only examined after a suitable anti-reoviral agent was determined.

3.3. Effects of various antiviral agents on reovirus replication. Antiviral agents can be classified based on where they act during the viral lifecycle (De Clercq 2002). In this study, seven antiviral agents with a variety of antiviral mechanisms of action were chosen

for examination. The main objective of this research arm was to determine an agent that inhibited reovirus replication and could have been used in the management of an infection with T2W. However, we also wanted to find an antiviral agent that could be used as a tool to help elucidate the mammalian reovirus lifecycle and characterize differences in mammalian and avian reovirus replication.

Compounds chosen for investigation were either known to have activity against reovirus (positive control), predicted not to have activity (negative control) and agents with unknown activity. Ribavirin was chosen as a positive control. It is a nucleoside analogue with activity against a broad spectrum of mostly RNA viruses and known activity against reovirus (Rankin, Jr. et al. 1989;Bodelon et al. 2002;Connolly and Dermody 2002;Labrada et al. 2002). There is debate regarding the mechanism of antiviral activity of ribavirin. In its 5'-monophosphate form, ribavirin inhibits IMPDH, which causes depletion of intracellular pools of GTP and dGTP. In its 5'-triphosphate form, ribavirin can also interfere with the viral RNA polymerase, capping guanylation and acts as a lethal mutagen. AZT is a nucleoside RT inhibitor targeted at the RT of HIV and thus, was chosen as a negative control. It acts as a chain terminator in the RT reaction in its 5'-triphosphate form and is incorporated at the 3'-end of the viral DNA chain when monophosphorylated. Amantadine was also chosen as a negative control. It is an anti-influenza agent which blocks the M2 ion channel in influenza A virus. This blockage prevents the passage of H^+ ions required for the endosomal acidification and viral uncoating. Ayclovir is an acyclic guanosine nucleoside analogue used primarily for treatment of herpes simplex and varicella zoster viruses. Similar to AZT, it acts as a chain terminator in its 5'-triphosphate form and is incorporated at the 3'-end of the viral DNA chain when monophosphorylated. Phosphorylation is catalyzed by the virus-encoded thymidine kinase which explains the specificity of acyclovir for herpes simplex virus and VZV. Foscarnet is a pyrophosphate analogue that does not need to be phosphorylated to interact with the pyrophosphate binding site of the viral DNA polymerase. Its principal indications for use are in cytomegalovirus retinitis in AIDS patients and ACV-resistant (i.e. viral thymidine kinase-deficient) HSV infections in immunocompromised patients; however it has been shown to inhibit rotavirus, another member of the family *Reoviridae*, replication in cell culture (Rankin, Jr. et al. 1989;Rios et al. 1995). Rifampin is generally

used clinically with other anti-tuberculosis drugs to prevent the emergence of drug-resistant mycobacteria, but also exhibits anti-pox virus actvity affecting the packaging and assembly stage in the poxvirus lifecycle. Other agents shown to decrease reovirus replication, such as neplanocin A (*S*-adenosylhomocysteince hydrolase inhibitor), acivicin (glutamine analog), cicloxolone sodium (Golgi apparatus inhibitor) and cyclopentenylcytosine (cytidine analogue) were unfortunately not available for examination. For review of antiviral compounds, see (De Clercq 2001b).

To screen compounds, subconfluent L929 cells were inoculated with T1L and T3D at an MOI = 0.5 PFU/cell and treated with the appropriate antiviral agent at various concentrations. Concentrations of antiviral agents tested ranged above and below previously reported active concentrations with the particular agent being tested (Esteban 1977;Hay et al. 1985;Furman et al. 1986;Rankin, Jr. et al. 1989;Sodeik et al. 1995;Rios et al. 1995;Field 2001). Virus was harvested at 72 hpi and viral titre determined.

Results were not promising as most of the drugs tested were not suitable for use against T2W. All antiviral agents tested showed minimal (less than 10%) inhibition of virus production or no inhibition at all, except rifampin and ribavirin (Figure 21). The decrease in viral titre with rifampin at 500μM was attributed to the cell toxicity of the solvent DMSO, despite minimizing the DMSO concentration (Figure 21E). At this concentration, cell monolayers lifted and cells shrivelled and died within 24 hours. At concentrations less than or equal to 100μM (where no cell toxicity was observed), there were no antiviral effects.

Unfortunately, antiviral activity was minimal with the agents discussed above and any differences between T1L and T3D or between mammalian and avian reoviruses were not significant enough to exploit for our purposes.

3.3.1. Antiviral effects of ribavirin. Ribavirin has been used to inhibit several viruses clinically (Davis et al. 1998;McHutchison et al. 1998;Wyde 1998) and *in vitro* (De Clercq 1993;Crotty et al. 2002) and has been used in the study of mammalian and avian reoviruses (Rankin, Jr. et al. 1989;Bodelon et al. 2002;Connolly and Dermody 2002;Labrada et al. 2002). In our experiments ribavirin inhibited the production of reovirus progeny less than expected based on previous inhibition studies with mammalian reovirus even under similar experimental conditions (Rankin, Jr. et al. 1989). At a

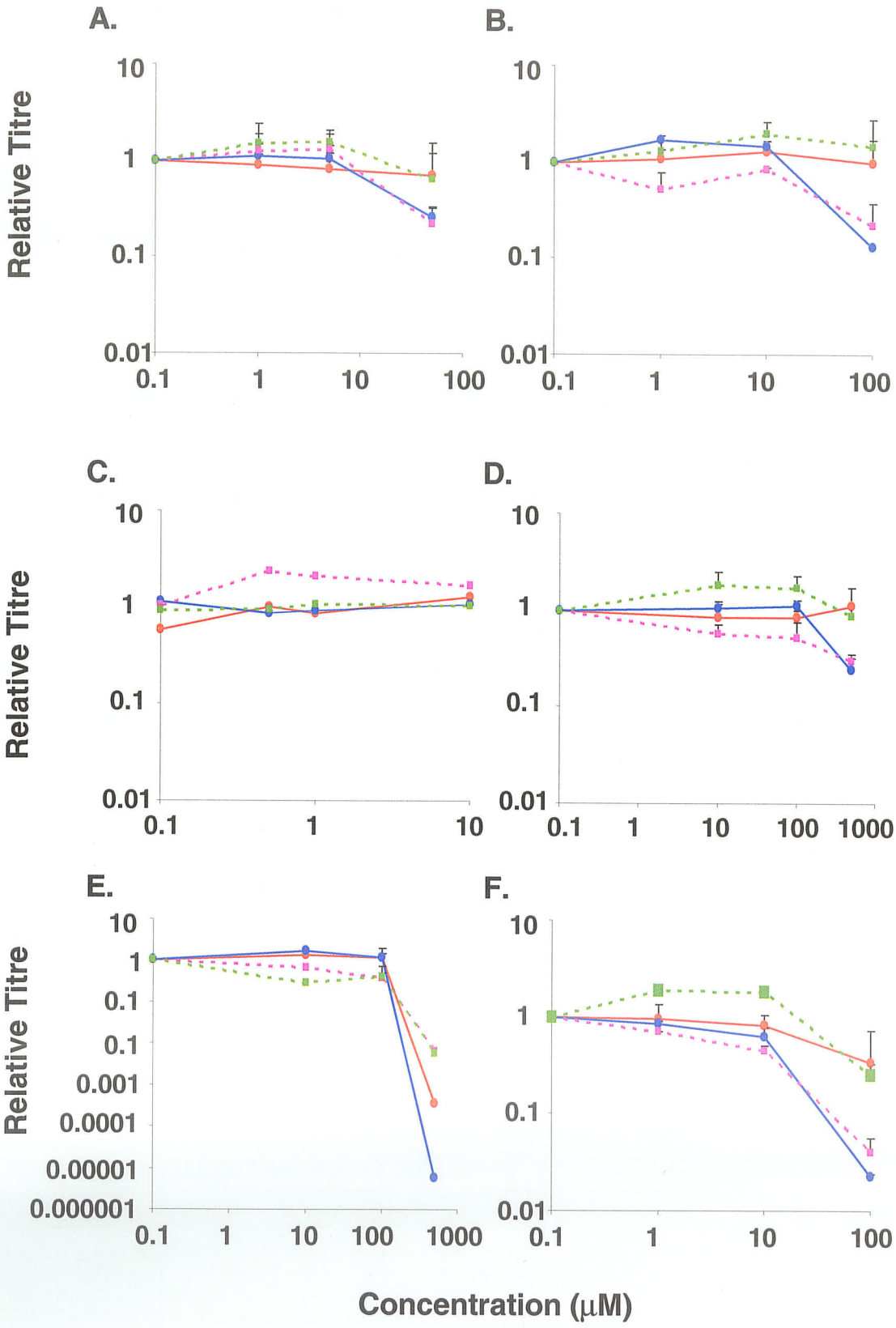

A.
B.
C.
D.
E.
F.
Relative Titre
Concentration (μM)
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.1
1
10
100
1000

Figure 21. Effect of various antiviral agents on production of infectious reovirus progeny

Either subconfluent L929 or QM5 cells were infected with T1L (●), T3D (●), avian reovirus 138 (■) or avian reovirus 176 (■) at an MOI = 0.5 PFU/cell. After virus adsorption, cells were overlaid with fresh MEM for MRV infections or M199 for ARV infections, which contained the indicated concentrations of the appropriate antiviral and were incubated at 37°C. Virus was harvested at 72 hpi for MRV and 48 hpi for ARV and viral titre was determined. **(A)** Amantadine. **(B)** AZT. **(C)** Acyclovir. **(D)** Foscarnet. **(E)** Rifampin. **(F)** Ribavirin. Results are displayed as a relative titre, with infectious progeny virus produced at each drug concentration expressed as a proportion of virus produced in the untreated control. The data represents the average of a minimum of three experiments (n=3) and the error bars represent one standard deviation.

concentration of 100μM, production of infectious progeny was decreased 8-fold for T1L and 45-fold for T3D at 72 hpi (Figure 21F). ARV also showed similar serotypic differences with ARV 138 being more sensitive than ARV 176 to 100μM ribavirin with replication inhibited 19- versus 5-fold, respectively at 72 hpi. There were no significant differences in the antiviral activity of ribavirin against MRV and ARV. Experiments at a concentration of 200μM ribavirin yielded similar results with a slight increase in inhibition while concentrations of 500μM ribavirin appeared toxic to the cells (data not shown). In subsequent experiments, a concentration of 100μM was used.

3.3.1.1.Growth curve of mammalian reovirus in the presence of ribavirin. Experiments to investigate the effect of ribavirin during the course of viral replication were conducted in order to help characterize the effects of ribavirin at various times throughout the reovirus infection. Subconfluent L929 cells were infected with T1L or T3D at an MOI ~ 1 PFU/cell and harvested at various time points to generate a growth curve (Figure 22). Maximum viral inhibition occurred between 18 and 24 hours with an average decrease in viral titre of 341- and 724-fold for T1L and T3D respectively. In non drug-treated control samples, viral titres increased dramatically after the initial 12 hour viral eclipse phase. In contrast, titres of ribavirin-treated samples remained relatively stable at input levels until 24 hpi and then increased significantly to levels similar to non drug-treated controls at 48 and 72 hours (Figure 22). There are two possible suggestions for this increase in titre. It is likely because the inhibitory effects of ribavirin are short lasting as ribavirin is metabolized or ribavirin-resistant mutants are being produced. Either possibility helps explain the small antiviral effect (8 to 45-fold decrease) seen when either T1L or T3D was harvested at 72 hours (Figure 21F).

3.3.1.2.Serial passage in ribavirin-treated reovirus. In order to determine if ribavirin-resistant mutants were being produced, an experiment was conducted where a virus sample taken after 48 hours of ribavirin exposure was used as the inoculum in a second experiment. Subconfluent L929 cells were inoculated with T1L or T3D previously grown in the presence of ribavirin for 48 hours. Media that contained 100μM ribavirin was added, virus harvested and titre determined at various time-points between 0 and 48 hours. Growth patterns for T1L and T3D were comparable when virus was passaged either once or twice in the presence of ribavirin when compared to the appropriate control

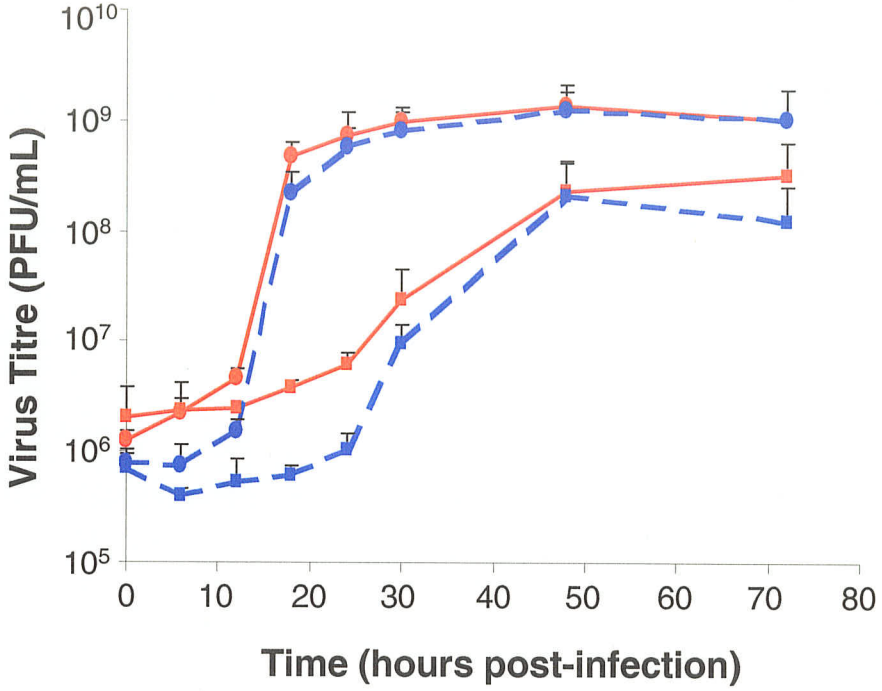

Virus Titre (PFU/mL)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0
10
20
30
40
50
60
70
80
Time (hours post-infection)

Figure 22. Virus production of reovirus over time in the presence of ribavirin

Subconfluent L929 cells were infected with T1L (solid lines) or T3D (dashed lines) at an MOI ~ 1 PFU/cell. Cells were overlaid with fresh MEM that contained 0 (●) or 100μM (■) ribavirin and incubated at 37°C. Virus was harvested between 0 and 72 hpi and viral titre was determined. The data represents the average of a minimum of three experiments (n=3) and the error bars represent one standard deviation.

(Figure 23). This suggests the increase in viral titres at 48 hours were due to the depletion of active phosphorylated ribavirin molecules within the cell rather than formation of resistant mutants.

3.3.1.3.Effect of pre-treated cells on viral growth. Since ribavirin must first be phosphorylated in the cell in order to be active as an antiviral agent (Richman et al. 1987;Rankin, Jr. et al. 1989), experiments were conducted where cells were pre-treated with ribavirin before infection. Subconfluent L929 cells were pre-treated with 0 or 100μM ribavirin 3 hours before infection with T1L or T3D at an MOI ~ 1 PFU/cell and media with the appropriate concentration of ribavirin was added to the cells. Virus was harvested and viral titre determined at various time-points between 0 and 72 hpi (Figure 24). There were no significant differences between the pre-treated and non pre-treated infections for both T1L and T3D. Maximum viral inhibition was between 18 and 24 hpi and ranged between 124- and 312-fold and between 350- and 608-fold for T1L and T3D respectively. These results show the amount of ribavirin that enters L929 cells is rapidly phosphorylated into its active form and at a level sufficient to cause inhibition.

3.3.2. Antiviral effects of mycophenolic acid. In an effort to characterize additional antiviral agents that could attenuate reovirus infection, MPA, a molecule that inhibits replication of other viruses (Malinoski and Stollar 1981;Ichimura and Levy 1995;Gong et al. 1999;Diamond et al. 2002;Morrey et al. 2002), was assessed for its ability to inhibit reovirus replication in mouse L929 fibroblasts. It was selected because like ribavirin, it is an IMPDH inhibitor and it has the ability to cross the blood-brain barrier.

3.3.2.1.Dose response of reovirus to mycophenolic acid treatment. The dose response of MPA on the production of infectious viral progeny was determined for concentrations of MPA between 0 and 100μg/mL (Figure 25A). Concentrations of MPA $\leq$ 30 ng/mL had no significant effect upon the replication of either T1L or T3D when cells were infected at a low MOI of 0.12 PFU/cell. An MPA concentration of 300ng/mL appeared to produce different effects in T1L infections as compared to T3D infections. On average, addition of 300ng/mL MPA led to a 3-fold increase in infectious T1L viral progeny which was not significant when compared to the untreated control ($p>0.1$). In some experimental trials, addition of this dose had no significant effect upon T1L production as compared to non drug-treated control infections, whereas in other trials addition of this dose of MPA led to

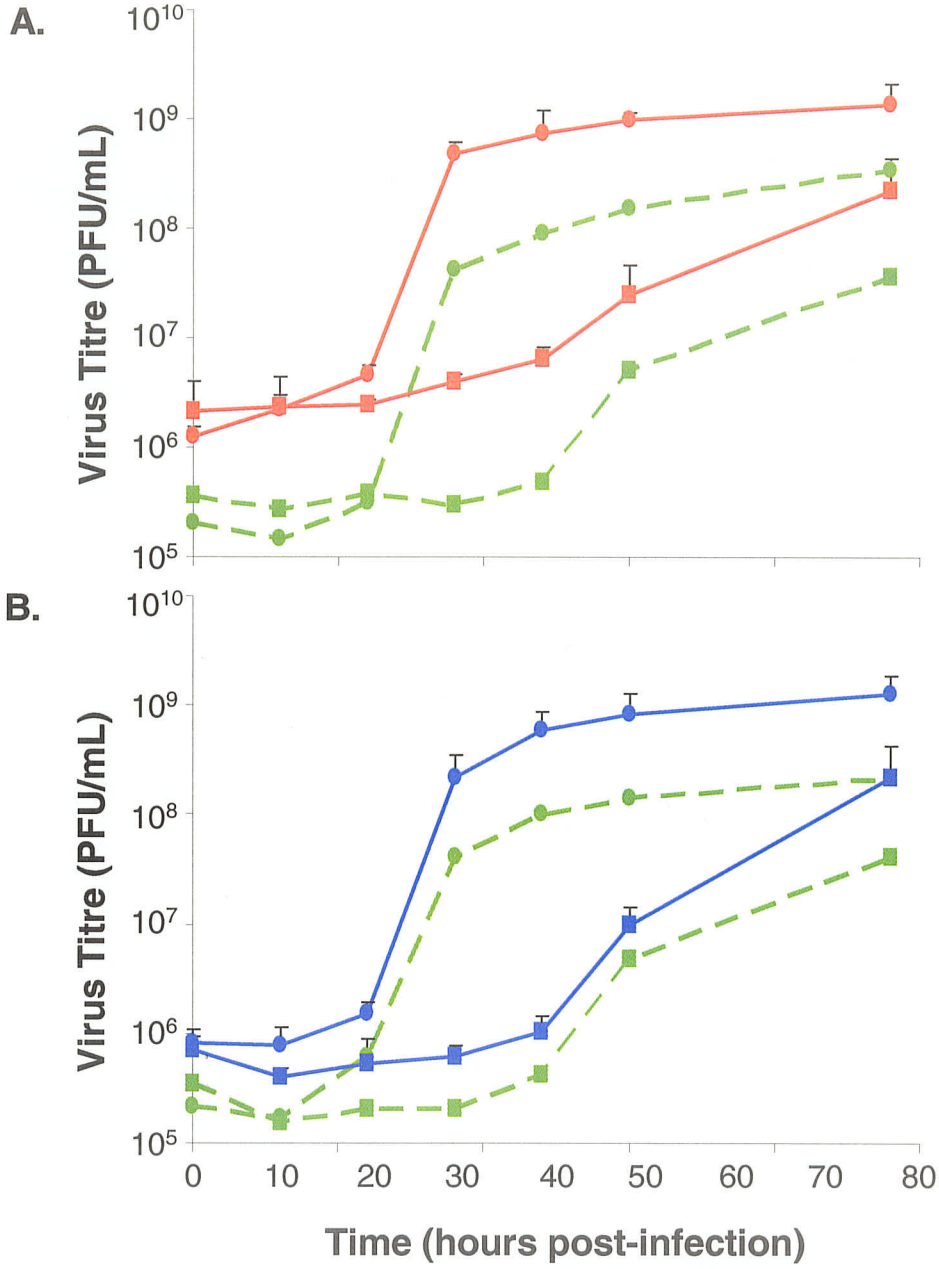

A.
B.
Virus Titre (PFU/mL)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0
10
20
30
40
50
60
70
80
Time (hours post-infection)

Figure 23. Serial passage of reovirus in the presence of ribavirin

Subconfluent L929 cells were infected with either **(A)** T1L or **(B)** T3D that had either been previously exposed to ribavirin for 48 hours (broken line) or not (solid line). After virus adsorption, cells were overlaid with fresh MEM supplemented to contain 0 (●) or 100μM (■) ribavirin and incubated at 37°C. Virus was harvested between 0 and 48 hpi and viral titre was determined. The data represents the averaged of a minimum of two experiments (n=2) and the error bars represent one standard deviation.

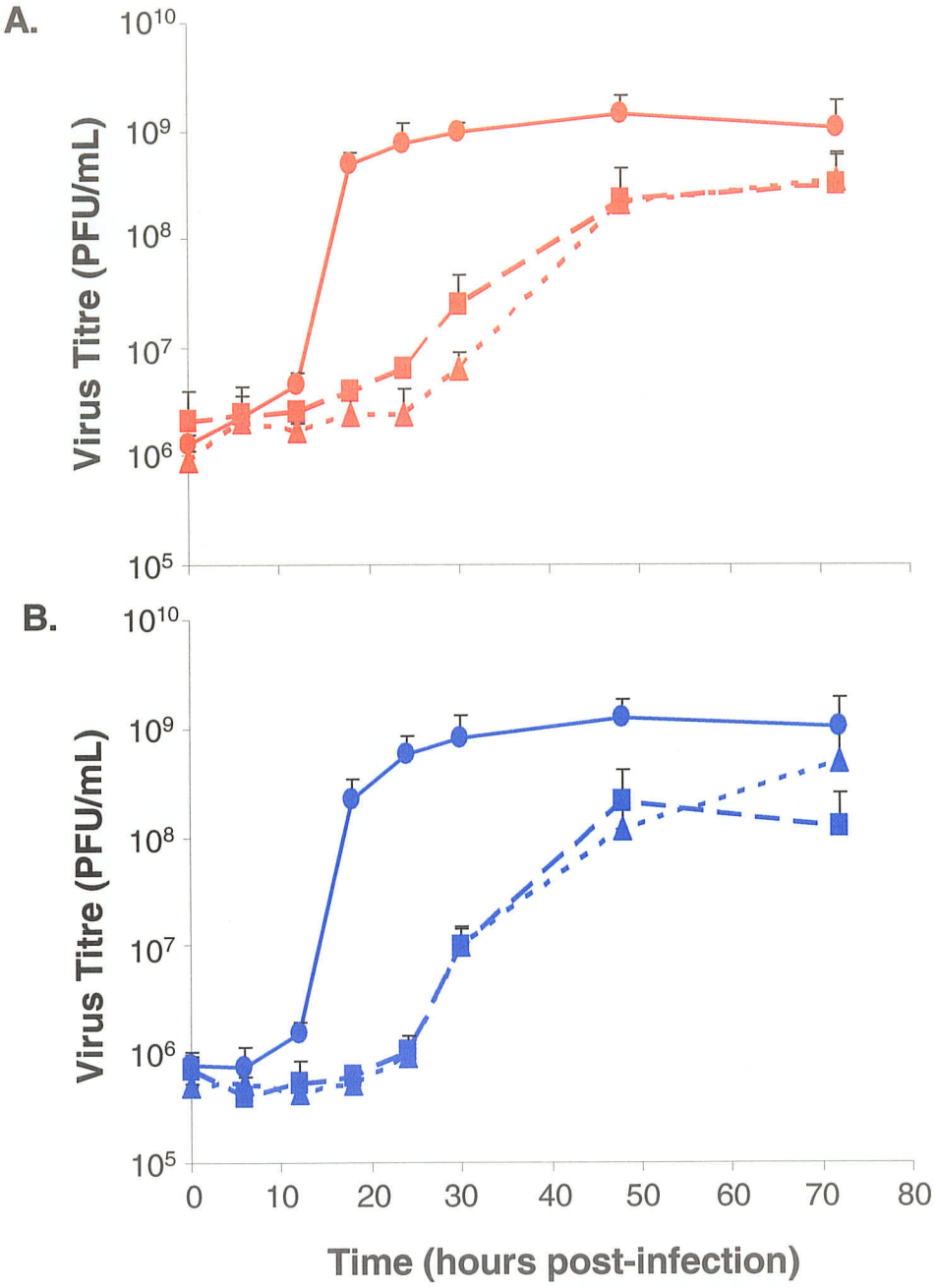

A.
B.
Virus Titre (PFU/mL)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0
10
20
30
40
50
60
70
80
Time (hours post-infection)

Figure 24. Effect of ribavirin pre-treatment on reovirus replication

Subconfluent L929 cells were infected with **(A)** T1L and **(B)** T3D at an MOI ~ 1 PFU/cell and harvested between 0 and 72 hpi. MEM supplemented with 0 (●) or 100μM (■) was added either at the time of infection (■) or 3 hours before infection (▲). Results are displayed as viral titre. The data represents the average of a minimum of two experiments (n=2) and the error bars represent one standard deviation.

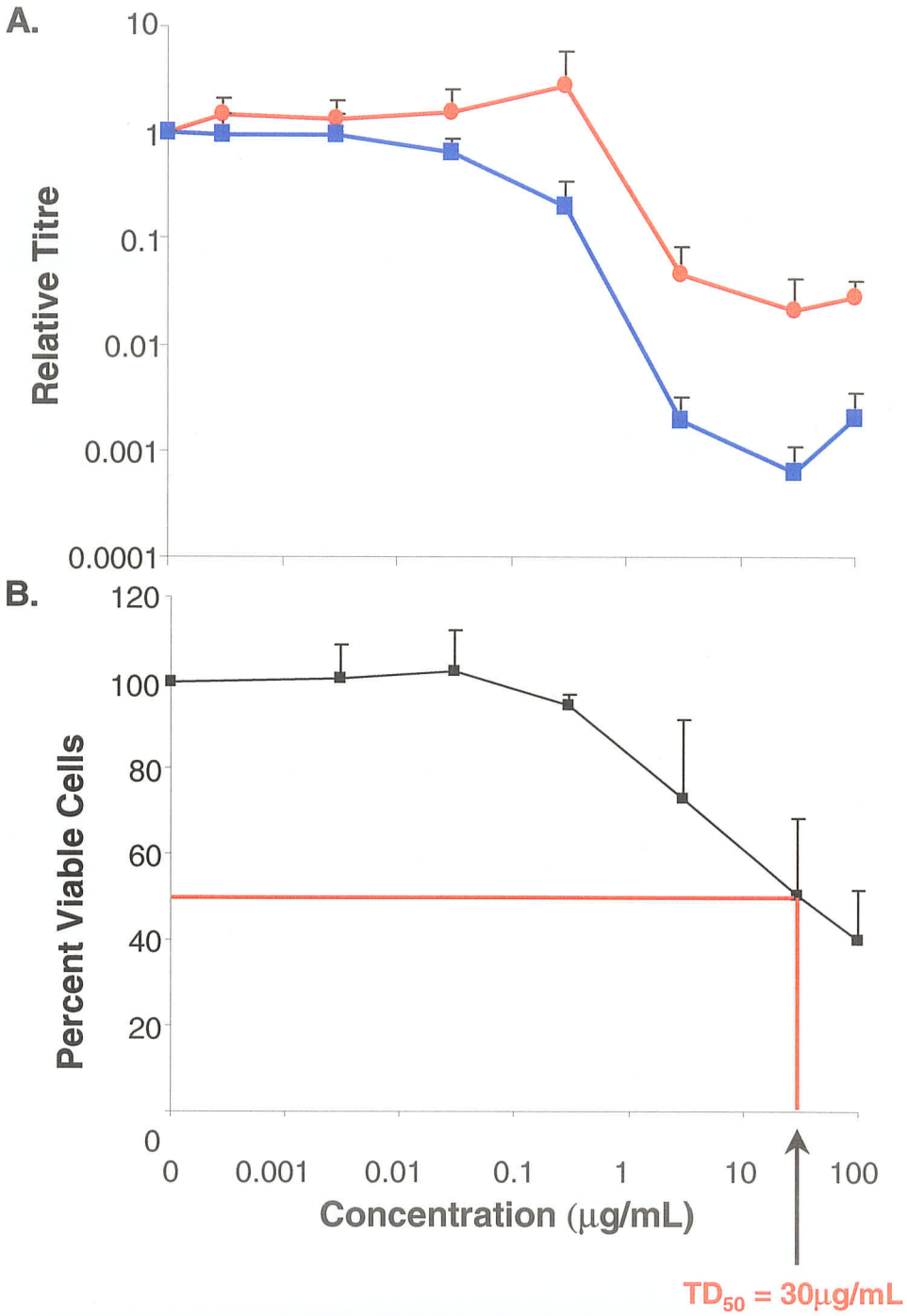

A.
Relative Titre
10
1
0.1
0.01
0.001
0.0001
B.
Percent Viable Cells
120
100
80
60
40
20
0
0
0.001
0.01
0.1
1
10
100
Concentration (µg/mL)
TD50 = 30µg/mL

Figure 25. Effect of mycophenolic acid on L929 cells

(A) Effect of MPA on production of infectious reovirus progeny. Subconfluent L929 cells were pre-treated with the indicated concentrations of MPA for 1 hour before infection with T1L (●) and T3D (■) at an MOI of 0.12 PFU/cell. After virus adsorption, cells were overlaid with fresh MEM that contained the indicated concentrations of MPA and incubated at 37°C. Virus was harvested between 65 and 72 hpi and viral titre was determined. Results are displayed as a relative titre, with infectious progeny virus produced at each MPA concentration expressed as a proportion of virus produced in the untreated control. The data represents the average of a minimum of three experiments (n=3) and the error bars represent one standard deviation. **(B)** L929 cell viability in the presence of MPA. L929 cells were added to a 96-well plate (5 x 10^3 cells/well) and incubated with various concentrations of MPA for 24 hours. Cellular response to MPA was measured with the Roche Cell Proliferation Reagent (WST-1) and plotted against MPA concentration. The data represents the average of a minimum of five experiments (n=5) and the error bars represent one standard deviation. TD_{50} is indicated.

an increase in infectious progeny production up to 8-fold. In contrast, application of a dose of 300ng/mL MPA to T3D infections consistently led to a decrease in progeny virus production that ranged from 2 to 17-fold (average of 7-fold) less than comparable non drug-treated control infections. Higher concentrations of MPA led to decrease in virus production for both strains and these plaque reduction assays demonstrated decreased production of infectious viral progeny was maximal at an MPA concentration of 30μg/mL. Strain-dependent differences in responses of T1L and T3D to different doses of MPA were observed, with T3D generally more sensitive to the inhibitory effects of MPA than T1L. In addition to the strain-dependent differential responses to 300ng/mL MPA noted above, the extent of viral inhibition was also different between T1L and T3D. The inhibition of infectious viral progeny production by MPA was greater in T3D with a maximum inhibition of 1560-fold compared to 46-fold for T1L at a dose of 30μg/mL MPA ($p<0.03$). Viral inhibition was not due to cell toxicity because there were only small differences in cell viability determined by cell doubling times and Trypan Blue exclusion at these MPA doses (data not shown). However, cell monolayers started to deteriorate after exposure to 100μg/mL MPA within 24 hours. Use of the more sensitive WST-cell viability assay determined the TD_{50} (50% toxic dose) to be 30μg/mL MPA (Figure 25B). Still higher doses (100μg/mL MPA) produced a paradoxical effect where production of infectious viral progeny was increased compared to lower MPA concentrations, i.e. less viral inhibition at higher MPA concentrations. For most subsequent experiments, a dosage of 3μg/mL MPA was used routinely. This concentration was selected because it is less than the clinically immunosuppressive level of 10μg/mL (Diamond et al. 2002) and cytotoxicity was less than at higher MPA concentrations, which would ensure any inhibition seen was due to the inhibitory effects of MPA on the virus rather than cytotoxic effects on the cells. Strain-dependent differences in inhibition were also seen at this concentration of MPA, with near-maximal levels of inhibition. Some studies were also performed using an MPA dosage of 300ng/mL.

3.3.2.2.Effect of treatment time and multiplicity of infection on reovirus growth in the presence of mycophenolic acid. In order to gain greater insight into the mechanism of action of MPA, the drug was added at different timepoints and MOIs (Figure 26). MPA was added to subconfluent L929 cells 1 hour pre-infection or at 0, 3, 6, 12 or 24

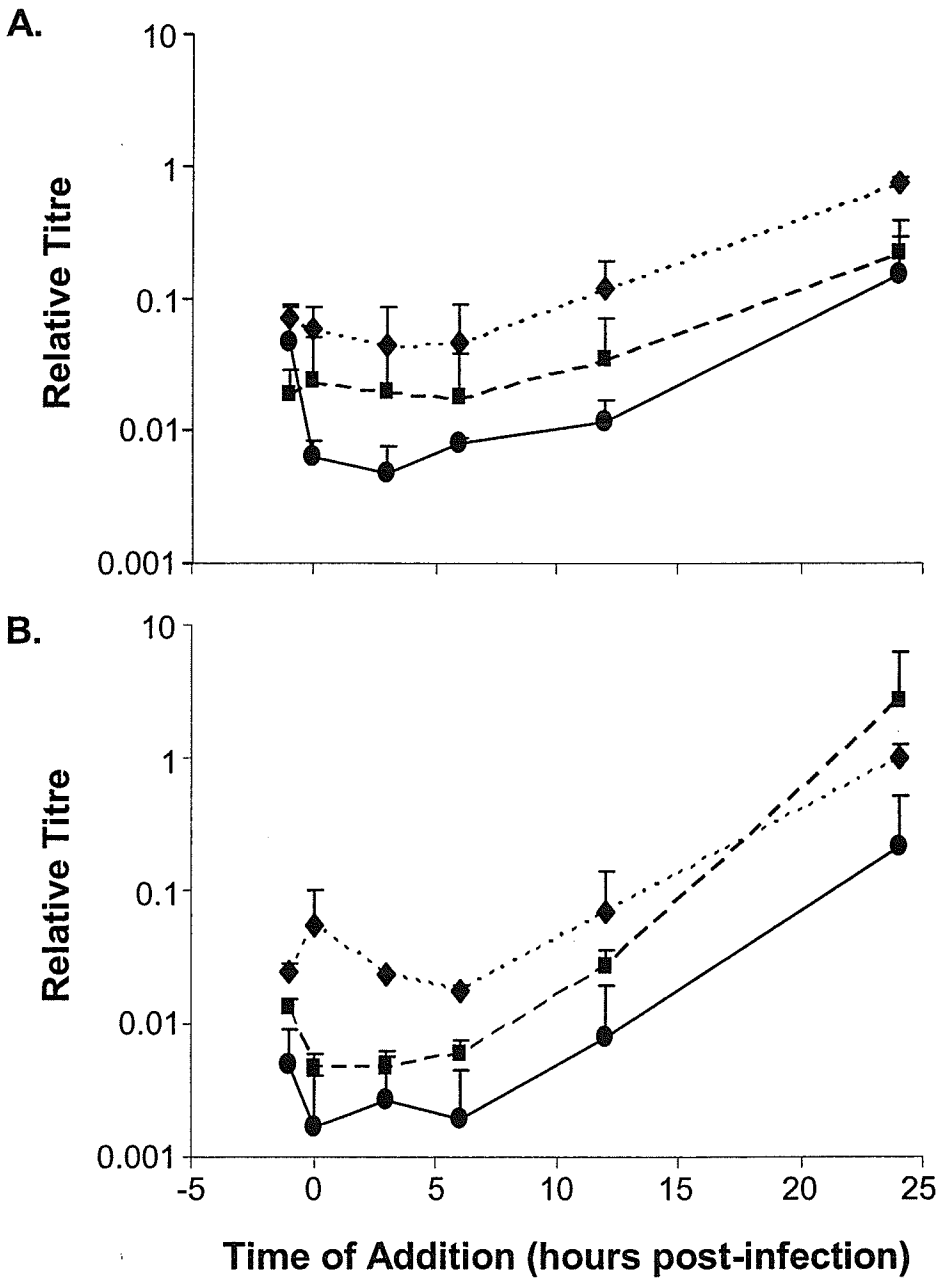

A.
B.
Relative Titre
Relative Titre
10
1
0.1
0.01
0.001
-5
0
5
10
15
20
25
Time of Addition (hours post-infection)

Figure 26. Effects of time of MPA addition and multiplicity of infection on inhibition of infectious progeny

L929 cells were treated with 3μg/mL MPA at indicated times prior to or during infection with T1L **(A)** or T3D **(B)** at MOIs of 0.12 (●), 1.2 (■) or 12 (◆) PFU/cell. Virus was harvested 72 hpi and viral titre was determined. Results are displayed as a relative titre with infectious progeny virus produced at each MPA concentration expressed as a proportion of virus produced in the untreated control. The data represnts the average of two experiments (n=2) and the error bars represent one standard deviation.

hpi. Cells were then infected with T1L or T3D virus at MOIs of 0.12, 1.2 or 12 PFU/cell and infections harvested 72 hpi. As MOI was increased, inhibition of T1L and T3D was decreased. Even though inhibition decreases slightly from an MOI of 0.12 to MOI of 12 PFU/cell, the differences in viral production were not statistically significant for either T1L or T3D ($p>0.05$). When MPA was added at later timepoints, virus inhibition was generally decreased. Production of infectious viral progeny was reduced the greatest when MPA was added between 0 and 12 hpi and cells were infected at an MOI of 0.12 PFU/cell (Figure 26). T1L replication was inhibited less in cells pre-treated for 1 hour with MPA (22-fold) than when MPA was added at the time of infection or 3, 6 or 12 hpi (range 86 to 208-fold) ($p<0.08$). Inhibition of virus production was minimal in cells treated with MPA at 24 hpi and was not significantly different from non drug-treated control infections. When cells were infected with T3D and MPA was added 1 hour pre-infection, at the time of infection or 3, 6 or 12 hpi, production of infectious viral progeny was always decreased by at least 100-fold (range 123-581-fold) as compared to non drug-treated controls. As in T1L infection, there was no significant decrease in T3D virus production if MPA was added 24 hpi.

3.3.2.3.Reassortant mapping of antiviral effect of mycophenolic acid. As indicated earlier, MPA has been shown to inhibit replication of several viruses (Malinoski and Stollar 1981;Ichimura and Levy 1995;Neyts and De Clercq 1998;Neyts et al. 1998;Gong et al. 1999;Margolis et al. 1999;Diamond et al. 2002;Morrey et al. 2002). However, viral factors, if any, that play role(s) in sensitivity to MPA have not yet been delineated. The strain-dependent differences seen in reovirus MPA sensitivity (Figure 25A) afforded a unique opportunity to undertake a genetic approach to identify viral factors involved in the differential effect of MPA on T1L and T3D virus production. A panel of 39 virus clones (the 2 parents T1L and T3D, plus 37 reassortant viruses) was investigated to determine the inhibitory effects of MPA (Table 10). All 39 virus clones were tested for their capacities to replicate in the presence or absence of MPA. A few reassortants behaved similarly to T1L with lower levels of sensitivity to MPA, while others behaved more like T3D with increased sensitivity to MPA. Some reassortants exhibited less sensitivity to MPA than T1L. The different reassortant behaviours prevented clones from being placed into only two distinct groups. Therefore, the relative sensitivities of each

Table 10. Genotypes of T1L X T3D intertypic reassortants tested for mycophenolic acid sensitivity

	Parenteral source for the following gene segments[a]										Fold	
Clone	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	Reduction[b]	Rank[c]
H14	1	1	3	1	1	1	1	3	3	1	26	39
EB143	3[e]	1	1	1	1	1	3	1	1	1	27	38
EB129	3	3	3	3	3	1	3	1	1	3	28	37
EB74	1	3	1	1	1	1	1	1	3	1	34	36
EB136	3	3	3	1	3	1	3	3	3	3	35	35
KC36	3	1	1	1	1	1	1	3	1	3	42	34
EB120	3	3	3	1	1	3	3	3	1	1	52	33
H24	1	1	1	1	1	1	1	1	1	3	58	32
EB85	1	1	1	1	1	3	1	3	1	1	61	31
H27	1	3	1	1	3	1	1	1	1	1	62	30
KC9	3	3	1	3	3	3	1	3	3	3	76	29
H60	3	3	1	1	3	3	3	3	3	1	81	28
EB93	1	1	1	1	1	1	1	1	3	1	90	27
T1L	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**91**	**26**
EB108	1	3	1	3	1	1	1	1	3	3	107	25
KC10	1	1	1	1	1	1	1	3	1	1	109	24
EB47	1	3	1	1	1	1	1	1	1	1	122	23
G2	1	3	1	1	1	1	3	1	1	1	127	22
EB118	3	3	1	1	3	3	3	3	1	1	128	21
KC28	3	3	1	3	3	1	1	3	3	3	130	20
EB146	1	1	1	3	1	1	1	1	1	3	140	19
EB96	1	3	1	3	1	1	1	1	3	1	148	18
EB18	3	3	1	3	3	3	1	1	3	1	149	17
G16	1	1	1	3	1	1	1	3	1	1	170	16
EB126	3	3	1	3	1	1	1	3	3	1	203	15
EB39	1	3	3	1	3	3	3	3	3	3	217	14
EB31	1	1	1	3	1	1	1	3	3	1	249	13
H15	1	3	3	1	3	3	3	3	3	1	273	12
KC35	1	3	1	1	3	1	3	1	3	1	290	11
E3	3	3	3	3	1	3	3	3	3	3	305	10
EB113	1	1	1	3	1	1	1	1	3	1	310	9
KC19	1	1	1	1	3	1	3	1	3	1	460	8
EB13	3	3	3	3	3	3	3	3	3	1	533	7
KC55	1	3	1	3	3	3	1	3	1	3	585	6
EB88	3	3	3	3	1	3	3	3	3	3	707	5
EB62	3	3	3	3	3	3	3	1	3	1	757	4
T3D	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**844**	**3**
EB28	3	3	1	3	3	3	3	1	3	3	1231	2
EB97	3	3	1	3	3	3	3	3	3	1	4968	1
p value[d]	0.2771	0.9524	0.2561	0.00025	0.0173	0.0028	0.0260	0.7505	0.9960	0.3961		

[a] Numbers indicate parental sources of gene, 1 – T1L; 3 – T3D.

[b] Fold reductions were calculated by comparing each clone's replication in the presence of 3μg/mL MPA to replication in the absence of MPA. Values represent the average from two or more trials.

[c] Ranking was used for statistical purposes.

[d] The λ3 protein of EB143 (encoded by L1) exhibits an aberrant mobility in an alternative gel system.

[e] Calculations performed using (SISA 2003).

clone to MPA were used to facilitate reassortant mapping analyses. These relative sensitivities, expressed as fold reduction in virus replication for each virus clone, generated a continuum from 26 to approximately 5000 (Table 10). Wilcoxon rank sum analysis of the ranked clones indicated the viral M1 gene, which encodes minor core protein μ2, was the primary determinant of the strain-dependent difference in MPA sensitivity. The Wilcoxon analysis also suggested the M2, M3, and S1 genome segments contributed to the strain-dependent differences; however Chi square and linear regression analyses indicated the M1 gene was the principle determinant of strain-dependent differences (Table 11). Similarly, reassortant mapping of the strain-dependent differences seen in the presence of 300ng/mL MPA implicated the M1 gene (data not shown).

3.3.2.4.Growth curve of reovirus in the presence of mycophenolic acid. To help characterize the effects of MPA at various times throughout the reovirus infection, time course studies were performed. Subconfluent L929 cells were pre-treated with MPA at 0 or 3μg/mL for 1 hour, infected with T1L or T3D at an MOI of 0.12 PFU/cell and harvested at various time points to generate a growth curve (Figure 27). In non drug-treated control samples, viral titres increased dramatically after the 12 hour viral eclipse phase. In contrast, T1L titres of MPA treated samples started to increase at 24 hours and slowly increased until 72 hours when infections were harvested. Titres were approximately 7-fold greater than input virus titre, which indicates a low level of progeny virus production. In contrast, T3D viral titres did not begin to increase until 48 hpi and titre at 72 hours was not significantly higher than input titres, suggesting limited progeny virus growth. This experiment shows MPA does not prevent reovirus infection, but decreases viral replication.

3.3.2.5.Guanosine rescue of reovirus replication in the presence of mycophenolic acid. The mechanism of antiviral activity of MPA likely occurs through the depletion of intracellular guanine nucleoside pools (Allison and Eugui 2000;Sintchak and Nimmesgern 2000); therefore, the addition of excess exogenous guanosine to virus infected cells treated with MPA should yield viral titres comparable to untreated controls. L929 cells were either mock- or pre-treated for 1 hour with 3μg/mL MPA, infected with T1L or T3D at an MOI of 0.12 PFU/cell and harvested between 65 and 72 hpi. Guanosine, added at a concentration of 50μg/mL at the time of infection, restored viral

Table 11. Statistical analyses of genes associated with differential MPA effects

	Individual Trials[a]		Averages from Table 10[b]	
Gene	**U[c]**	**M[d]**	**U**	**M**
L1	0.8		0.8	
L2	0.6		0.2	
L3	0.8		0.3	
M1	**0.007**	**0.006**	**0.005**	**0.04**
M2	0.4		0.2	
M3	0.1		0.1	
S1	0.2	0.08	0.7	
S2	0.4		0.8	
S3	**0.06**	0.3	**0.02**	0.13
S4	0.8		0.8	

[a] p values determined from analysis of complete data set of all individual experiments.
[b] p values determined from analysis of average values (from Table 10).
[c] Univariate Chi square analysis performed on distribution of each gene.
[d] Multiple logistic regression analysis of significant genes.

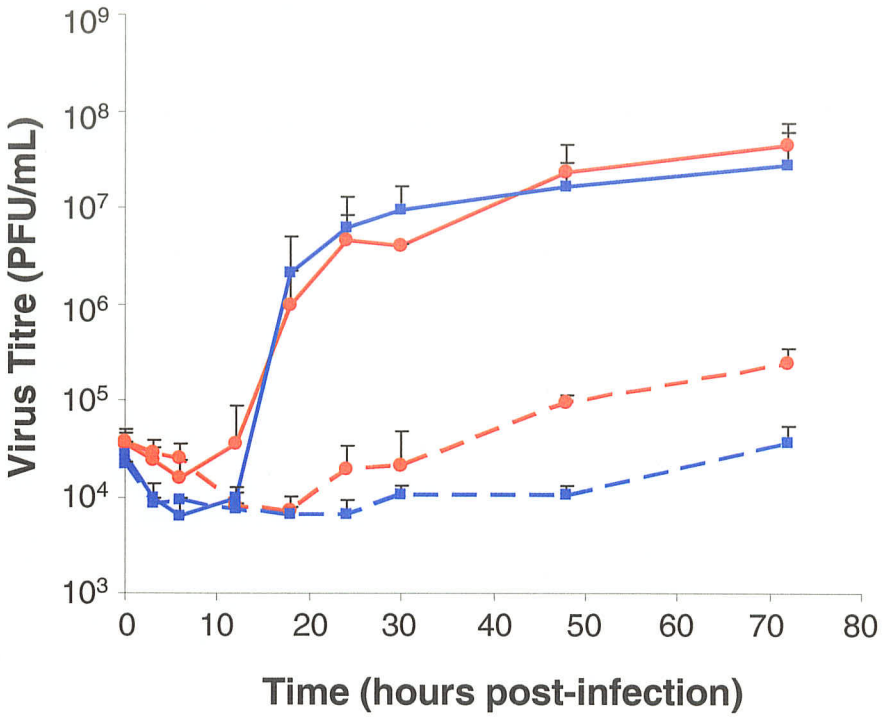

Virus Titre (PFU/mL)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
0
10
20
30
40
50
60
70
80
Time (hours post-infection)

Figure 27. Virus production over time in presence of mycophenolic acid

Subconfluent L929 cells were pre-treated with 3μg/mL MPA for 1 hour before infection with T1L (●) and T3D (■) at an MOI of 0.12 PFU/cell. After virus adsorption, cells were overlaid with fresh MEM that contained 0 (solid line) or 3μg/mL (broken line) MPA and incubated at 37°C. Virus was harvested between 0 and 72 hpi and viral titre was determined. The data represents the average of a minimum of two experiments (n=2) and the error bars represent one standard deviation.

replication to near-normal levels (Figure 28). These results are in agreement with previous studies (Neyts and De Clercq 1998;Diamond et al. 2002).

3.3.2.6.Viral RNA and protein production in the presence of mycophenolic acid. Although the antiviral activity of MPA is due to decreased levels of intracellular GTP, it is not clear which guanosine- or GTP-dependent step in the viral lifecycle (e.g. intracellular signalling, translation or replication) is most susceptible; therefore, we examined earlier stages in the viral life cycle that might be susceptible to MPA inhibition. Since MPA reduced the production of infectious viral progeny, but did not decrease levels of virus below input levels, two possible mechanisms of inhibition were hypothesized: 1) MPA inhibited viral RNA synthesis or 2) MPA inhibited the translation of the infectious viral RNA. We investigated the effect of MPA on both viral RNA production and protein production.

To investigate whether MPA affects viral RNA production, L929 cells were infected with T1L or T3D virus in the presence or absence of different concentrations of MPA, biosynthetically labelled with ^{32}P-orthophospate and incubated at 37°C. Virus was harvested at 24 or 72 hpi and RNA was extracted and separated in agarose gels. Viral RNA production for both T1L and T3D was significantly decreased compared to the non drug-treated control at 24 hpi and the decrease was still evident at 72 hpi (Figure 29A). However, some progeny RNA production was seen in T1L infections, especially with 300ng/mL MPA at later timepoints, consistent with growth curve results (Figure 27).

To investigate whether MPA affected translation, L929 cells were infected with T1L or T3D in the presence of absence of MPA and biosynthetically labelled with [^{35}S]methionine-cysteine. Infections were incubated at 37°C for 24 or 72 hours, harvested and protein was immunoprecipitated and separated by SDS-PAGE. Synthesis of viral proteins in infections treated with MPA was substantially decreased in cell lysates harvested at both 24 and 72 hours for both T1L and T3D (Figure 29B). The identities of viral proteins were confirmed by comparison with in-house viral markers.

3.3.2.7.Effect of mycophenolic acid on reovirus in various cell lines. The sensitivity of virus/cell culture systems to ribavirin and MPA are often dependent on the host cell line being used (Richman et al. 1987;Rankin, Jr. et al. 1989;Neyts et al. 1998); therefore, the effect of MPA on the production of infectious reovirus progeny in other cell lines was

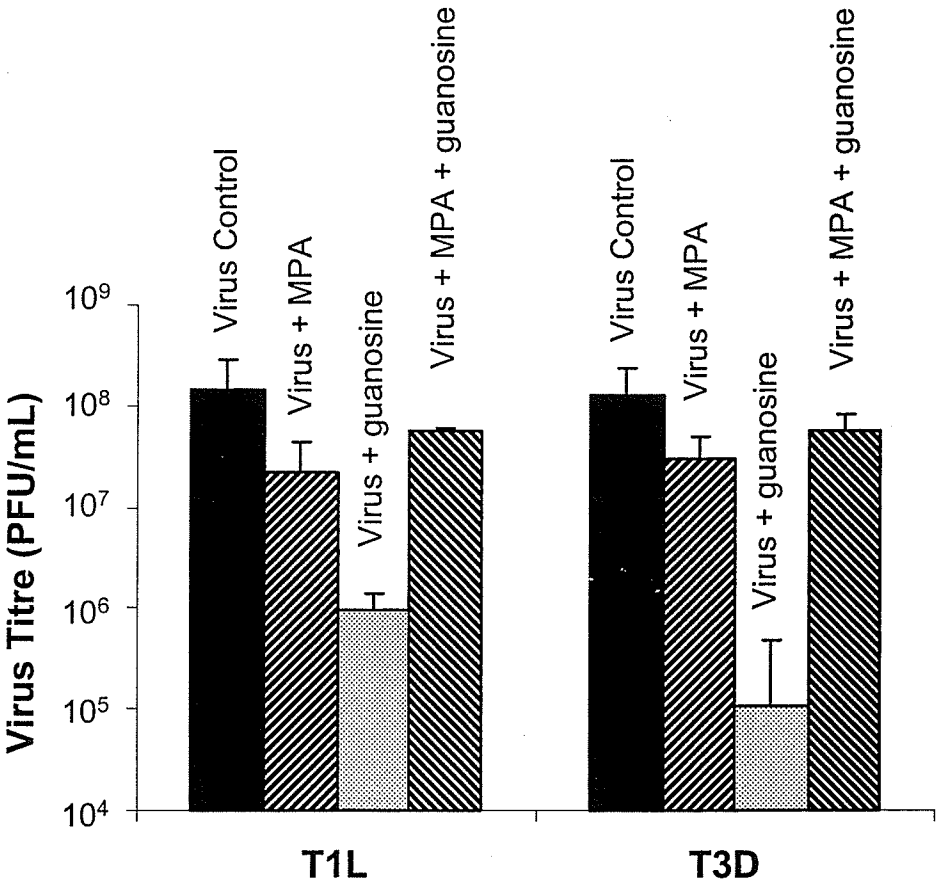

Virus Titre (PFU/mL)
10^9
10^8
10^7
10^6
10^5
10^4
Virus Control
Virus + MPA
Virus + guanosine
Virus + MPA + guanosine
T1L
T3D

Figure 28. Effect of exogenous guanosine on virus production in the presence of mycophenolic acid

Subconfluent L929 cells were pre-treated with MPA for 1 hour before infection with T1L and T3D at an MOI of 0.12 PFU/cell. After virus adsorption, cells were overlaid with fresh MEM that contained no supplements, 3μg/mL MPA, 50μg/mL guanosine, or a combination of MPA plus guanosine. Virus was harvested at 72 hpi and viral titre was determined. The data represents the average of a minimum of two experiments (n=2) and the error bars represent one standard deviation.

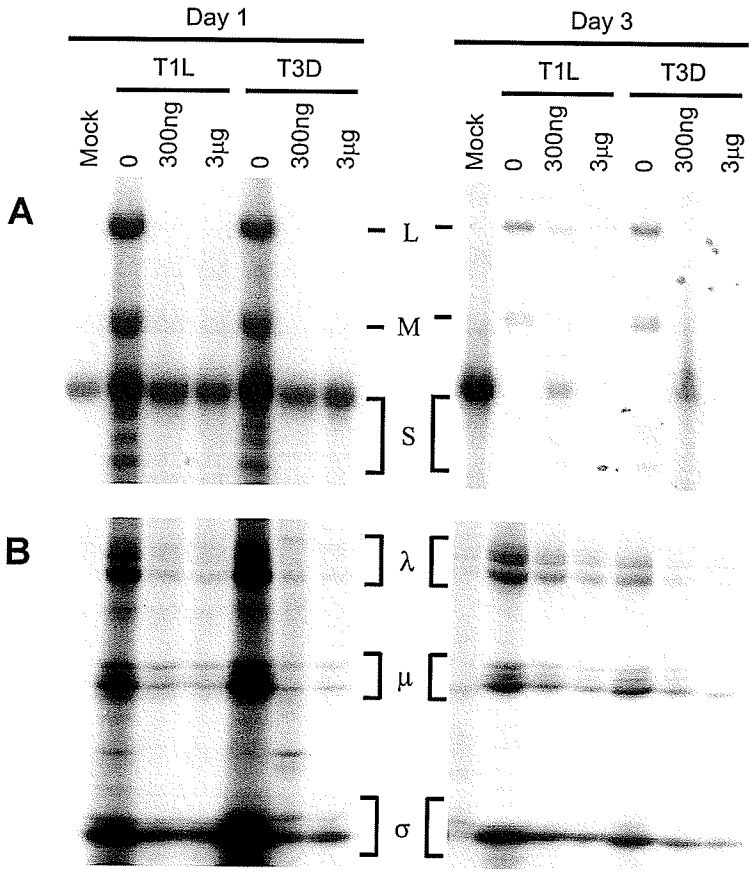

Day 1
Day 3
T1L
T3D
Mock
0
300ng
3μg
A
B
L
M
S
λ
μ
σ

Figure 29. Effect of mycophenolic acid on reovirus RNA and protein production

(A) Agarose gel analysis of ^{32}P-orthophosphate labeled viral RNA. Pre-treated L929 cells were either mock infected (M) or infected with T1L or T3D in the presence of 0, 300ng, or 3μg/mL MPA, labeled with ^{32}P-orthophosphate and incubated at 37°C as detailed in sections 2.10 and 2.11. At 24 or 72 hpi, dsRNA was purified and agarose gels were run at 125V for 2 hours, dried and exposed to X-ray film. The location of the L, M and S gene segments are indicated between the Day 1 and Day 3 panels. **(B)** Immunoprecipitation fluorograph of [^{35}S]methionine-cysteine-labeled cell extracts mock infected (M) or infected with T1L or T3D and treated as described in "A". Extracts were precipitated with anti-T3D polyvalent anti-serum conjugated to Protein-A Sepharose. Labeled proteins were resolved in 5-15% SDS-PAGE gels (16.0 by 12.0 by 0.1 cm) at 5mA for 18 h and the gels were fixed, dried and exposed to X-ray film. The location of the major λ, μ and σ classes of reovirus proteins are indicated between the Day 1 and Day 3 panels.

examined. Vero and U373 (human astrocytoma) cells, which are known to support reovirus replication (Keirstead and Coombs 1998;Butler et al. 2000), were pre-treated with various concentrations of MPA for 1 hour before infection with T1L or T3D at an MOI of 0.12 PFU/cell. Cells were incubated in the presence of various MPA concentrations at 37°C, harvested between 65 and 72 hpi and viral titre determined (Figure 30).

Results of infections in MPA-treated Vero cells (Figure 30A) were similar to results seen in MPA-treated L929 cells (Figure 25A). Concentrations $\leq$ 30ng/mL MPA had no significant effect on either T1L or T3D virus production. An MPA concentration of 300ng/mL appeared to produce a different effect in T1L infections as compared to T3D. Addition of 300ng/mL MPA had no significant inhibitory effect on T1L production compared to non drug-treated controls, whereas it caused a 10-fold decrease in production of T3D infectious viral progeny as compared to untreated controls. Higher concentrations of MPA led to a decrease in virus production for both strains with maximal inhibition of viral production at 30μg/mL. Viral production was decreased 535- and 1379-fold for T1L and T3D respectively as compared to non drug-treated controls. In addition to strain-dependent differential responses at 300ng/mL, we also observed T3D was more sensitive to the effects of MPA than T1L. These differences were greatest at 3μg/mL MPA, unlike in L929 cells where strain-dependent differences were greatest at 30μg/mL (Figure 25A).

The effects of MPA on reovirus infection in U373 cells were similar to L929 and Vero cells in that MPA concentrations $\leq$ 30ng/mL had no significant effects on viral titre compared to non drug-treated controls (Figure 30B). However, in contrast to L929 and Vero cells, strain-dependent differences in viral inhibition were minimal with T3D slightly more sensitive than T1L to the inhibitory effects of MPA at a concentration of 300ng/mL. Maximum inhibition of virus production for both T1L and T3D was around 250-fold and occurred between 3μg/mL and 30μg/mL MPA. These results have been published (Hermann and Coombs 2004a).

3.3.2.8.Effect of mycophenolic acid on T2W. We chose to investigate the ability of MPA, not ribavirin, to inhibit T2W replication in mouse L929 fibroblasts for a few reasons. Inhibition with MPA was greater than with ribavirin and at lower concentrations,

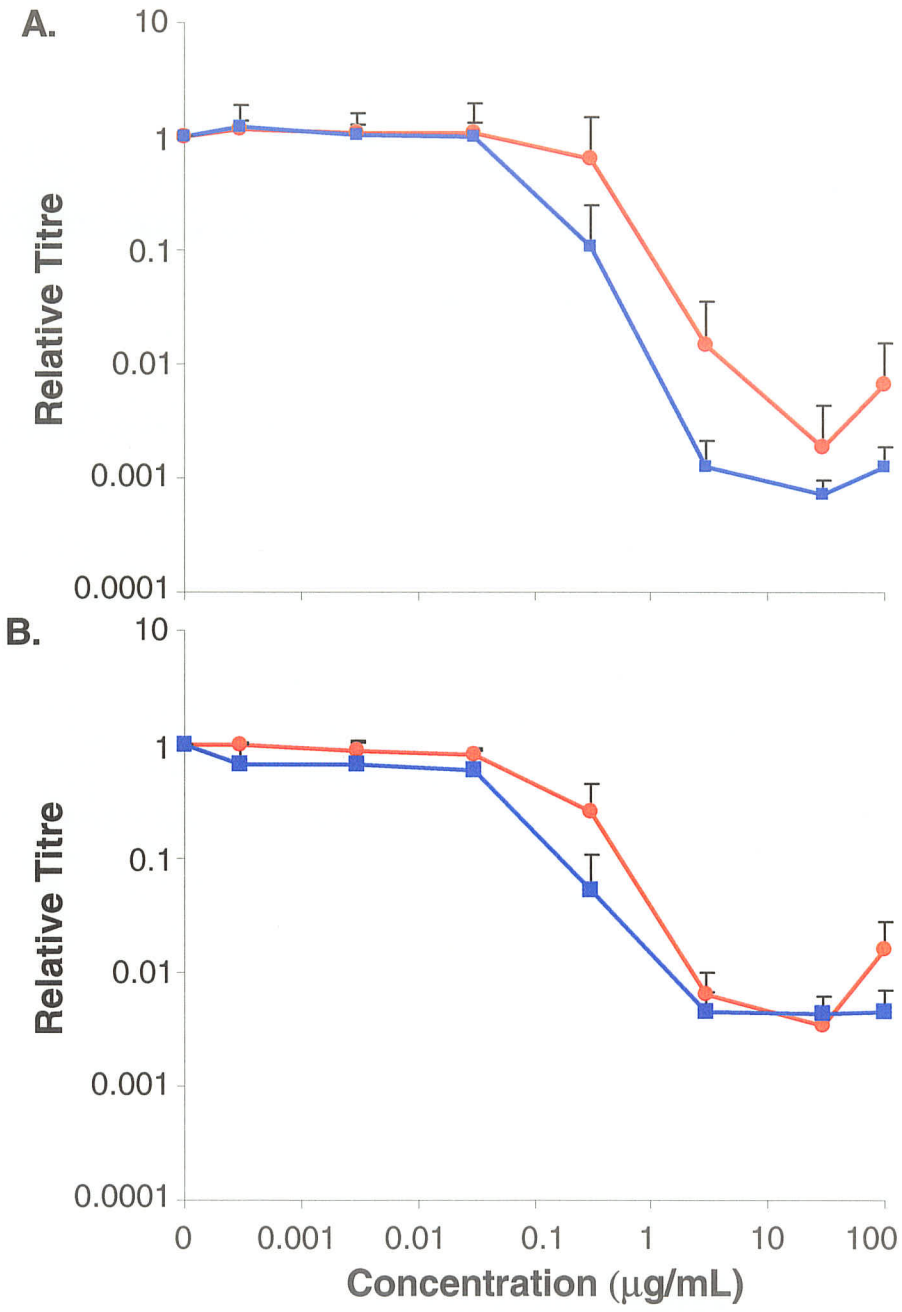

A.
B.
Relative Titre
10
1
0.1
0.01
0.001
0.0001
0
0.001
0.01
0.1
1
10
100
Concentration (μg/mL)

Figure 30. Effect of mycophenolic acid on reovirus production in Vero and U373 cells

(A) Vero and **(B)** U373 cells were pre-treated with indicated concentrations of MPA for 1 hour before infection with T1L (●) and T3D (■) at an MOI of 0.12 PFU/cell. After virus adsorption, cells were overlaid with fresh MEM that contained the indicated concentrations of MPA and incubated at 37°C. Virus was harvested between 65 and 72 hpi and viral titre was determined. Results are displayed as a relative titre compared to progeny virus production in untreated control. The data represents the average of a minimum of two experiments (n=2) and the error bars represent one standard deviation.

production of infectious viral progeny in the presence of MPA was decreased throughout the 72 hour infection period as compared to ribavirin (compare figures 22 and 27) and most importantly because it appears to distribute slowly into the CSF over four to seven weeks (McEvoy 2003). Dose response, time course and guanosine studies were completed with T2W as for the prototype reoviruses (see above). Similar to T1L and T3D, production of T2W infectious progeny was inhibited by MPA (Figure 31A). Viral replication began to decrease above a MPA concentration of 30ng/mL. The greatest inhibition occurred at 3μg/mL MPA with a 184-fold decrease in viral replication. Higher doses (100μg/mL MPA) produced a paradoxical effect in T2W replication; also seen with T1L and T3D prototype viruses. In non MPA-treated control samples, viral titres increased dramatically after 12 hours. In contrast, T2W titres of MPA-treated samples continued to decrease to a low at 24 hpi after which point viral titre slowly begin to increase to a level slightly higher than input titre (Figure 31B). Guanosine rescue studies yielded results similar to those seen with T1L and T3D where the addition of 50μg/mL of guanosine to infections returned viral titres to levels comparable to untreated controls (Figure 31C). The results have been published (Hermann and Coombs 2004b).

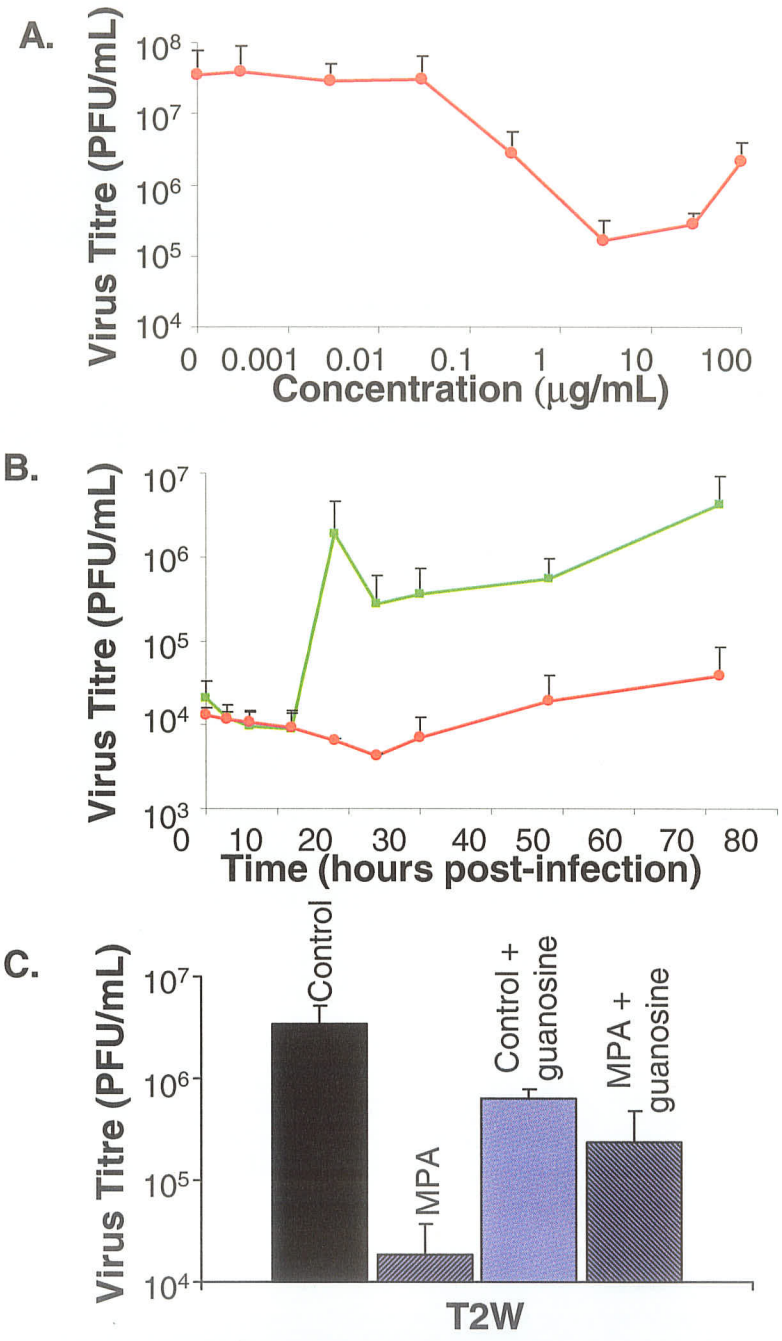

A.
Virus Titre (PFU/mL)
10^8
10^7
10^6
10^5
10^4
0 0.001 0.01 0.1 1 10 100
Concentration (μg/mL)
B.
Virus Titre (PFU/mL)
10^7
10^6
10^5
10^4
10^3
0 10 20 30 40 50 60 70 80
Time (hours post-infection)
C.
Virus Titre (PFU/mL)
10^7
10^6
10^5
10^4
Control
MPA
Control + guanosine
MPA + guanosine
T2W

Figure 31. Effect of mycophenolic acid on T2W virus production

(A) Dose response of mycophenolic acid on production of infectious T2W virus progeny. Subconfluent L929 cells were pre-treated with the indicated concentrations of MPA for 1 hour before infection with T2W at an MOI of 0.12 PFU/cell. After virus adsorption, cells were overlaid with fresh MEM that contained the indicated concentrations of MPA and incubated at 37°C. Virus was harvested between 65 and 72 hpi and viral titre was determined. Results are displayed as viral titre. **(B)** Production of infectious T2W virus progeny over time in the presence of 3µg/mL MPA. Subconfluent L929 cells were pre-treated with MPA for 1 hour before infection with T2W at an MOI of 0.12 PFU/cell. After virus adsorption, cells were overlaid with fresh MEM that contained either 0 (■) or 3µg/mL (●) MPA and incubated at 37°C. Virus was harvested between 0 and 72 hpi and viral titre was determined. **(C)** Effect of exogenous guanosine on T2W virus production in the presence of MPA. Subconfluent L929 cells were pre-treated with 3µg/mL MPA for 1 hour before infection with T2W at an MOI of 0.12 PFU/cell. After virus adsorption, cells were overlaid with fresh MEM that contained no supplements, 3µg/mL MPA, 50µg/mL guanosine, or a combination of MPA plus guanosine. Virus was harvested at 72 hpi and viral titre was determined. All data represents the average of a minimum of two experiments (n=2) and the error bars represent one standard deviation.

4. *DISCUSSION*

There are two main foci of the research presented in this thesis; the characterization of T2W, a reovirus clone isolated from the CSF of an immunocompromised infant and the investigation of molecules with antiviral activity against reovirus replication. Reoviruses are not commonly associated with symptomatic human infection. Despite extensive CNS disease seen in neonatal mice infected with reovirus, there have only been a handful of reports that associate reovirus and CNS disease in humans. Results shown here provide evidence a novel reovirus clone was isolated from the CNS of an immunocompromised infant. Furthermore, studies of various antiviral molecules show reovirus replication can be inhibited by the IMPDH inhibitor, MPA.

4.1. A comparative analysis of Freon substitutes in the purification of reovirus.

Characterization of the novel clone, T2W, often required purification of virus from infected cell lysates. For many non-enveloped viruses, such as reovirus, purification is by organic solvent extraction. Historically Freon has been used in many viral purification methods (Gomatos and Tamm 1963;Gschwender and Traub 1978;Berman et al. 1981;Richt et al. 1993;Liebermann and Mentel 1994). This compound is an ideal solvent for extraction of lipids and lipid bilayers without extraction of proteinaceous (or polar) material, such as viruses, due to its unique physicochemical properties. Following solvent extraction, virus can be concentrated and purified by ultra-centrifugation gradients and used in biochemical and structural studies. Unfortunately, the use of Freon has been implicated in ozone depletion and its use is prohibited. The unavailability of Freon, when laboratory stores have been depleted, would remove an essential step in virus purification. A logical solution to this obstacle was to find a replacement solvent for Freon in viral purification. The ideal substitute should behave like Freon, yet have low toxicity and no associated environmental threats.

As discussed earlier (section 3.1), various organic solvents to purify infectious reovirus from cell lysates were tested in an attempt to identify a suitable substitute for Freon. Organic solvents can be characterized by Hansen's three solubility parameters. These are dispersion energy (D), polar energy (P) and hydrogen bonding energy (H) (Hansen and Beerbower 1971;Goldschmidt 1993). In order to find a replacement for

Freon, solvents with similar solubility parameters were examined (Table 7). There are a few alternate halogenated hydrocarbons with very similar solubility parameters to Freon, yet other characteristics make them unsuitable for viral purification (e.g. carbon tetrachloride with its high toxicity and 1,2-dichloro-1,1,2,2-tetrafluoroethane with a boiling point of 4°C). Many other organic compounds have some solubility parameters similar to Freon, but differ substantially in other parameters (e.g. hexane and toluene). In these instances, solvents were combined in various ratios in order to create solvent mixtures with solubility parameters that closely matched Freon. Hexane/1-chlorobutane (7:3 v/v mixture), isopentane/1-chlorobutane (7:3), toluene, chloroform/isobutanol and Vertrel were tested as suitable replacement solvents. Other combinations of solvents (3:7 and 1:1) were also tested to determine whether the ratios of each solvent were important.

Results indicate both Vertrel and isopentane/1-chlorobutane (7:3) are suitable solvent substitutes for Freon. Both solvents extracted a similar amount of infectious virus particles as compared to Freon. Most other solvents tested were partially effective at viral purification, but the virus recovered was less infectious and the quantity was decreased. In addition, more contamination was seen in these preparations, in particular with the hexane/1-chlorobutane (7:3) mixture. The poor results seen for chloroform/isobutanol (1:1) may be explained by the examination of their solubility parameters. Compared to Freon, isopentane/1-chlorobutanol (7:3) has a similar D value, a slightly higher P value and a significantly higher H value. A higher H value would result in increased hydrogen bonding capabilities. This promotes a strong interaction between the solvent and both polar and nonpolar molecules, which may produce an environment not conducive for extraction of intact virions away from cellular debris and may have a detrimental effect on the virions.

Although Vertrel and isopentane/1-chlorobutane (7:3) appear the best substitutes for Freon on a functional basis, other considerations suggest Vertrel to be a better substitute. Vertrel consistently yielded larger amounts of purer and more infectious virus than the other solvents (Table 8). Isopentane and 1-chlorobutane are flammable and volatile liquids monitored by various environmental agencies. Due to their flammability, they are not recommended for use in any type of centrifugation process (a key step in viral purification), as vapours may be ignited by exposure to electrical contacts. In

addition, solvent mixtures have to be made fresh for each use because the differential volatility of each liquid may affect the mixture ratio and decrease the effectiveness of the solvent mixture. This makes Vertrel a better option, since there is no need for preparation before use. Furthermore, Vertrel appears to be an attractive solvent substitute for other reasons. Like Freon, it is denser than the aqueous phase and does not require a semi-solid solvent layer to be punctured in order to collect the aqueous phase. Vertrel is also compatible with plastic pipettes (both polystyrene and polypropylene), nontoxic, chemically and thermally stable (up to 300°C), non-flammable and has low global warming potential with "zero" ozone depletion (DuPont 2001). Vertrel has been accepted as a replacement for hydrochlorofluorocarbons currently in use (e.g. Freon) and is exempted from classification as a volatile organic compound by various environmental protection agencies.

Extraction with Freon is also a key step in medical and environmental virology for purification of viral nucleic acids from non-enveloped fastidious agents found in clinical and environmental samples (Atmar et al. 1995;Le-Guyader et al. 1996;Dix and Jaykus 1998;Traore et al. 1998;Mignotte et al. 1999;Monpoeho et al. 2001). Work in our laboratory showed purification of calicivirus nucleic acid from stool samples with the selected test solvents with Vertrel and isopentane/1-chlorobutane (7:3) was as sensitive as Freon at extracting viral RNA (Mendez et al. 2000). Work by another group has found organic extraction with Vertrel in combination with filtration through an electropositive filter membrane an acceptable method for obtaining highly purified rotavirus concentrates from environmental samples suitable for RT-PCR without the need of extra purification steps (Queiroz et al. 2001). These results have led to Vertrel as the organic solvent of choice in our laboratory for all viral purification of non-enveloped viruses needed for viral studies and was used when purified T2W was required.

4.2. Characteristics of the novel reovirus T2W. The role of reoviruses in human disease remains as uncertain as when Sabin (Sabin 1959) first coined the term ***reo***virus (respiratory, enteric, orphan virus). It is difficult to provide evidence to link reovirus infection to specific human disease processes (Table 4). Despite the presence of antibody to reovirus in the majority of the population (Selb and Weber 1994), symptomatic disease appears rare. Most identified cases are sporadic in nature, with no seasonal predilection.

In fact, most infections appear to be asymptomatic or produce minor upper respiratory or gastrointestinal symptoms that are indistinguishable from other illnesses seen in infancy or early childhood.

Electron microscopy (Figures 5A and 14) and NT studies completed by Cadham Provincial Laboratory (Winnipeg, Manitoba) and in our laboratory identified a reovirus serotype 2 clone from the CSF of an infant with gastrointestinal, respiratory and CNS symptoms. The electrophoretic gel pattern of the virus' RNA indicated the isolate was different from all reovirus strains examined (Figure 16B) and suggested it was a novel reovirus clone. At this point, the clone was named Type 2 Winnipeg (T2W), after the city where it was isolated. A reovirus serotype 2 strain was isolated from two different CSF samples taken from the patient approximately 6 weeks apart. As well, a stool sample taken midway between the two CSF samples was also culture-positive for a reovirus serotype 2 strain. This strongly supports persistent reovirus infection during the patient's illness. Although this case is quite complicated, as the patient had other concomitant infections, it is possible reovirus was partially responsible for the CNS symptoms. In general, additional proof reovirus is pathogenic would include detection of antibodies against the virus in the CSF, evidence of seroconversion against the isolated virus and a biopsy to definitively identify reovirus in the brain. Unfortunately, the child was unable to mount an immune response and produce antibodies because she was severely immunocompromised and brain samples were not taken at autopsy to look for reovirus.

In an attempt to characterize T2W on a molecular level, the S gene segments were sequenced. The S gene segments were chosen because they are small and the nucleotide sequences for the prototype viruses and many field isolates are known and are readily available in Genbank for comparison.

4.2.1. Sequence Analysis of T2W S gene segments. The reovirus S1 gene segment has been determined for each of the prototype strains (Cashdollar et al. 1985;Duncan et al. 1990;Nibert et al. 1990) and a variety of field isolates (Dermody et al. 1990;Nibert et al. 1990). The S1 gene segment is bicistronic and encodes the σ1 protein and σ1s protein in overlapping reading frames. The σ1 protein is present in the virion outer capsid in 36 copies in T1L, i.e. one σ1 trimer per icosahedral vertex (Coombs 1998) and interacts with the λ2 protein and perhaps with the outer capsid protein, σ3. It likely adopts two very

different conformations – a folded/retracted form or an elongated fiber which extends from the surface of the virion (Furlong et al. 1988;Dryden et al. 1993). It has been implicated as the reovirus cell-attachment protein (Lee et al. 1981;Yeung et al. 1987;Banerjea et al. 1988), viral hemagglutinin (Weiner et al. 1978;Yeung et al. 1987) and determinant of serotype-specific humoral immune response (Weiner and Fields 1977;Virgin et al. 1991). It is also responsible for tissue- and cell-specific tropism (Weiner et al. 1977) and the pattern of viral spread (Tyler et al. 1986). Sequence analyses of the σ1 protein has yielded a model with a head-and-tail morphology with distinct structural units, or domains (Bassel-Duby et al. 1985;Furlong et al. 1988;Nibert et al. 1990). The globular head and fibrous tail are located at the C- and N- terminal ends of the σ1 protein respectively.

In this study, the nucleotide sequence of the T2W S1 ORF was found to have 99.4% identity to the prototype T1L S1 sequence and the deduced aa sequence of the T2W σ1 protein had 99.6% identity. This was unexpected since the σ1 protein, encoded by the S1 gene segment, is the protein against which serotype-specific neutralizing antibodies are directed (Weiner and Fields 1977;Yeung et al. 1987;Virgin et al. 1991). Neutralization of the clinical sample with serotype specific polyclonal antisera indicated it was a reovirus serotype 2 strain and had no reaction to serotype 1 polyclonal antisera. It is surprising that with an almost identical deduced aa sequence to T1L σ1 protein, T2W was found to be serotype 2, not serotype 1. Neutralization studies by the provincial laboratory confirmed T2W was a reovirus serotype 2 strain on at least two occasions (B. Wells, Cadham Provincial Laboratory, personal communication). Polyclonal antisera, in particular antisera directed against T2J, has been previously reported to yield heterotypic neutralization, i.e. neutralizes more than one serotype (Virgin et al. 1988;Goral et al. 1996). Type-specific neutralizing monoclonal antibodies, directed against type 1 or type 3 σ1 proteins (5C6 and 9BG5 respectively) are available and have been shown to have minimal heterotypic neutralization when used to serotype a variety of field isolates (Goral et al. 1996). Unfortunately, neutralization studies with monoclonal antibodies were not able to be completed due to lack of purified virus.

The most obvious difference between T1L and T2W σ1 proteins was the insertion of 3 nucleotides which lead to the insertion of a lysine molecule at the 7^{th} aa position of

the T2W σ1 protein. This insertion occurs in the tail (T(i) morphological region) of the σ1 protein which is hydrophobic and forms a short α-helical coiled coil. In T3D, aa residues between 3 and 24 are required for the attachment of the σ1 protein to the λ2 protein in the viral particle (Mah et al. 1990;Leone et al. 1991). Despite no significant changes in the hydropathy of T2W σ1 protein as compared to T1L σ1 protein, it is possible the insertion of a lysine molecule in this region will affect the attachment of σ1 to the viral particle and overall stability of the virion. Conformational changes in the N-terminal tail region may also affect the stability of T2W σ1 trimers and affect viral entry.

The other aa acid change between T1L and T2W of a tryptophan to leucine occurred at aa 410. Despite the significant structural differences between the amino acids, the change is likely not responsible for significant differences between T1L and T2W and this aa is not highly conserved, in general, within reovirus clones.

The σ1s protein is a 14 to 15 kDa nonstructural protein that is highly variable between reovirus isolates, even within the same serotype (Dermody et al. 1990;Duncan et al. 1990). Its function is unknown; however, evidence indicates this protein is dispensable for reovirus growth in L and MDCK cells as well as for induction of apoptosis in L cells (Rodgers et al. 1998). It remains to be determined whether σ1s is required for replication in certain cells or whether it performs another function related to growth and/or spread in host animals. Since σ1s is a second translation product of the S1 gene segment, it is possible σ1s might be responsible for some of the phenotypic differences mapped to S1 and attributed to the σ1 protein. The results presented here show the deduced aa sequence of σ1s for T2W was identical to T1L; thus, σ1s is not responsible for differences between T1L and T2W.

The 1,331 base S2 gene segment encodes the structural σ2 protein (47 kDa) which interacts with the λ1 protein to form the core shell of the inner capsid. Crystal structures show the σ2 protein forms 150 nodule-like features that reside externally to the λ1 protein on the core surface (Reinisch et al. 2000). The σ2 protein has also been shown to interact with the λ2 protein and likely binds to the base of the major outer capsid protein μ1 (Reinisch et al. 2000). The functions of σ2 protein remain poorly understood; however, it has been shown to bind reovirus dsRNA (Schiff et al. 1988;Dermody et al.

1991) and may be involved in selective packaging of virion RNA or regulation of transcription or translation (Chappell et al. 1994). In recombinant form, σ2 must be co-expressed with the λ1 core protein for assembly of icosahedral particles (Xu et al. 1993). An experiment with temperature-sensitive mutant *ts*C447, whose lesion maps to the S2 gene segment (Ramig et al. 1978), suggests a role for σ2 protein in one or more steps in replicase particle assembly (Coombs et al. 1994b).

Unfortunately, we were unable to amplify the S2 gene segment of T2W. Primers were initially designed based on the T1L S2 gene segment sequence (Figure 7). Changes in primer design (e.g. degenerate primers or primers based on areas of sequence homology between known sequences), temperature gradient PCR varying annealing temperature and changes in Mg^{2+} concentration were also not successful. This was unexpected since the S2 gene segment is highly conserved among reovirus strains and can be folded into a complex secondary structure (Chappell et al. 1994). Thus, the inability to amplify the gene segment suggests the T2W S2 gene segment is significantly different from previously reported clones. This was sufficient to show T2W is a novel reovirus isolate; however, further efforts to sequence the S2 gene segment are still required for a more complete characterization.

Without sequence data for the T2W S2 gene segment and deduced aa sequence of σ2, one can only speculate the effect sequence differences between T2W and other known reovirus clones will have. It is likely T2W σ2 protein has an altered conformation because of the obvious significant difference in sequence from known reovirus clones. Due to the location of the σ2 protein in reovirus virions, this could lead to virion instability or affect formation of icosahedral particles during infection. It may also affect the formation of replicase particle assembly. The effects any of these changes may have on T2W replication are not known.

The reovirus S3 gene segment is 1,198 bp and encodes the 41 kDa σNS protein. When isolated from infected cells and expressed *in vitro*, σNS binds ssRNA nonspecifically. A region at the extreme N-terminus is important for this binding (Gillian and Nibert 1998). A temperature sensitive mutant whose lesion maps to the S3 gene segment shows severely decreased dsRNA synthesis, which suggests a role in replicase particle assembly (Cross and Fields 1972;Ito and Joklik 1972). Immunoprecipitation

studies show σNS binds to reovirus mRNAs shortly after they are synthesized and remain bound through negative strand synthesis and the generation and selection of the 10 dsRNA genome segments (Antczak and Joklik 1992). It was proposed σNS was required to nucleate viral inclusions in the cell based on evidence σNS localized to factories throughout infection and the viral mutant, *ts*E320, with a temperature-sensitive σNS protein, is defective for factory formation at restrictive temperatures (Becker et al. 2001).

In this study, the nucleotide sequence of the T2W S3 nucleotide gene segment ranged between 68.1 and 73.6% identity to other reovirus clones examined. The deduced aa sequence of the T2W σNS protein was between 86.3 and 89.9% identity. The amount of variability between T2W and the other reovirus clones was comparable to variability seen between T2J and other clones (Figure 19A). This was reflected in phylogenetic analyses which showed T2W had is own distinct lineage for the S3 gene segment (Figure 19B).

Thirteen cysteine residues previously identified in the deduced aa σNS sequence of T1L and conserved in field isolates were also conserved in T2W (Goral et al. 1996). Changes in aa were random and were not concentrated in a particular area of the protein, but occurred throughout (data not shown). Recent work suggests that functional σNS and μNS are the minimal viral components required to form viral inclusions, which then recruit other reovirus proteins and RNA to initiate viral genome replication (Becker et al. 2003). Thus, changes in T2W σNS protein may affect the ability of T2W to form viral inclusions and produce progeny particles.

The S4 gene segment of mammalian reovirus encodes the 41 kDa σ3 protein. It is present in 600 copies in 1:1 complexes with μ1 protein in the outer capsid of the reovirus virion (Dryden et al. 1993). It also interacts with λ2 and possibly σ1 proteins. It serves a number of distinct structural roles in the reovirus lifecycle such as viral stability to inactivating agents such as heat and detergents (Drayna and Fields 1982b) and it has been hypothesized that σ3 stabilizes the outer capsid in harsh environmental conditions (Nibert et al. 1991a). Removal of σ3 yields ISVP particles and is essential for reovirus infection to proceed beyond receptor binding and endocytic uptake (Baer and Dermody 1997;Jane-Valbuena et al. 1999). Removal likely occurs within endocytic vacuoles mediated by

acidic cysteine proteases from lysosomes, which allows viral particles to enter the cytoplasm of the host cell (Sturzenbecker et al. 1987;Baer and Dermody 1997). However, the σ3 protein may also be removed by alkaline proteases of pancreatic origin in the lumen of the small intestine (Bodkin et al. 1989;Bass et al. 1990). Studies with temperature-sensitive mutant *ts*G453, which fails to assemble outer capsids at nonpermissive temperature, also suggest σ3 plays a critical role in viral assembly and may be required to interact with μ1 in order to assemble onto core particles (Shing and Coombs 1996). Recoating experiments have provided further evidence that preformed complexes of σ3 and μ1 are required for assembly into particles and can occur independently of σ1 (Chandran et al. 1999;Farsetta et al. 2000). In addition to a structural role, the σ3 protein may also serve a regulatory function within the infected cell as differences in the capability of reovirus strains to affect cellular RNA and protein synthesis map to the S4 gene segment (Sharpe and Fields 1982;Nibert and Schiff 2001). Also, through its ability to bind dsRNA (Olland et al. 2001), σ3 is thought to counteract reovirus induced host antiviral defence mechanisms. In the presence of dsRNA, interferon-induced dsRNA-dependent protein kinase (PKR) will phosphorylate the α-subunit of initiation factor eIF-2, which shuts off translational initiation, ultimately interfering with viral protein synthesis (Huismans and Joklik 1976;Lloyd and Shatkin 1992;Beattie et al. 1995;Schmechel et al. 1997;Yue and Shatkin 1997). By binding with dsRNA, σ3 removes PKR's potent inducer and σ3 has been shown to inhibit activation of interferon-induced PKR *in vitro* (Imani and Jacobs 1988).

The T2W S4 ORF nucleotide sequence was identical to T1L S4 ORF. These results were unexpected, since the RNA electropherotype of T2W indicated the 10 gene segments in T2W were unique from all clones examined (Figure 16B). There is no explanation for this result at this time. There is always the possibility the clinical sample was contaminated with T1L, after the serotype and electropherotype were determined, but this is not likely the case. Sequencing studies were repeated numerous times with the same results on each occasion. Also, a repeat electropherotype with T2W RNA used in the sequencing studies was identical to RNA gels obtained early in the investigation (data not shown). This does not explain the incongruities of the results. Only the ORFs of the T2W gene segments were analyzed and compared to sequences of known clones.

Sequence variability in the non-coding regions was not evaluated because all PCR products were prepared using primers representing the T1L gene sequence. It is possible the non-coding regions of the gene are responsible for the different mobilities seen on SDS-PAGE. This is less likely, since among S4 gene segments from different isolates, the lengths of the terminal nontranslated regions are generally conserved and the nucleotide sequences are less variable than the internal coding sequence (Kedl et al. 1995;Nibert and Schiff 2001). However, inserted or deleted bases have been shown near the segment ends for gene segments L2, M1, M3 and S1 (Breun et al. 2001;Yin et al. 2004). Determination of the 3'- and 5'-untranslated terminal regions would need to be done to test whether differences in non-coding regions explains gel mobility differences. It is also important to note that relative genome segment-length does not strictly correlate with relative electrophoretic mobility (Ramig et al. 1977). These differences are not well explained, but likely reflect differences in conformation of the dsRNA helices, which affect their migration in gels.

T2W is definitely a novel reovirus isolate. In addition to the identical sequence of T2W S4 ORF compared to T1L ORF, the T2W S1 ORF was almost identical to the T1L S1 ORF, despite having a different serotype (discussed above). Ideally, repeat serotype and sequencing studies to verify results should be done. Unfortunately, it was often difficult to amplify T2W virus to high titres in order to obtain purified virus for these studies. Despite optimization of growth conditions, viral growth was not consistent. Growth of virus in suspension culture followed by viral purification was particularly troublesome. Purified virus was obtained less than half the time by this method. Attempts to grow T2W in nervous system cells, U373 (human astrocytoma) and HCN (human cortical neuron) cell lines, followed by virus concentration were successful at obtaining high titre cell lysates (data not shown); however, we were still not successful in extracting purified T2W and viral RNA. The lack of consistent high titre T2W viral stocks, purified virus and viral RNA limited the characterization of T2W. It is not known why viral purification and extraction was so particular. Perhaps T2W is less hardy than other reovirus clones. This would make it more difficult to grow in tissue culture. Virus bands collected from cesium chloride gradients of T2W from early passages of original clinical sample, often contained decreased amounts of whole virion particles with

increased amounts of viral particle debris compared to other reovirus clones, which suggests T2W is more susceptible to fragmentation during virus manipulations, such as virus extraction, than other reovirus clones.

The novel reovirus clone, T2W, strengthens the evidence reovirus may be occasionally pathogenic to humans. There are a few isolated reports presented in the literature, which associate reovirus with CNS disease. The best characterized isolate associated with CNS symptoms is a type 3 reovirus isolated from a 6.5-week old infant with meningitis (Tyler et al. 2004). S1 sequence analysis and pathogenesis mice studies conducted suggest this is a novel reovirus strain and can cause meningitis in humans. Another persuasive case is a report of a previously healthy 3-month-old girl who developed aseptic meningitis (Johansson et al. 1996). The child seroconverted and serotype 1 reovirus was isolated after inoculation of CSF onto green monkey kidney cells. There are other less well-documented cases, which associate reovirus with human CNS disease. In some cases virus was isolated only from stool and not CSF and seroconversion was not documented (Krainer and Aronson 1959). This raises the possibility reovirus infection may have been coincidental rather than causal. In some other cases, virus has been isolated from brain tissue (Krainer and Aronson 1959;Joske et al. 1964). The case presented in this thesis research seems to constitute one of a few cases where reovirus has been associated with meningitis in humans. This is similar to the situation in neonatal mice where CNS disease is one of the features of reovirus infection (Tyler 1998;Tyler 2001). The pattern of injury produced after intracerebral inoculation into neonatal mice between T1L and T3D is quite distinct. (For review, see (Tyler 1998)). T1L infects the ependymal cells lining the ventricles and produces ependymitis with associated hydrocephalus. Serotype 3 reoviruses infect neurons and produce a lethal meningoencephalitis. This pattern of spread is serotype specific. The genetic basis for the differences in CNS tropism has been analyzed using reassortant viruses (Weiner et al. 1977). The capacity of reoviruses to infect neurons (T3 pattern) or ependymal cells (T1 pattern) is determined by the S1 gene. This was confirmed with subsequent experiments with monoreassortants, i.e. T3D S1 gene on a T1L genetic background and T1L S1 gene on a T3D genetic background (Weiner et al. 1980). The difference in neurotropism is also reflected in cultured cell lines. In primary cultures derived from fetal rat cortex, T3D, not

T1L infects neurons, whereas T1L, not T3D, binds isolated human and murine ciliated ependymal cells (Tardieu and Weiner 1982). Both strains are able to infect astrocytes. Serotype 2 reovirus infection has been studied much less, but T2J (originally described as serotype 2 strain D5) has been reported to infect neurons (Walters et al. 1965)

Although T2W was isolated from the the CSF of an immunocompromised infant with respiratory, gastrointestinal and CNS symptoms, it is not entirely clear if reovirus infection was responsible for her CNS symptoms. Two strains of *Staphlococcus aureus* were also recovered in the CSF. As well, the patient had severe active varicella infection, which may have contributed to her meningitis. Unfortunately, it was not considered when CSF was examined, so it is unknown if varicella zoster virus was present in the CSF. Any of these pathogens may be responsible for the infant's symptoms and it is possible T2W at least partially contributed to her meningitis. It is also entirely possible T2W did not contribute to the CNS symptoms, however its presence in the CSF was persistent over time.

Reoviruses are normally gastrointestinal or respiratory pathogens in humans and have only rarely been described in the CNS in the literature (Johansson et al. 1996;Tyler et al. 2004). The question then arises; how did a reovirus clone enter the CNS? There are a few possible explanations. Perhaps the underlying immune deficiency or meningeal infection allowed for penetration of the blood-brain barrier and entry into the CSF. Another alternative explanation is reoviruses can infect the CNS, but do not cause CNS symptoms. Hence, it is possible reovirus is often present in the CSF during an infection, just not detected as there is no need to examine the CSF of an asymptomatic person.

T2W was also found in fecal samples of the infant. Reovirus has been associated with gastrointestinal infection in and it is possible reovirus was partially responsible for these symptoms. However, rotavirus particles were also identified in the stool sample and its contribution to the infant's diarrhea cannot be discounted. Unfortunately, the clinical case presented here is not simple and does not allow for a clear association between T2W and the CNS and gastrointestinal symptoms seen. Nevertheless, T2W is one of only a handful of reovirus clones to be isolated from the CNS of humans and warrants further investigation.

Even if T2W was responsible for only a small portion of the gastrointestinal and CNS symptoms in the infant, it would have be useful to have a possible treatment especially as the infant was immunocompromised and unable to mount a significant immune response of its own to fight this infection. The next phase of the research presented here, was to find an antiviral compound that could inhibit reovirus and thus, T2W replication. Because T2W viral stocks were limited, potential antiviral agents were initially screened with prototype reoviruses, T1L and T3D. Inhibition of T2W was only examined after a suitable anti-reoviral agent was determined.

4.3. Effects of antiviral agents on reovirus replication. In the past viral diseases have been difficult to treat with selective antiviral chemotherapy. Due to a greater understanding of viral life cycles and the elucidation of virus-specific events as targets for antiviral agents over the past decade, today there are more than 30 antiviral drugs approved for treatment of viral infections and several others are in advanced phase III clinical trials (De Clercq 2001c). Half of these agents are for the treatment of HIV, while the remainder are primarily for hepatitis B virus, influenza viruses and herpesviruses (De Clercq 2002). Unfortunately, effective therapies for several important viral infections are still lacking and current treatments are not always well tolerated. These deficiencies highlight the need for further refinement of antiviral drug design and development.

Inhibition of *in vitro* reovirus replication has been achieved with various antiviral compounds, including neplanocin A and some of its derivatives (De Clercq et al. 1989;Obara et al. 1996;Robins et al. 1998), acivicin (Keast and Vasquez 1992), cicloxolone sodium (Dargan et al. 1992), cyclopentenylcytosine (De Clercq et al. 1990), ribavirin (Rankin, Jr. et al. 1989;Connolly and Dermody 2002;Labrada et al. 2002) and MPA (Gong et al. 1999;Diamond et al. 2002). In an effort to characterize antiviral agents that could attenuate reovirus infection amantadine, AZT, acyclovir, foscarnet, rifampin, ribavirin and MPA were examined. Of all the agents examined only ribavirin and MPA inhibited reovirus replication to any great extent. This was not totally unexpected since a number of the agents investigated exhibit antiviral activity towards a specific virus family, e.g. amantadine is an anti-influenza A agent which targets the M2 ion channel in influenza A virus, AZT is an anti-HIV agent which targets the RT of HIV and acyclovir targets herpesvirus replication and requires herpes virus-encoded thymidine kinase for

activation (De Clercq 2001b). Unfortunately, other antiviral agents reported to have reovirus activity (neplanocin A, acivicin, cicloxolone sodium and cyclopentenylcytosine) were unavailable for study. An antiviral agent that targets virus specific mechanisms (i.e RdRp) would be ideal for antiviral therapy against reovirus. Since reovirus is generally thought of as a nonhuman pathogen, significant research into antiviral agents against reovirus is limited and no agent has been found that directly targets reovirus.

Ribavirin was first described as a broad-spectrum antiviral agent in 1972 (Wikowski et al. 1972). In humans, it is currently used in combination with interferon-α to treat hepatitis C virus infections and as a monotherapy for respiratory syncytial virus infections (Davis et al. 1998;McHutchison et al. 1998;Wyde 1998). It has been demonstrated to have two main activities *in vivo* or in cell culture: inhibition of IMPDH and incorporation as a mutagenic nucleoside by the viral RNA polymerase. (For review, see (Crotty et al. 2002)). The mechanism of antiviral activity is under debate. IMPDH, the enzyme that catalyzes the conversion of IMP to XMP in the *de novo* purine biosynthesis pathway (Figure 4) has been recognized as a target for the antiviral action since 1973 (Streeter et al 1973). This inhibition is dependent on the intracellular conversion of ribavirin to its 5'-monophosphate. However, ribavirin may also interfere directly with influenza (Eriksson 1977) and vesicular stomatitis viral RNA polymerase (Fernandez-Larsson et al 1989) and HIV reverse transcriptase (Fernandez-Larsson & Patterson 1990), capping guanylation (Goswami 1979, Schiedel & Stollar 1991) and primer generation/elongation (Wray 1985) in its 5'-di- or triphosphate form. The other likely mechanism of ribavirin activity is as an RNA-virus mutagen, which forces RNA viruses to accumulate a lethal number of errors, called 'error catastrophe' (Crotty et al. 2002). The antiviral activity of ribavirin might result from the lethal mutagenic effect after incorporation as a 5'-triphosphate into the viral genome.

Ribavirin has been shown to effectively inhibit growth of a number of viruses, including mammalian (Rankin, Jr. et al. 1989;Connolly and Dermody 2002) and avian reovirus (Bodelon et al. 2002;Labrada et al. 2002). In this study, ribavirin maximally inhibited T1L and T3D mammalian reovirus replication between 341- and 724-fold at a concentration of 100μM ribavirin between 18 and 24 hpi. This inhibition is greater than was seen in a previous report with T3D grown in L929 cells (Rankin, Jr. et al. 1989).

Inhibition of reovirus replication decreased over the course of the infection and virus production in ribavirin-treated cells increased to levels just below non drug-treated controls by 48 hpi (Figure 22). This could not be explained by the production of ribavirin-resistant mutants. One possible explanation for this result, is active ribavirin (i.e. phosphorylated ribavirin) molecules are no longer available to inhibit reovirus production. Based on the possible mechanism of ribavirin's antiviral activity, the decreased levels of ribavirin could be due to metabolism of ribavirin molecules by the cell or the incorporation of ribavirin triphosphate into the reovirus genome. Previous work with reovirus found two components of transcription of reovirus mRNA molecules were sensitive to ribavirin: elongation (more sensitive) and initiation (less than half as sensitive) (Rankin, Jr. et al. 1989). They also demonstrated ribavirin triphosphate was not incorporated into RNA sequences; thus, it likely does not act as a chain terminator or RNA mutagen in reovirus.

The decrease in inhibition of mammalian reoviruses at 48 hpi was not seen in growth studies with ARV (Robertson et al. 2004). Ribavirin pharmacokinetics in the cell lines used to grow mammalian (L929) and avian (QM5) reoviruses may explain the differences. Ribavirin enters cells by facilitated diffusion (Paterson et al. 1975) and must be phosphorylated in the cell in order to exert its antiviral effects (Richman et al. 1987;Rankin, Jr. et al. 1989). The rate of diffusion and ratios of the mono-, di- and triphosphate forms may be different in the two cell lines and may affect the ability of ribavirin to inhibit reovirus replication. The pharmacokinetics of ribavirin in QM5 cells needs to be evaluated and compared to data in L929 cells (Rankin, Jr. et al. 1989) to determine if differences in inhibition could be attributed to kinetics. Alternatively, it is possible ribavirin may have different mechanisms of action against mammalian and avian reoviruses. This could be evaluated by examination of the antiviral effects of ribavirin in a cell line that supports both mammalian and avian reovirus replication, such as Vero cells. Any differences seen could then be attributed to differences in mammalian and avian reoviruses not differences in cell metabolism.

MPA, another IMPDH inhibitor, has been shown to have antiviral activity (Malinoski and Stollar 1981;Ichimura and Levy 1995;Gong et al. 1999;Diamond et al. 2002;Morrey et al. 2002). It was also assessed for its ability to inhibit mammalian

reovirus replication in mouse L929 fibroblasts. MPA is used clinically as an immunosuppressive agent to prevent organ rejection in kidney and heart transplants (Sollinger 1995;Behrend et al. 1997;Kobashigawa et al. 1998). In this capacity, it is believed to act through inhibition of T cell proliferation. The proliferative response of human lymphocytes is highly dependent on *de novo* purine biosynthesis, whereas the major salvage pathway catalyzed by HGPRTase is not used in lymphocyte proliferation (Figure 4). Thus, inhibition of IMPDH, which results in a depletion of the intracellular GTP and dGTP pools, would be more potent on lymphocytes than on other cell types (For review, see (Allison and Eugui 2000)).

Previous studies have reported MPA inhibited several viruses *in vitro* in a guanosine-dependent manner likely by a similar mechanism (Gong et al. 1999;Diamond et al. 2002). However, we are not aware of previous studies that examine viral factors that contribute to this inhibition. Although some previous studies indicate that MPA inhibits reovirus T1L infection (Gong et al. 1999;Diamond et al. 2002), the primary sources and original data were not found and could not be adequately evaluated.

In this study, the ability of MPA to inhibit reovirus replication in L929, Vero and U373 cells was investigated. Using plaque reduction assays, growth studies and biosynthetic labelling, it was demonstrated MPA decreases reovirus replication, prevents an increase in reovirus titre beyond input titre and inhibits production of reovirus progeny by reducing levels of viral RNA and protein. This data convincingly shows MPA is an anti-reovirus agent and although it cannot prevent infection, it can attenuate reovirus replication. In the presence of 3μg/mL MPA, viral replication was suppressed for at least 24 and 48 hpi in T1L and T3D infections respectively as compared to non drug-treated controls where viral titres increased dramatically after 12 hpi (Figure 27). To test whether this represented a decrease in infectious viral progeny or a true decrease in viral production, the effect of MPA on RNA and protein production was investigated. Both RNA and protein production of both virus clones were suppressed in the presence of 3μg/mL MPA, and only T3D progeny RNA and protein production were substantially reduced in the presence of 300ng/mL MPA, when infections were harvested at 72 hpi (Figure 29). The inhibitory effect of MPA was not due to cell toxicity and was reversed by the addition of exogenous guanosine (Figure 28). These observations support the

major mechanism of action of MPA is to deplete intracellular GTP and inhibit viral polymerase activity and is consistent with reports of *in vitro* antiviral activity in other virus systems (Williams et al. 1968;Ichimura and Levy 1995;Gong et al. 1999;Smee et al. 2001;Diamond et al. 2002;Morrey et al. 2002).

Despite the evidence the antiviral activity of MPA is attributable to inhibition of IMPDH, it is possible MPA has other mechanisms of antiviral activity. Work has shown a concentration of 0.33μg/mL MPA decreases intracellular GTP and dGTP pools in T lymphocytic cells (Allison et al. 1991). It is possible reduction of GTP levels is maximized at low concentrations of MPA, however higher concentrations of MPA (i.e. 3μg/mL) were required to inhibit reovirus replication to any extent. This raises the possibility that MPA possesses additional mechanisms of antiviral activity at higher concentrations. This has been seen with ribavirin where a series of cell culture studies demonstrated ribavirin reduced intracellular GTP levels to 65% normal at a concentration of 25μM ribavirin (Wray et al. 1985). Although 25μM ribavirin exhibited antiviral activity against influenza at this level, the effect was small and higher concentrations were required for maximal antiviral activity. This is also reflected in the work presented here where 100μM ribavirin was required to inhibit reovirus replication. Measurement of GTP levels in the presence of MPA in conjunction with antiviral studies are required in order to further investigate the antiviral activity of MPA.

Inhibition of infectious reovirus progeny was greatest between 3 and 30μg/mL MPA. Interestingly, a higher dose of MPA (100μg/mL), which was also shown to be cytotoxic to L929 cells, lead to increased production of viral progeny. To our knowledge, this is the first time a paradoxical effect has been described for MPA or any antiviral agent. Paradoxical effect of antibiotics is defined as a substantially reduced bacterial killing at antibiotic levels above the minimal bactericidal concentrations *in vitro* (Holm et al. 1991). This phenomenon was originally described for β-lactam antibiotics, but has also been seen with aminoglycosides and quinolones. The explanation of the paradoxical effect is unknown. It is uncertain whether the decrease in inhibition when higher doses of MPA are used is a true paradoxical effect since the dose was also cytotoxic to the cells; however, the paradoxical effect seen with MPA and reovirus replication was consistently seen with mammalian and avian reoviruses in multiple cell lines. It is possible viral titre

determined after infection in cells treated at higher MPA doses may be indicative of input titre (since a cell lysate is used) and may not reflect production of infectious viral progeny. Further investigation is required in order to sort out the exact mechanism and clinical significance. MPA is used clinically in kidney and heart transplantation (Sollinger 1995;Behrend et al. 1997;Kobashigawa et al. 1998) at higher levels than used to inhibit reovirus infection. Patients are immunosuppressed in order to prevent graft rejection; thus, are susceptible to a variety of infections. If the antiviral MPA effects are truly paradoxical, i.e. viral replication is inhibited to a lesser extent at higher MPA concentrations, this may have considerable implications.

Mycophenolate mofetil (MMF), the morpholinoethyl ester of MPA, has increased bioavailability in humans compared to MPA. After oral administration MMF is rapidly hydrolyzed by esterases in the intestine and blood to release MPA (Allison and Eugui 1996). There are a number of studies that have investigated viral infections in transplant patients receiving MMF and viral infections, in particular cytomegalovirus infections. Generally results indicate MMF does not decrease the incidence of cytomegalovirus disease after transplantation, but reduces the severity and complications from the disease, which leads to increased graft survival (Paterson et al. 1998;Nashan et al. 1999;Giral et al. 2001). However, a study in a pediatric population found renal transplant recipients had increased susceptibility to varicella infection (Rothwell et al. 1999). The increase in varicella infection was attributed to the enhanced immunosuppression achieved with MMF, but in light of the results above (i.e. paradoxical effect), there is the possibility other factors, besides immunosuppression, may be involved.

The current results revealed reovirus strains T1L and T3D differed in their sensitivity to MPA in L929 cells, with T3D more sensitive to the antiviral effects of MPA than T1L. Maximum inhibition was seen at a level of 30μg/mL MPA, where inhibition of T3D was 1560-fold compared to 46-fold for T1L in L929 cells (Figure 25A). Genetic reassortant analysis of strain-dependent differences identified the M1· gene, which encodes the μ2 protein, as the primary determinant of the difference in inhibition between T1L and T3D in L929 cells (Table 10 and Table 11). Some reassortant clones (e.g. EB129, KC19 and others) appeared exceptions to the presumed M1 mapping and a few other gene segments i.e. M2 ($p=0.017$), M3 ($p=0.0028$) and S1 ($p=0.026$) appeared to

contribute to the phenotypic differences by Wilcoxon rank sum analysis. Other studies have found some phenotypes do not map to the M1 gene segment in distinct groups (Sherry and Fields 1989;Yin et al. 1996). The reasons for this are not known. To further evaluate the contribution of each gene segment in the differential MPA sensitivity phenotype, a variety of statistical analyses were performed. Division of the reassortant data set into halves and a Chi square analysis of the distribution of each gene in each half indicated the M1 gene was the most significant ($p \leq 0.007$) and that S3 played a minor role ($p \leq 0.06$) (Table 11). Multiple linear regression analyses were also performed to determine whether the above genes truly contributed to the phenotypic differences or whether apparent contribution was caused by gene distributions in the available reassortants. Irrespective of whether multiple linear regression was performed on the complete data set of all individual experiments or whether it was performed on the average values (Table 11 column 2 and column 4), the M1 gene segment was the only major determinant of the MPA strain-dependent differences ($p \leq 0.04$).

The reovirus M1 gene is 2,304 bp long (Wiener et al. 1989;Zou and Brown 1992b), except in T2J where it is 2,303 bp long (Yin et al. 2004), and encodes the minor reovirus structural protein, μ2. It is a minor component of the inner capsid, present in only 20-24 copies per particle (Coombs 1998). Protein μ2 has not been definitively localized within the reovirus particles, but is thought to associate with the RNA-dependent-RNA-polymerase and reside near the icosahedral five-fold axes (Dryden et al. 1998;Reinisch et al. 2000). The functions of μ2 are only partially understood. The M1 gene segment is genetically associated with viral strain differences in the severity of CPE in mouse L929 cells, the frequency of myocarditis in mice, the levels of viral growth in cardiac myocytes and endothelial cells, the degree of organ-specific virulence in SCID mice and the level of interferon induction in cardiac myocytes (Moody and Joklik 1989;Sherry and Fields 1989;Matoba et al. 1991;Haller et al. 1995;Sherry et al. 1998). The μ2 protein has also been shown to bind RNA and to be involved in formation of viral inclusions through microtubule binding in infected cells (Brentano et al. 1998;Mbisa et al. 2000;Parker et al. 2002). Other genetic studies have associated the M1 gene with viral strain differences in the *in vitro* transcriptase and nucleoside triphosphatase activities of viral core particles (Yin et al. 1996;Noble and Nibert 1997), which has been used to

suggest that μ2 is a transcriptase cofactor, but μ2 remains the most poorly understood of the eight structural proteins found in reovirus virions. The complete sequence of the M1 gene segment has been reported for a variety of reovirus field isolates (Wiener et al. 1989;Zou and Brown 1992b;Parker et al. 2002;Yin et al. 2004). The T1L and T3D M1 sequences are among the most highly conserved among different reovirus strains, showing about 98% nucleotide and >98.5% amino acid identity between T1L and T3D. This indicates 1 or more of the 10 amino acid differences found between the T1L and T3D's μ2 proteins are responsible for the phenotypic differences. Computer-based comparisons of the M1 gene and μ2 protein sequences to others in GenBank have failed to show significant homology to other proteins, so that no clear indications of the function of μ2 were revealed. However, small regions of sequence similarity to NTP-binding motifs have been identified near the middle of μ2.

The results of this study provide further insight into reovirus minor core protein μ2, which remains the most functionally and structurally enigmatic of the reovirus proteins. The strain-specific difference in antiviral activity of MPA was genetically associated with the M1 gene segment (Table 10 and Table 11). Based on the mechanism of action of MPA, this suggests the μ2 protein is involved in the uptake and processing of GTP in viral transcription and strengthens the evidence that the μ2 protein can function as an NTPase and is likely a transcriptase cofactor. Results from work with a MPA-resistant Sindbis virus mutant are consistent with the idea that an alteration of the RNA guanyltransferase is responsible for the MPA-resistant phenotype (Scheidel and Stollar 1991). An N-terminal domain of the reovirus λ2 protein has been defined as the reovirus guanyltransferase (Cleveland et al. 1986;Luongo et al. 1998); however this does not rule out the possibility the μ2 protein may also play a role in guanyltransferase activity. Generation and study of a MPA resistant reovirus mutant may help elucidate the roles played by the minor core protein μ2 in reovirus replication.

Both ribavirin and MPA have IMPDH inhibitory activities and possess similar antiviral effects between 18 and 24 hours on reovirus infectious viral progeny production (compare Figures 22 and 27). Strain-specific differences in antiviral activity, with T3D more sensitive than T1L, were seen for both antiviral agents. This supports the argument

both agents inhibit production of infectious reovirus progeny in a similar manner likely by inhibition of IMPDH. Inhibition of virus production over time was different between the two antiviral agents. Production of infectious reovirus progeny with ribavirin was no longer inhibited at 48 hpi; whereas, virus titres still hovered near input levels in the presence of MPA. Also a significantly greater concentration (25μg/mL=100μM) of ribavirin was needed in comparison to MPA (3μg/mL=9μM) for inhibition. This suggests dosing with ribavirin would need to be more frequent and with higher concentrations; thus, with an increased risk of undesirable side effects.

The objective of the antiviral studies was to find an antiviral agent that could have been used as treatment for infection with T2W. The ability of ribavirin to inhibit T2W replication was not examined for several reasons that include the high concentrations required for inhibition, poor antiviral activity at 48 hpi and more importantly because it appears to distribute slowly into the CSF over four to seven weeks (McEvoy 2003). MPA was found to inhibit the production of infectious T2W viral progeny. Maximum inhibition was 184-fold (Figure 31A) and was intermediate between inhibition seen for the prototype viruses, T1L and T3D. However, it occurred at a concentration of 3μg/mL versus the 30μg/mL for the prototype viruses. The addition of guanosine to restore viral replication to non drug-treated levels supports viral inhibition is likely due to inhibition of IMPDH, which leads to decreased intracellular GTP.

Although the inhibitory effect of MMF on reovirus replication was not examined in this research, work with herpesvirus showed a comparable reduction in virus yield between MPA and MMF in Vero cells (Neyts et al. 1998) and it is likely inhibition of reovirus replication with MMF would be comparable to that seen with MPA. Unlike ribavirin, MPA is able to penetrate the CNS (Mizobuchi et al. 1997;Wennberg et al. 2001); therefore at low doses, MMF may have been effective for the treatment of CSF T2W infection.

4.4. Conclusions and future directions. In this research, a novel reovirus clone isolated from the CSF of a severe combined immunocompromised infant, was characterized. This report is only one of a few in the literature which have isolated reovirus from the CNS (Krainer and Aronson 1959;Joske et al. 1964;Johansson et al. 1996). Serologic, electron microscopic and molecular studies showed T2W was a novel

reovirus clone; however, there were some anomolous results. Despite a unique RNA electropherotype, the S4 ORF nucleotide sequence was identical to T1L. It is possible differences in the 5'- and 3'-untranslated ends may explain differences in RNA mobility. Determination of the S4 gene segment ends would need to be examined to test this hypothesis. In addition, T2W was shown to be serotype 2 despite the deduced aa sequence of T2W σ1 protein being almost identical to T1L σ1. Repeat serotyping with monoclonal antibodies directed against type 1 and type 3 reoviruses may help to clarify the situation. To gain insight into the potential CNS pathogenesis and virulence of this unique reovirus isolate, differences in cytopathic effect kinetics and phenotypic changes that differentiate this isolate from prototypic reovirus strains should be completed. Mice studies could also be employed to study CNS interaction. Unfortunately, further characterization of T2W is limited by the inability to consistently obtain high titre stocks and purified virus.

The small amount of T2W virus stock lead to the examination of potential antiviral agents with the prototype viruses, T1L and T3D. This resulted in the observation of strain-specific differences in sensitivity to MPA between T1L and T3D, which mapped to the M1 gene segment. Different inhibition patterns of mammalian and avian reoviruses for ribavirin were also observed. Ribavirin and perhaps other IMPDH inhibitors could be used as a tool to exploit the differences in order to elucidate variations in mammalian and avian reovirus replication and/or different mechanisms of antiviral activity for a particular molecule.

In general, viral diseases are difficult to treat with selective antiviral chemotherapy. In the studies presented here, ribavirin and MPA, both IMPDH inhibitors, were found to inhibit reovirus replication *in vitro*. The antiviral effects of ribavirin have been studied extensively in the past and are still studied in many viral systems. The antiviral effects and mechanisms of other IMPDH inhibitors, such as MPA, have only begun to be discovered. Future research needs to be directed at explotation of MPA and other novel IMPDH inhibitors, such as VX-497 and VX-148 (Markland et al. 2000;Jain et al. 2001;Jain et al. 2002) as potential broad-spectrum antiviral agents.

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