MOLECULAR STUCTURE/FUNCTION STUDIES OF MAMMALIAN REOVIRUS AND VIRUS-INDUCED GADD45α RESPONSE IN P53-INDEPENDENT PATHWAYS

By

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

DOCTOR OF PHILOSOPHY

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FACULTY OF GRADUATE STUDIES

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Finally, I would like to thank my wife, my son, and my parents, my brothers and sisters in China who always support me, believe me and understand me for all the years. I love them dearly.

FOREWORD

I would like to dedicate this thesis to my late mother, Fenghua Li. Her strong belief and kindly assistance in the value of education inspired me to pursue my academic career to its fullest.

LIST OF ABBREVATION

aa	amino acid
7-AAD	7-aminopanctinomycin
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
cDNA	complementary DNA
CPE	cytopathic effect
dADP	deoxyadenosine diphosphate
dATP	deoxyadenosine triphosphate
D-buffer	dialysis buffer
ddH_2O	double-distilled water
DEPC	diethyl pyrocarbonate
dGDP	deoxyguanosine diphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DOC	deoxycholate
dsRNA	double-stranded RNA
DTT	dithiothreitol
EM	electron microscopy
ESB	electrophoresis sample buffer
FCS	fetal calf serum
Freon	1,1, 2-trichloro-1,2,2-trifluoroethane
GADD45α	growth arrest and DNA damage 45α
GDP	guanosine diphosphate
GMP	guanosine monophosphate
HO-buffer	honogenization buffer
hpi	hour post-infection
ISVP	intermediate subviral particle
MEM	minimum essential medium
MOI	multiplicity of infection
mRNA	messenger RNA
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming units
PI	post-infection
RNA	ribonucleic acid
RPA	RNA protection assay
rpm	revolutions per minute
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
T1L	Type 1 Long
T2J	Type 2 Jones
T2W	Type 2 Winnipeg
T3D	Type 3 Dearing
TBE	Tris/borated/EDTA buffer
ts	temperature sensitive
UV	ultraviolet
VSP	viable serum protein
Vertrel® XF	1,1,1,2,3,4,4,5,5,5-decafluoropentane

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ABSTRACT

Mammalian reovirus is the type virus of the family *Reoviridae* and consists of 10 segments of dsRNA genome and 8 structural proteins. Although the role of reovirus infection in human disease is not clear, it has served as an interesting model to understand the molecular biological mechanisms between virus infection and cellular responses. I now confirmed that reovirus infection induced apoptotic changes in L929 cells and Vero cells, but H1299 cells were arrested; and further found that the reovirus induced cell responses depended on the doses of reovirus infection. Reovirus grew very well and reached to very high titers in the three cell lines. Reovirus infection at MOI $\geq$10 PFU/cell resulted in apoptosis or cell arrest, but no effects were seen if the dose was 0.5 PFU/cell.

Results show the cell responses to reovirus infection are unrelated to the p53 pathway. Reovirus infection does not induce the transcription of p53, and the expression of p53 in cells also did not change reovirus titers. The expressions of cellular genes GADD45α, mcl 1 bax and p21 were induced by infection with reovirus strains T1L and T3D at MOI=100 PFU/cell. However, other genes, such as TRAIL, DR3, caspase-8, RIP, FAS, FASL mRNAs were not increased in the infected cells. Furthermore, the expression of Bcl-x gene mRNA in H1299 cells depended upon the reovirus strain; it was positively induced in the cells infected with reovirus T3D but inhibited in cells infected with reovirus T1L.

The expression of GADD45α was significantly and stably induced in the H1299 cells and L929 cells infected with reoviruses T1L and T3D. GADD45α expression levels were decided by the infectious doses of reovirus; GADD45α was induced by reovirus infection at MOI $\geq$ 10 PFU/cell. UV-inactivated reovirus particles did not induce the expression of GADD45α. Thus, expression of GADD45α is probably by a p53-undependent pathway and is probably regulated by NF-κB pathway in reovirus infection because these NF-κB and GADD45α proteins are similarly expressed in infected cells. In addition, the expression of NF-κB occurs a little earlier than that of GADD45α in infected cells. GADD45α expression was partly inhibited by siRNA; and GADD45α inhibition decreased the virus titers at various times post infection.

By using transfection, non-infectious reovirus core particles entered cells and the cores replicated as efficiently as reovirus virions do. Comparison of two transfection

methods showed that Lipofectamine reagent transfected reovirus cores more efficiently than Calcium phosphate ($CaPO_4$) methods. These transfection methods may pave the way to improve genetic manipulations.

For reovirus siRNA experiments, two sets of reovirus siRNAs did not inhibit the reovirus replication. It is possible that the selection of these parts of siRNAs was not satisfactory or the siRNAs did not function on the virus replication, suggesting that siRNA function is very complex and needs more clarification. However, introduction of full-length reovirus dsRNA into cells inhibited subsequent reovirus replication very efficiently, and this inhibition was not class size specific.

Finally, a newly isolated clinical sample of reovirus, designated T2W was further characterized. Sequence analysis of the T2W S genes showed this virus had high identity to other serotype 2 reoviruses in the S1, S3 and S4 gene sequences; thus T2W is more closely related to T2J than to T1L or T3D. However, The T2W S2 gene is more closely related to T1L or T3D S2 genes than T2J S2. The virus σ protein sequences were also analyzed by mass spectrometry to further confirm this reovirus is a new type 2 virus strain.

Chapter 1. *INTRODUCTION.*

Reoviruses are the prototype member of the virus family *Reoviridae*. The viruses consist of 10 segmented double-strand RNA (dsRNA) genomes and 2 protein capsids. These viruses were originally isolated and identified in the early 1950s in the respiratory and enteric tracts of healthy individuals. Thus the acronym *reo* (for respiratory, enteric, orphan meaning not associated with human disease) was proposed and named (Sabin, 1959).

The dsRNA viruses:

Currently known dsRNA viruses have 1 to 12 segmented dsRNA genomes and the majority of these viruses have icosahedral capsid structures (Mertens, 2004). The dsRNA viruses perhaps come from a common ancester. They share many similar structural and biochemical properties such as: replication strategy, an innermost icosahedral (T=1) capsid layer, the internal virion-associated enzymes, and the similar secondary structure of the innermost caspid layer proteins. However, it is the outer capsid layer proteins and some non-structural proteins that appear to be adapted for virus transmission and initiation of infection in different host, these show much more diversity in the component sequences and/or their structural patterns (Mertens, 2004), and suggest all these viruses come from a common ancestry.

The dsRNA viruses represent a very large group of different pathogens, affecting a wide variety of vertebrates, invertebrates, plants, fungi, and prokaryotes. So far, a total of 135 dsRNA virus species have already been identified (plus 52 tentative or unassigned) and are classed into eight distinct families, including the family *Reoviridae* which contains the largest number (74 species plus more than 30 tentative or unassigned) by the International Committee for the Taxonomy of Viruses (ICTV) (Table **1.1**).

1.2. *Reoviridae*:

The *Reoviridae* currently contains a total of 12 different genera, shown in Table **1.2** (Mertens, 2004). Many of these agents are pathogenic, either only infect humans (*e.g.* coltiviruses) or only infect animals (*e.*g. orbiviruses, avian reoviruses, seadornaviruses, and aquareoviruses), or infect both humans and animals (e.g. reovirus and rotaviruses).

Table 1.1. The families of dsRNA viruses.

Family	Number of genome segments	Type of virus particle	Host
Hypoviridae (Hillman et al, 2002))	1 (unpackaged)	~50-80nm diameter, pleomeophic vesicles (no capsid)	Fungi
Totiviridae (Wickner et al, 2000)	1 (packaged singly)	~30-40nm diameter, icosahedral	Fungi
Birnaviridae (Leong et al, 2000)	2 (co-packaged)	~60nm diameter icosahedral, single shell	Fish, insects, birds, mollusks
Varicosavirus (genus) (Mayo, 2000)	2 (separately packaged)	~18 x 320-360nm, rod shaped	Plants
Partitiviridae (Glasbrial et al, 2000)	2 (separately packaged)	~ 30-40nm diameter, icosahedral protein capsid	Fungi, plants
Cystoviridae (Banford, 2000)	3 (co-packaged, equimolar)	~85nm diameter, three layer structure with an envelope surrounding a two layered icosahedral nucleocapsid	Bacteria
Chrysoviridae (Gihabrial et al, 2003)	4 (packaged separately)	~30-40nm diameter icosahedral protein capsid	Fungi
Reoviridae (mertens et al, 2000)	10, 11 or 12 (co-packaged)	~70-80nm diameter icosahedral (one, two or three layered protein capsid)	Insects, plants, fish, reptiles, birds, ammals, anachnids, fungi, arthropods, crustacea

Copied from Mertens, 2004: Seventh report of the International Committee for the Taxonomy of Viruses.

Table 1.2. The *Reoviridae* Family:

Genera	Number of Gene segments	Number of member species (+ tentative or unassigned isolates)	Total number of types (serotypes) (+ tentative or unassigned isolates)
Cypovirus[a]	10	16 (+3)	16 (+3)
Fijivirus	10	8	8
Orbivirus	10	20 (+12)	157 (+12)
Orthoreovirus	10	4	6
Oryzavirus[b]	10	2	4
Proposed new genus of insect reoviruses	10	2 (+7)	2 (+)
Aquareovirus	11	6	23 (+5)
Rotavirus[c]	11	6 (+2)	
Coltivirus	12	2	5 (+1)
Phytoreovirus	12	3 (+1)	3 (+1)
Seadornavirus	12	3	7 (+15)
Mycoreovirus	11 or 12	3	3
Unassigned viruses	9,10, 11 or 12	(6)	(6)

Modified from Mertens, 2004. Seventh report of the International Committee for the Taxonomy of Viruses.

[a] A large number of *cypovirus* isolates (230) has been recovered from a range of different insect species.

[b] The serological relationships between different isolates of some distinct *Orbivirus* species has not been fully explored.

[c] The identity of Rotavirus serotypes has not been fully explored.

Mammalian reoviruses are ubiquitous in nature. They infect a variety of mammalian species including humans, monkeys, rats, mice, insects, fish and reptiles (Tyler and Fields, 1996). They are easily isolated from the environment –rivers, stagnant water and sewage (Stanley, 1967; Matsuura et al., 1993). Orthoreovirus, Cypovirus, Idnoreovirus, Mycoreovirus, Aquareovirus and Rotavirus are transmitted horizontally or vertically between individual hosts, often by an oral/fecal route. However, many of the plant reoviruses (Fijiviruses, Oryzaviruses and Phytoreovirues) and plant arboviruses are transmitted between their hosts by vector insects (Attoui et al., 2005a, 2005b; Mertens et al., 2005). 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 **1.3**). A putative new genus that contains 9 segmented dsRNA genomes was recently isolated and identified (Attoui et al., 2005c), and the family may be expanded.

1.3. Orthoreovirus:

The genus mammalian *Orthoreovirus*es are the prototype members of the family *Reoviridae*, and contains the type species, the nonfusogenic mammalian orthoreoviruses (reoviruses). It also includes the fusogenic avian orthoreoviruses and an assortment of other orthoreoviruses (*e.g.* Nelson Bay virus and baboon reovirus). Viral isolates are assigned to the *Orthoreovirus* genus based on similar traits (Table **1.3**) including the genome 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 reaction and the ability of these viruses to assort (mix) their genome segments upon co-infection of cells to yield reassortant progeny. Three serotypes of mammalian reoviruses have been determined by hemagglutination-inhibition (HAI) and neutralization studies (NT) (Sabin, 1959; Rosen, 1962; Atanley, 1967). An isolate (strain Lang) from a healthy child is the prototype for reovirus serotype 1 (Ramos-Alvarez and Sabin, 1958; Sabin, 1959), and abbreviated as T1L; an isolate (strain Jones) from a child with diarrhea is the prototype virus for

Table 1.3. General characteristics of the *Reoviridae:*

Structure
About 70-85 nm in diameter
Icosahedral
Nonenveloped
Multiple concentric protein capsids
Innermost capsid serves as transcriptase complex
Outermost capsid serves as gene delivery system
Genome
Linear double-stranded RNA (dsRNA)
Total genome size 18-29 kbp
10-12 gene segments
Segmented genome capable of assortment to produce hybrid reassortant viruses
Most gene segments monocistronic
Replication
Cytoplasmic
Proteolytic processing of intact virion to produces subviral particles
Uncoating is incomplete; innermost core capsid serves to transcribe mRNA

Copied from Coombs, 2002.

serotype 2 (Sabin, 1959) (T2J), and an isolate (strain Dearing) from a child with diarrhea (Ramos-Alvarez and Sabin, 1958; Sabin, 1959) is the prototype for serotype 3 (T3D).

1.3.1. Reovirus composition:

Reovirus particle is relatively simple, and consists of two concentric protein capsids and 10 segmented dsRNA genomes (Figure **1.1** and Table **1.3**).

1.3.1.1. Genome:

The 10 segmented dsRNA are classified according to size (Table **1.4** and Figure **1.1b**): Three size classes exist: large (L) segments consist of about 3800 base pairs each, medium (M) segments consist of about 2200 base pairs each, and small (S) segments of about 1100-1500 base pairs each. Each virion contains three L (L1, L2, L3), three M (M1, M2, M3), and four S (S1, S2, S3, S4) segments. All reovirus RNA possess several conserved nucleotides at their 5' and 3' ends. This conservation of nucleotides is also present in the RNA of the other members of the *Reoviridae* family, with all the genome segments of a specific virus possessing identical nucleotides at their 5' and 3' termini, but with these nucleotides being different from genera to genera in numbers and composition.

The genome of 10 discrete segments of double-strand RNA has: (I) a $^{m7}GpppG^{m}$ cap structure at the 5'end of the plus-sense strand and conserved terminal elements: 5 -GCUA and UCAUC-3`; (II) each gene has short non-translated regions (UTRs) at both ends; (III) the UTRs among the viral segments range in size from 13-33 nt at the 5` end to 32-80 nt at the 3` end of the plus-sense strand, and the UTRs of different reoviruses vary in the length and composition (Table **1.5**); (IV) virtually every segment contains a single ORF encoding a single protein with exception that one or two genes contain two ORFs (Nibert and Schiff, 2001; Coombs, 2002).

Specific nucleotide mutations at the –3 and +4 positions relative to the AUG initiation codon did not significantly influence the amount of translation product produced (Munemitsu and Samuel, 1988). Reovirus S4 UTRs contained a translational operator sequence that serves to regulate translational efficiency of the S4 mRNA (Mochow-Grundy et al., 2001). The T1L RdRp (λ3) could recognize capped viral RNA segments by conserved terminal elements: 5`-$N^7$-MeG-GCUA and UCAUC-3 (Tan et al., 2002). Binding of the cap at one position on the polymerase surface and insertion of the 3` end

Figure 1.1. Mammalian reovirus structure, genome, and proteins.

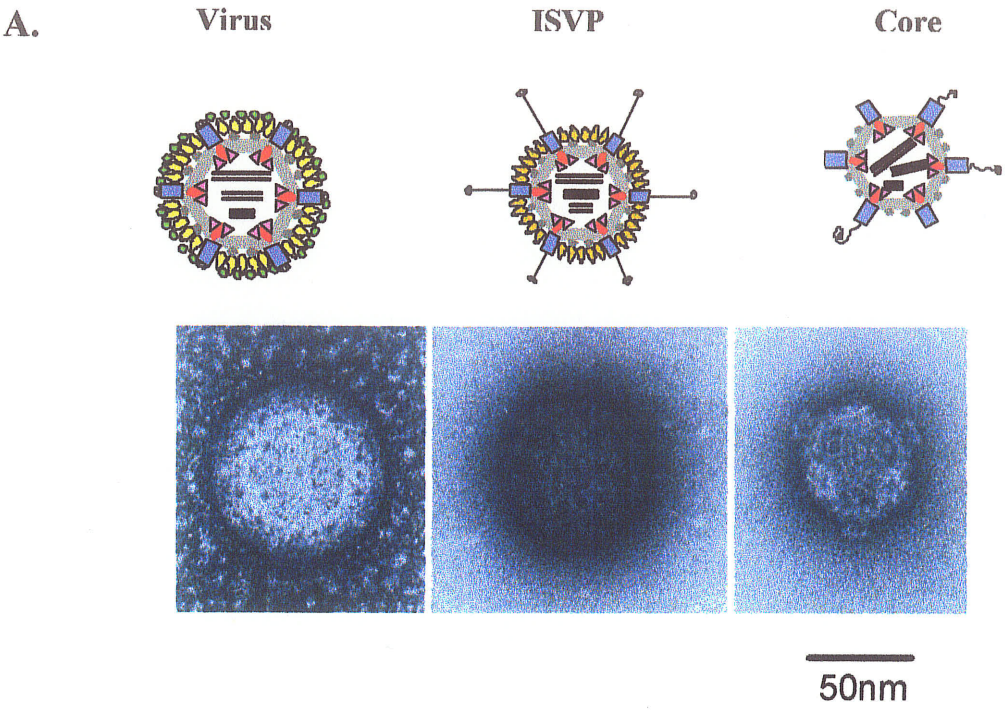


Figure 1.1. Mammalian reovirus genome, proteins and structure.

A. Schematic representation (on the top) of the three reoviral morphological forms: virion, ISVP and core. Proteins in outer and inner capsid are color coded to match the proteins resolved in the cartoon protein SDS-PAGE (see **B**). Middle lower portion of A shows real electron micrographs of the virus, ISVP and core. **B**. Cartoon representation of the genome and proteins. Genome consists of ten dsRNA segments that are clustered into three groups: L (large) genes, the M (medium) genes, and the S (small) genes that encode eight different structural proteins. Virus particles have all of the eight structural proteins. ISVP particles have σ3 and μ1 cleaved. δ and ϕ are cleavage products of μ1 generated during proteolysis of virions and are present in ISVPs only (Nibert and Fields, 1992). Core particles have only five structural proteins (λ1, λ2, λ3, μ2, σ2).

Table 1.4. dsRNA genome sequences and proteins of mammalian reovirus 3 (MRV-3):

dsRNA (size, bp)	ORFs(bp inclusive)	Proteins (proteins structure/function)	Protein size aa (kDa)	protein copy number per particle	Location	Protein function
L1(3854)	19-3819	λ3 (Pol)	1267 (142)	12	Core	Fully conservative RNA dependent RNA polymerase
L2(3916)	14-3880	λ2(CaP)	1289 (144)	60	Core spike	Guanylytransferase, methytransferase "turret" protein. Comparable to the cypovirus turret protein VP3 (CaP).Virus species specific antigen
L3 (3901)	14-3838	λ1 (Hel)	1275 (143)	120	Core	Inner capsid structural protein, binds dsRNA and zinc, putative NTPase, helicase and 5' triphophase phosphohydrolase
M1 (2304)	14-2221	μ2	736 (83)	12	Core	NTpase, influences the morphology of inclusion bodies, interacts with cytoskeleton
M2 (2203)	30-2153	μ1	708 (76)	30	Outer capsid	Multimerizes with σ3, CLeaved to μ1C and μ1 N, which assume T=13 symmetry in the outer capsid
		μ1C (T13)	667 (72)	600		μ1C is cleaved to δ and ϕ during the entry process. Myristoylated N-terminus, membrance penetration
		δ	539 (59)			
		ϕ	128 (13)			
		μ1N	42 (4)	600		
M3 (2241)	19-2181	μNS	721 (80)	0	N/S	Binds ssRNA and virus core, primary determinant inclusion body formation, interacts with μ2 and σNS, Phosphoprotein, coiled coil motifs, trannscriptase interaction, genome packaging?
		μNSC	681 (75)	0	N/S	μ1NSC is from alternate translation start size, unknown function
S1 (1416)	13-1377	σ1	455 (49)	36	Outer capsid	Cell attachment protein, homo-trimer, haemagglutinin, type-specific antigen, possible glycosyl hydolase activity, induces apoptosis, Interacts with neutralizing antibodies, induce cytotoxic T-cell response.
	71-430	σ1s	120 (16)	0	N/S	Basic protein, nonessential, blocks cell cycle progression, Induce cytotoxic T-cell response.
S2 (1331)	19-1272	σ2	418 (47)	150	Core	Inner capsid structural protein, weak dsRNA- binding, morphogenesis?
S3 (1198)	28-1125	σNS	366 (41)	0	N/S	ssRNA-binding, associates with μNS during inclusion body formation, genome packaging?
S4 (1196)	33-1127	σ3	365 (41)	600	Outer capsid	dsRNA-binding, multimerizes with μ1C, nuclear and cytoplasmic localization, translation control. Virus species specific antigen.

Copied from Mertens, et al. 2000 with updates by Roy Duncan, Jim Chappell and Terry S. Dermody.

Table 1.5. Nucleotide numbers of UTRs of the genome of mammalian reovirus:

		S1	S2	S3	S4	M1	M2	M3	L1	L2	L3
T3D	5`	12	18	27	32	13	29	18	24	13	13
T3D	3`	36	56	70	66	80	47	57	32	33	60
T1L	5`	13	18	27	31	13	29	18	18	12	13
T1L	3`	37	56	70	66	80	47	57	32	33	60
T2J	5`	13	18	27	32	13	29	18	18	12	13
T2J	3`	38	56	70	66	73	47	56	32	33	60

Summary of sequences from GenBank.

into the template channel nearby could ensure association of a single polymerase molecule with each segment of viral plus-strand RNA (Starnes and Joklik, 1993). The degree of the sequence conservation and the specific characteristics of these UTRs suggest that the UTRs probably contain important regulatory elements that determine the fate of nascent viral RNA and virus replication.

Like other viruses of *Reoviridae*, MRV has the specific pattern of replication different from other RNA viruses that enter the cytoplasm; the 10 segments of viral genome remain packaged with the core throughout the replication cycle. The newly synthesized viral mRNA is capped and methylated within the core, is extruded into the cytoplasm as template to translate viral proteins. The viral mRNA then assembles with newly synthesized viral proteins to form nascent virus particles and viral dsRNA synthesis is carried out in these particles.

1.3.1.2. Proteins:

Reovirus encoded proteins can be divided into two types: non-structural proteins and structural proteins (Table **1.4** and Figure **1.1b**.). There are 8 structural proteins in mature reovirus virions that have specific functions. The outer capsid proteins appear to be very important in determining the susceptibility of viruses to inactivation by a variety of physical and chemical agents. For example, the σ3 outer capsid protein determines the relative susceptibility of different reovirus strains to sodium dodecyl sulfate (SDS) and high temperature, and the μ1 outer capsid protein determines the sensitivity to phenol and ethanol (Drayna and Fields, 1982a, and 1982b). T3D mutants that are 100- to 1000-fold less susceptible than wild-type virus to inactivation by ethanol contain point mutations in the amino acids 425 to 459 region of μ1 protein (Wessner and Fields, 1993). The σ1 outer capsid protein has several functions such as determining the serotype of reovirus, serving as the viral hemagglutinin and attaching to the host cells. The 5 core proteins (λ1, λ2, λ3, μ2 and σ2) are mainly responsible for viral transcription and replication, and construct the inner capsid structure and prevent the viral genome and enzyme from digestion by host cellular enzymes. Examination of proteins in reovirus-infected cells also revealed two other virally encoded proteins that are not present in mature viruses; these two viral proteins are called nonstructural proteins and named μNS and σNS (Zwerink et al., 1971). A smaller form of μNS, μNSC is also found in infected cells (Lee

et al., 1981). There is another nonstructural protein σ1s identified by nucleotide sequencing and in vitro translation of viral mRNA that is encoded by the second ORF in the S1 genome segment (Joklik, 1985). All proteins are summarized in Table **1.4** and some of them related with host cell response or some specific functions that are relevant to this thesis are discussed below in detail.

1.3.1.2.1. Reovirus σ3 protein:

The σ3 protein is encoded by the S4 genome segment. The protein consists of 365 amino acids in all of three prototypes (T1L, T2J, T3D) as well as wild isolates of mammalian orthoreoviruses (Figure **1.2**), and has a predicted molecular weight of 41kDa; 600 copies form the bulk of the virion outer capsid together with 600 copies of μ1 in a ratio of 1:1, σ3 also interacts with reoviral protein λ2.

The σ3 protein exhibits a tightly packed structure (Figure.**1.2**); the σ3 monomer is a two-lobed structure, organized around a central helix spanning the length of the protein. The two motifs are rich in basic amino acids which are located in the carboxyl terminal third of the protein and are responsible for the affinity of the protein to double-stranded RNA (dsRNA) (Schiff et al., 1988; Miller and Samuel, 1992; Denzler and Jacobs, 1994; Mabrouk et al., 1995; Wang et al., 1996). The smaller lobe contains ~140 residues in two segments (residues 1-90 and 287-336), and contains a CCHC zinc-binding motif. The zinc site is Cys51 on strand 3 and Cys54 on the turn between strand 3 and 4 coordinate the zinc, together with residues His71 and Cys73. The small lobe also consists of helix A, a four-stranded anti-paralled β-sheet and the N-terminal part of the long central helix B. A loop from the large lobe completes the small lobe with a hairpin of bent helices F and G. The larger lobe contains ~225 residues in two segments (residues 91-286 and 337-365). It includes the C, D and E helices, two-, three-, and four-stranded anti-parallel β-sheet all composed of non-consecutive strands. The C-terminus is nearly at the middle strand of three-stranded sheet (Olland, et al. 2001). The amino-terminal third of the protein encompasses a zinc finger domain that is required for the intracellular stability of the protein; mutational analysis shows that loss of the zinc-binding site results not only in decreased intracellular stability, but also in loss of the ability of σ3 to interact with μ1 (Mabrouk and Lemay, 1994a; Shepard et al., 1996). Loss of zinc does not appear to affect dsRNA binding (Shepard et al., 1996). The multiple copies of σ3 molecules are organized

Figure 1.2.

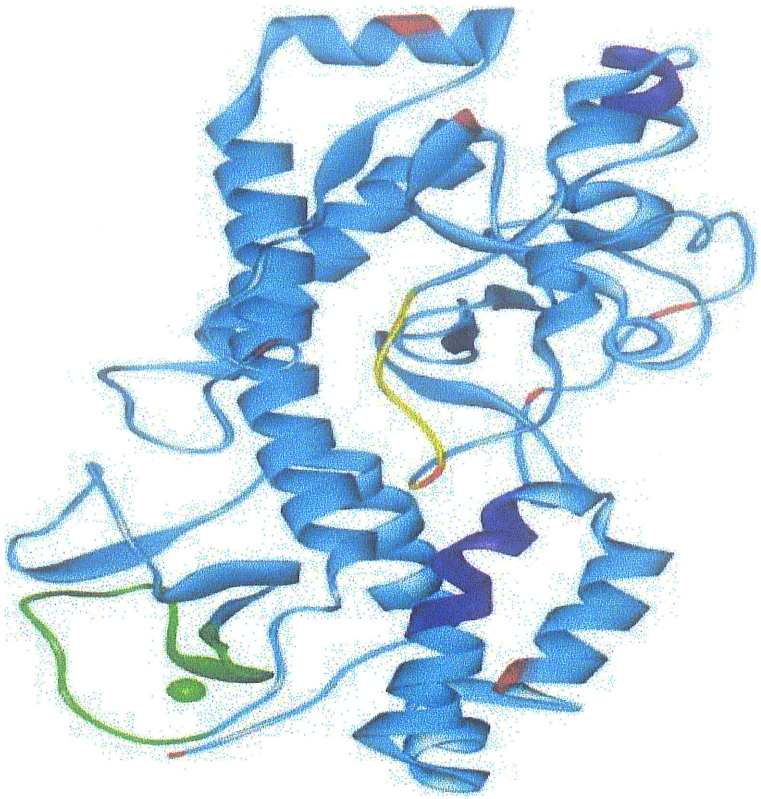


Figure 1.2. The crystallized structure of reovirus outer capsid and dsRNA-binding protein σ3. The folded structure of the reovirus σ3 polypeptide chain. β-strands are represented by green ribbons with arrowheads, α-helices by blue coiled ribbons and connecting loops by thin orange tubes. The zinc atom is represented by red points. Copied from Olland et al., 2001.

in flower-like structures exposed at the virion's outermost surface (Metcalf et al., 1991; Dryden et al., 1993; Centonze et al., 1995). Mutagenesis studies previously showed that the amino-terminal domain is involved in σ3–μ1 interaction (Mabrouk and Lemay, 1994; Shepard et al., 1996; Bergero et al., 1998). Using the monoclonal antibody specific to reovirus σ3 protein to bind reoviruses can induce identical conformation changes in outer-capsid proteins σ3 and μ1 and result in inhibiting σ1-mediated hemagglutination by steric hindrance (Nason et al., 2001).

During viral disassembly in cellular endosomes, σ3 is removed from virions by acid-dependent proteolysis (Sturzenbecker et al., 1987; Baer and Dermody, 1997). Removal of σ3 during viral disassembly also is to allow a change in the conformation of σ1 to a more extended form (Nibert et al., 1995; Nason et al., 2001). Mutations in T3D σ3 determine the sensitivity of virions to proteolysis by the intestinal protease chymotrypsin (Wetzel et al., 1997) and the endocytic protease cathepsin L (Ebert et al., 2001). Reovirus variants are isolated from persistently infected L-cell cultures treated with either ammonium chloride (Dermody et al., 1993, Wetzel et al., 1997), or E64 that inhibits virion replication and not ISVP replication (Baer and Dermody, 1997). By reassortant experiments with reovirus T1L, the E64-adapted viruses have mutations of the S4 gene encoding σ3. The mutation of σ3 contains one or two amino acid substitutions and a mutation at amino acid 354 (Y354H) is identical to mutations found in reovirus mutants selected during persistent infection. The sequences in the σ3 carboxy terminus influence the susceptibility of reovirus virions to proteolysis by endocytic proteases, and the region of σ3 including amino acid 354 play a key regulatory role in removal of the reovirus outer capsid during viral entry (Wilson et al., 2002). The σ3 recoated ISVPs (rcISVPs) retain full infectivity in murine L cells and behave identically to virions prior to exponential growth and in being inhibited from entering cells by either the weak base NH_4Cl or E64 (Jane-Valbuena et al., 1999).

The susceptibility of σ3 to proteolytic cleavage affects virion stability and initial uncoating. The central domain of amino acid region 208–214, in the serotype 3 Dearing protein, is apparently most sensitive to the action of proteases (Schiff et al., 1988; Miller and Samuel, 1992; Schiff, 1998; Jané-Valbuena et al., 2002); cleavage in this region separates the amino-terminal zinc-binding domain from the carboxyl-terminal dsRNA-

binding domain (Schiff et al., 1988; Miller and Samuel, 1992). Many different proteases, including alkaline and acid proteases, cleave first within a hypersensitive region between residue 210 and 242 with prototype difference. The T1L σ3 is first cut mainly at residues 238 to 244; but T3D is cut between residues 208 to 214. Cleavage also appears at different rates with reovirus strains, and cleavage of T1L σ3 is faster T3D σ3. Further site-directed mutation of recombinant σ3 identifies the carboxy-proximal residues 344, 347 and 355 as the primary determinants of these strain differences (Jane-Valbuena, et al. 2002). Time-course digestions of reovirus T1L particles with trypsin, Glu-C, pepsin and chymotrypsin result in the initial generation of two peptides analyzed with mass-spectrometry (Mendez et al., 2003). Most tested proteases can digest σ3 within a hypersensitive region between amino acids 217 and 238. Trypsin digestion initially releases a peptide of the C-terminus of μ1C, followed by a peptide of amino acids 214-236 of the σ3. Other regions of μ1C are not digested until the σ3 protein is completely cut. Similar experiments with Glu-C indicate the hypersensitive region of σ3 is cut first when virions are treated at pH of 4.5 or 7.4, but treatment of virions with pepsin at pH 3.0 releases different σ3 fragments. These results suggest acid-induced conformational changes in the outer-capsid σ3 protein. The conformational change of σ3 may be important for activation of the viral enzymes during replication (Mendez et al., 2003).

1.3.1.2.2. Reovirus cell attachment protein (σ1).

The σ1 protein is encoded by the S1 gene and consists of about 420-470 amino acids with predicted molecular weight between 49-51kd depending upon different reovirus strains. In general, the σ1 protein consists of an elongated fibrous domain-the tail-that inserts into the virion and virion-distal globular domain-the head (Banerjea et al., 1988; Furtong et al., 1988; Fraser et al., 1990) (Figure **1.3**). Sequence analysis and structural modeling have suggested that the N-terminal half of the tail is formed from a α- helical coiled coil (Bassed-Duby et al., 1985; Duncan et al.,1990; Nibert et al., 1990) and the C-terminal half is formed from a triple β-spiral (Chappell et al., 2002; Stehle and Dermody, 2003). A crystal structure of the C-terminal half of reovirus T3D σ1 revealed that the head contains three β-barrel domains (one from each trimer), each of which is constructed with eight antiparallel β-strands (Chappell et al., 2002).

Figure 1.3.

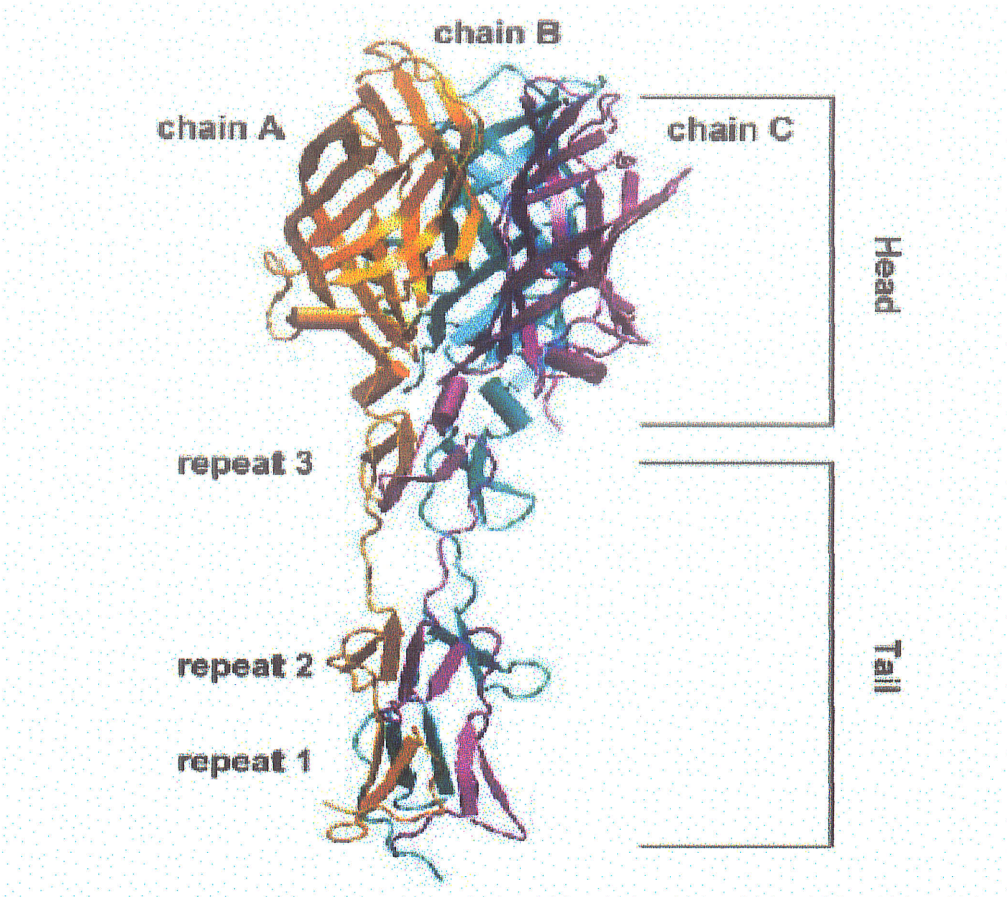


Figure 1.3. The crystallized structure of reovirus attachment protein σ1. Monomers A, B, and C of σ1 are shown in orange, cyan, and purple, respectively. Each monomer consists of a compact head domain and a fibrous tail. The different protein regions are annotated.

Copied from Cavalli et al., 2004.

Attachment to specific host cell molecules is the initial step in virus infection and plays a key role in target-cell selection in the infected host. Generally, reovirus infections are initiated by the binding of viral attachment protein σ1 to receptors on the surface of host cells. Sequence polymorphisms in reovirus attachment protein σ1 play an important role in determining sites of reovirus infection in the infected host (Weiner et al., 1977; 1980; Kaye et al., 1986; Barton et al., 2001). The pathogenesis of reovirus infection has been studied using newborn mice and serotype-specific patterns of disease have also been identified (Tyler, 2001). One of the best characterized of these models is reovirus pathogenesis in the murine central nervous system (CNS), in which type 1 reovirus strains spread by hemotogenous routes to the CNS where they infect ependymal cells, leading to nonietha and hydrocephalus (Weiner et al., 1977; 1980; Tyler et al., 1986). In contrast, type 3 reoviruses spread primary by neural routes to the CNS and infect neurons, causing fetal encephalitis (Weiner et al 1977; 1980; Tyler et al., 1986; Morrison et al., 1991). These distinct patterns of disease segregate genetically with the viral attachment protein (σ1) (Weiner et al., 1977; 1980; Tyler et al., 1986). The protein has several important functions, including that σ1 protein determines the phenotype of reovirus strains. T3 σ1 protein triggers signaling events that lead to apoptosis of infected cells. The interaction of σ1 head with distinct T1 and T3 receptors determines route of spread, tissue tropism, and resultant diseases (Weiner et al., 1977; 1980; Kaye et al., 1986; Barton et al., 2001) and induces cell apoptotic death (Clarke and Tyler, 2005; Clarke et al., 2005).

There are two distinct receptor-binding regions in both the tail and head regions of T3D σ1. A region in the fibrous tail domain binds to α-linked sialic acid (Dermody et al., 1990; Chappell et al., 1997; 2000; Barton et al, 2001), whereas a domain in the head binds junctional adhesion molecule 1 (JAM1) (Barton et al., 2001). A distinct region in the type 1 σ1 tail domain also binds to cell surface carbohydrates (Chappell et al., 2000), and some evidence suggests that the sialic acid may be also involved in the binding of T1L to intestinal cells (Heleander et al., 2003). Truncated forms of σ1 protein (forming only the head domain) are capable of specific cell interaction (Duncan et al., 1991; Duncan and Lee, 1994), and the head is responsible for the binding reaction. Proteolysis of T3D virion leads to release of a C-terminal receptor-binding fragment of the σ1

protein (residues 246-455) (Chappell et al., 1998) and loss of infectivity (Nibert et al., 1995). An expression-cloning approach was used to identify junctional adhesion molecule A (JAM-A) as a receptor for the prototype strains T1L and T3D (Barton et al., 2001). 13 reovirus strains including 5 type 1, 3 type 2 and 5 type 3 were used to determine whether JAM-A is used as a receptor recently, and the results were very interesting; each of the prototype and field-isolate reovirus strains is capable of utilizing JAM-A as a receptor regardless of their serotype, species, or geographical region of isolation (Campbell et al., 2005). Further experiments show that the digested fragment of σ1 protein can bind with JAM-A on a biosensor surface. Sequencing data of different reovirus serotype σ1 proteins show that the conserved residues are scattered throughout the molecule; examination of the σ1 protein surface reveals there is a single extended patch of conserved residues at the lower edge of the protein head. The special structure is mostly the residues in the vicinity of a long loop connecting β-strands D and E of eight-stranded β-barrel. Only the residues in the single monomer contribute to the putative JAM-A binding region and its borders; and they are not involved in σ1 intersubunit contacts. The location of conserved residues indicates that each σ1 monomer can independently bind to a JAM-A molecule. This strategy of cell attachment is strikingly similar to that used by adenovirus fiber to bind to the coxsackievirus and adenovirus receptor (CAR) (Bewley et al., 1999; van Raaij et al., 2000), an immunoglobulin superfamily member that shares considerable structural homology with JAM-A (Stehle and Dermody, 2004).

The capacity of T3 reovirus to bind sialic acid can determine the cell type of infection in cell culture. Both T1 and T3 reoviruses can grow in L929 cells, but only T3 reoviruses can replicate in murine erythroleukemia (MEL) cell, and this restriction is decided by sialic acid molecules (Robin et al., 1992; Chappell et al., 1997). Serial passage of non-sialic acid binding T3 strains in these cells results in selection of viruses which acquire the ability to bind sialic acid (Chappell et al., 1997). Gene sequence analysis showed that the prototype is conferred by single point mutation (Chappell et al., 2000). HeLa cells are permissive for reovirus T3SA+(strong apoptotic) and T3SA- (minus apoptotic) infection; however, virus yields from a single cycle are significantly higher for T3SA+ when Hela cells are infected with equivalent multiplicities of infection of these strains (Barton et al.,

2001). This enhanced growth is sialic acid dependent and removal of cell surface sialic acid by enzymes significantly decreases the yields of T3SA+ to the same level as the yield of T3SA- (Barton et al., 2001). The capacity of reovirus binding to sialic acid also influences tissue tropism and disease phenotypes. Both T3SA+ and T3SA- grow well in the intestine following peroral incubation of newborn mice and disseminate to distant sites, however, T3SA+ produces higher titers in the brain and liver at early times after infection than those produced by T3SA- (Barton et al., 2003).

A new murine IgA monoclonal antibody (MAb), 1E1, was recently raised against reovirus T1L and binds to T1L σ1 (Hutchings et al., 2003). This anti-σ1 IgA prevents infection at an early stage, most likely by blocking the σ1-mediated adherence of viral particles to intestinal M cells. 1E1 also neutralizes T1L infection of cultured L929 cells in vitro, blocks viral adherence to apical membranes of polarized Caco-2 cells (Hutchings et al., 2003). Another IgG MAb 5C6, binding to the σ1 proteins of type 1 isolates, recognizes the σ1 head domain (Chappell et al., 2000). 5C6 blocks T1L binding and uptake in several cultured non-epithelial cell systems (Verdin et al., 1988; 1989; Barton et al., 2001), when introduced into the blood circulation of mice either by intravenous injection (Tyler et al., 1993) or from subcutaneous hybridoma tumors (Hutchings et al., 2003). When 5C6 is administered perorally to mice along with T1L, it is capable of preventing Peyer's patch infection (Silvey et al., 2001). Thus, IgG MAb 5C6 and IgA MAb 1E1 share certain protective capacities. However, their respective binding sites on σ1 could be distinct because induction of serum IgG and secretory IgA occur in different immune-sampling environments, function in different compartments, and may protect by different specific mechanisms.

1.3.2. Virus morphology

Reoviruses exist in three forms: the mature virion, intermediate (or infectious) subviral particle (ISVP) and core (Figure **1.1a**), which differ in morphology, protein composition, and physicochemical and biological properties (Table **1.6**).

The mature virions are the predominant form released from infected cell cultures or infected animals or after artificial disruption of infected cells in culture; and consist of all eight structural proteins that comprise two radially concentric, separable protein shells

Table 1.6. Structural composition of three types of reoviral particles:

	Virion	ISVP	Core
Diameter (nm)			
Negative-stain EM	73	64	51
Cryoelectron microscopy	85	80	65
Comparison			
(dsRNAs)	10	10	10
Proteins	8 (λ1, λ2, λ3, μ1, μ2, σ1, σ2, σ3)	7 (λ1, λ2, λ3, μ1 cleave δ, ϕ μ2, σ1, σ2)	5 (λ1, λ2, λ3 μ2, σ2)
Function			
infection	Yes	Yes	No
replication	Yes	Yes	No
transcription	No	No	Yes

Modified from Nibert and Schiff, 2001.

(inner and outer capsid) (Dales et al., 1965; Mayor et al., 1965; Luftig et al., 1972). The viruses are about 85nm in diameter and have T=13 icosahedral symmetry shown by cryoelectron microscopic analysis (Metcalf et al., 1991; Dryden et al., 1993); and x-ray diffraction (Harvey et al., 1981). The icosahedral symmetry is formed with each of the two majorstructural proteins of the outer caspid. Two of the proteins in mammalian reoviruses are 600 copies of the protein σ3 and μ1 (and its cleavage product μ1C, the major form of this protein in mature virions) (Dryden et al., 1993), and the third outer capsid protein is 36 copies of the σ1 cell attachment protein (Strong et al., 1991; Coombs, 1998a). The outer capsid functions as a gene delivery system, containing proteins responsible for allowing the virus to recognize and bind to appropriate host cells.

Infectious sub-virus particle (ISVP) is about 80nm in diameter and lacks the σ3 protein. The μ1C protein also has undergone cleavage (to generate 2 peptides called δ and ϕ). In addition, the σ1 proteins undergo different changes in different prototype strains of mammalian reovirus. In T1L, the σ1 protein trimers take on an extended "Lollipop" conformation, with the amino-terminal fibrous coiled coil portion tethered inside the λ2 spike channels and the carboxyl-terminal globular head extended as much as 40nm from the surface. This extended conformation is thought to increase accessibility of the cell attachment protein and hence increase infectivity of the virion. The σ1 proteins of T3D are cleaved by proteolysis and can not be observed by EM (Nibert et al., 1995). The ISVP may be formed by intestinal proteases such as pepsin, chymotrypsin or trypsin (Bass et al., 1990; Amerongen et al., 1994). The ISVP possibly facilitates the viral particle attaching to the host cell during infection.

The cores (the innermost capsid) consist of five structural proteins. These are known as λ1, λ2, λ3, μ2, and σ2. The lattice of inner capsid is made up of 120 copies of λ1. The λ1 proteins are associated as 60 assymetric dimers to form a thin T=1 symmetry shell surrounding the viral genome RNA. 150 globular monomers of σ2 proteins decorate the outside of the λ1 shell and stabilize it. Pentamers of the λ2 proteins sit on top of the λ1 shell at each of the 12 icosahedral 5-fold axes, forming prominent spike structures that extend outward from the about 60nm diameter core shell by an additional 9nm and the λ2 pentamers have 8nm-wide channels running through them (Coombs, 1998b; Reinisch et

al., 2000). Protein λ3, the RNA-dependent RNA polymerase (RdRp), is associated with one or two copies of μ2, the alleged polymerase cofactor (Yin et al., 1996; Noble and Nibert, 1997; Hermann and Coombs, 2004), which are thought to underlie the core shell at the vertices (Dryden et al., 1998). This core complex (all 5 proteins + all 10 dsRNA genes) serves as the transcriptionally active metabolic machine that produces mRNA during viral replication. By transfection, the cores are confirmed to replicate in several cells and produce very high titer of infectious viruses (Jiang and Coombs, 2005).

There are also several other forms of reovirus particles observed in experiments, which may lack viral genome or proteins and account for a large proportion of the particles purified from reovirus-infected cells and a particle-to-pfu ratio of from 50:1 to 500:1 (Joklik, 1983; Epstein et al., 1984). One type of these particles is "empty" virions with decreased amounts of dsRNA genome and oligonucleotides (Rhim et al., 1961; Smith et al., 1969). Core-like particles were obtained from infections with temperature-sensitive (ts) group B and G mutants at nonpermissive temperature (Morgan and Zweerink, 1974). The particles containing core proteins λ1, λ3, and σ2, but lacking other core components, were the larger parts of the progeny in the cells infected with one L2-segment ts mutant at nonpermissive temperature (Hazelton and Coombs, 1999).

The purified ISVPs and cores generated from virions by in vitro protease digestion have served as platforms for research of reovirus assembly and the functions of the viral proteins, such as the binding to ISVPs of σ3 proteins expressed by baculovirus to yield recoated particles that are very similar to virions in structural and biological properties (June-Valbaena et al,. 1999). The core recoating approaches have also been performed to address the functions of the viral proteins expressed by recombinant μ1, σ3, or σ1 in vitro (Chappell et al., 1998; Faretta et al., 2000).

1.3.3. Reovirus Life cycle

The replication of reovirus, just like all viruses, must rely on the host cell to supply the appropriate place and material because a single virus particle (virion) is in and of itself essentially inert and lacks needed components that cells have to produce. Viruses are intracellular obligate parasites; they cannot reproduce or express their genes without the help of a living cell. Reovirus is the prototypical virus of the dsRNA viruses and infects a

wide range of cells, in vivo and in vitro. The replication of the virus has some specific characteristics different from other (+/-) RNA viruses or DNA viruses. These include that the viral genomes are 10 segments of dsRNAs; viruses have the enzymes such as RNA-dependent RNA polymerases (RdRps) and cofactors, and methyltransferase for transcription and replication of the viral genes; transcription and replication occurs in the viral core rather than naked in the cytoplasm; and two phases of transcription (primary transcription and secondary transcription) may occur within infected cells. The virus life cycle is shown in Figure **1.3**.

1.3.3.1. Entry into cells. Reoviruses infect their animal hosts via respiratory and enteric routes. The first step is reovirus must enter the cells. Binding of reoviruses to the surface of different cells in vitro or in vivo is by the viral proteins attaching to surface receptors of host cells. The cell-attachment protein, σ1, is responsible for the binding (Choi et al., 1990; Lee et al., 1981; Paul and Lee, 1987; Strong et al., 1993). 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). The sialic acid on cell surface proteins is one of the cell receptors for reovirus binding and critically important for infection (Paul and Lee, 1987; Paul et al., 1989). There are other cell receptors such as 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). Some reoviruses use carbohydrate-based co-receptors for cellular attachment (Chappell et al., 2000). The junction adhesion molecule 1(JAM 1), an integral tight junction protein, has been identified as a reovirus receptor (Barton et al., 2001; Forrest et al., 2003). An expression-cloning approach was used to identify junctional adhesion molecule A (JAM-A) as a receptor for the prototype T1L and T3D and further tests prove that JAM-A serves as a receptor for prototype and field-isolate strains of mammalian reovirus (Campbell et al., 2005). The σ1 protein undergoes a dramatic conformational change from a retracted to an elongated form during viral disassembly (Furlong et al., 1988; Dryden et al., 1993; Nibert et al., 1995). Recently, a molecular dynamics study of reovirus attachment protein σ1 reveals conformational changes in structure, with three

Figure 1.4.

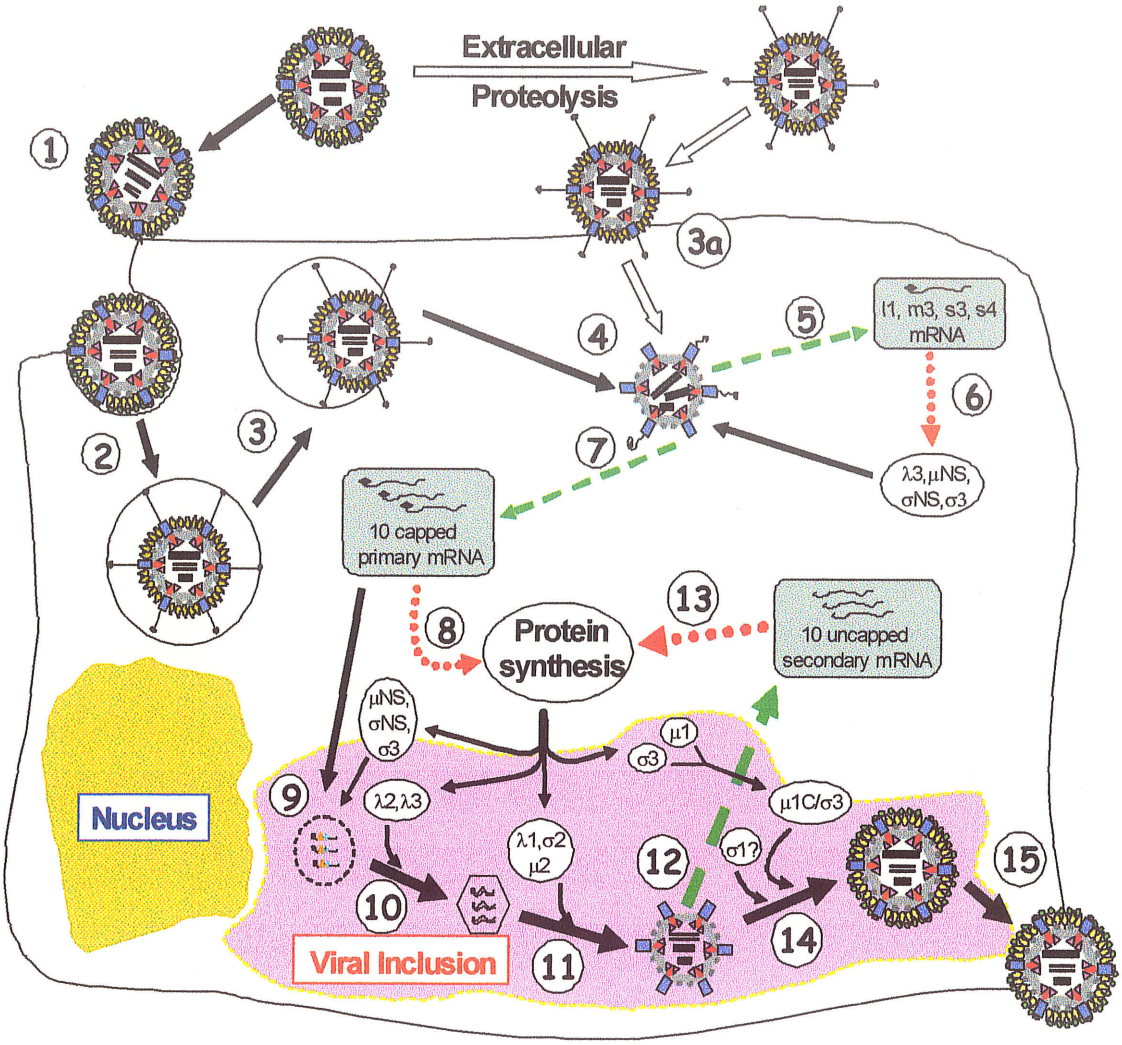

Extracellular
Proteolysis
1
2
3
3a
4
5
6
7
l1, m3, s3, s4 mRNA
λ3,μNS, σNS,σ3
10 capped primary mRNA
8
Protein synthesis
13
10 uncapped secondary mRNA
μNS, σNS, σ3
μ1
σ3
μ1C/σ3
Nucleus
9
λ2,λ3
λ1,σ2 μ2
σ1?
12
10
Viral Inclusion
11
14
15

Figure 1.4. Reovirus life cycle. The cartoon represents the replication cycle of a reovirus particle within a host cell. A brief description follows as; **(1)** the first step in reovirus replication is *attachment* of the virion to a cell surface; **(2)** following internalization by *receptor-mediated endocytosis*, reovirus particles enter into the cell; **(3)** removal of the outer capsid in the endocytic compartment, the particles undergo specific acid-dependent *proteolytic cleavages* and become ISVPs. The ISVPs generated by *Extracellular proteolysis* in intestinal conditions may directly cross the cellular membrane into the cytosol; **(4)** once in the cytosol of the cell, the ISVPs are likely further processed to become *the core particles*; **(5)** the transcriptional machinery in the cores begin *Primary transcription* and synthesize some capped viral mRNAs; **(6)** cellular ribosomes *translate viral proteins* according to the viral mRNA templates and the viral proteins are added to viral cores; **(7)** the viral cores produce 10 capped primary mRNA; **(8)** cell ribosomes syntheze viral proteins according to the 10 capped primary mRNA as the templates; **(9)** the μNS, σNS and σ2 as well as some cellular proteins construct into viral factories (*viral inclusions*); **(10)** the viral mRNA (+RNA) and primary translated viral proteins form *new replicase complexes*; **(11)** viral RdRps *synthesize minus-strand RNA* from the viral mRNAs as templates in these complexes, resulting in nascent genomic dsRNA; **(12)** the *secondary transcription* occurs in the newly synthesized viral cores; the majority of the viral mRNA (non-capped) transcripts are synthesized; **(13)** more viral proteins synthesized; **(14)** the outer-capsid proteins are added to progeny particle *(virus assembly)*, and silence transcription and complete virion morphogenesis; **(15)** Mature virions are *released* from infected cells via cell lysis (Coombs, 2002).

polar residues (Ser291, Thr292, and Ser293) located within an insertion between the second and third β-spiral repeats of the crystallized portion of the σ1 tail interacting with water molecules of the solvent. This is responsible for an oscillating movement of the head of 50° per 5 ns; this flexibility may facilitate viral attachment and also function in cell entry and disassembly (Cavalli et al., 2004). But, it will still be important to determine whether attachment to these potential receptors, alone or in combination, leads to virus uptake and a productive infection. It is unknown now whether interactions of cell surface proteins with other reovirus proteins occur and how they interact. Differences in the attachment process for virions and ISVPs also remain to be determined.

When reovirus particles attach to a cellular receptor, the viral capsid undergoes a conformational change. The cell-bound virions are more resistant to pepsin digestion than unbound virions (Fernandes et al., 1994). 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; 1981; Burstin et al., 1982; Sturzenbecker et al., 1987). Acidic proteases degrade outer capsid σ3 protein and convert μ1C protein to its stable cleavage product to generate ISVP-like particles (Borsa et al., 1981; Chang and Zweerink, 1971; Nibert and Fields, 1992; Stuzenbecker et al., 1987). It remains to be determined whether penetration through the plasma membrane by extracellular generated ISVPs occurs routinely.

1.3.3.2. Transcription and replication. After entering the cytoplasm, virions or ISVPs are digested into cores. Core particles in the cytosol are transcriptionally active and contain all activities necessary to produce capped methylated mRNAs (Furuichi et al., 1976). 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. 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; Yamakawa et al., 1981; 1982; Zarbl et al., 1980a). The λ3 core protein combined with μ2 core protein are the RNA-dependent-RNA-polymerase (Drayna and Fields, 1982a; Koonim et al., 1989; Morozov, 1989; Starnes and Joklik, 1993) and outative

putative polymerase cofactor (Wiener et al., 1989; Yin et al., 1996) respectively and are 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 (pi) (Zweerink and Joklik, 1970). The nascent mRNAs are translated by host cellular ribosomes to generate viral proteins, which associate with primary transcripts (mRNAs) to form replicase complexes. At this moment, 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). Assortment likely involves signals that distinguish between viral and cellular RNA as well as signals that identify each of the 10 segments as unique. The plus strand of each genomic segment contains a 5' cap identical to the cap on mRNAs made by primary transcription (Furuichi et al., 1975), thus the cap might serve as the bonding sites for RdRps and a packaging signal. Once mRNA transcripts and viral translated proteins are packaged to form new viral core-like structure, the viral *genome replication* can be carried out; each plus-strand serves as template for synthesis of the minus-strand, and regenerates genomic dsRNA (Acs et at., 1971; Sakuma and Watanabe, 1971; 1972; Schonberg et al., 1971; 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. These particles may contain a latent capping enzyme, and produce uncapped viral mRNAs (Skup and Millward, 1980; Zarbl et al., 1980b). These uncapped mRNA may be translated preferentially late in infection. Secondary transcription is the source of 95% of all transcripts formed throughout infection (Ito and Joklik, 1972) and also serve as template for translation into proteins. It is possible that the synthesis of viral proteins is sequentially controlled because large

amounts of viral outer capsid proteins σ3 and μ1 are translated when viral replicating complexes reach a high level during later periods of reovirus life cycle.

1.3.3.3. Assembly and release. 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 other viral proteins in this step. 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 these proteins come together to form nascent infectious particles still remains to be determined. A system for recoating reovirus core particles (rcCores) with recombinant outer capsid proteins has provided some evidence that preformed complexes of μ1 and σ3 are required for assembly into particles and do not require participation of σ1 (Chandran et al., 1999; Farsetta et al., 2000). Virion release is after the host cell death and the associated breakdown of the plasma membrane.

Reovirus *ts* mutants are also very useful materials for reovirus life cycle research. For example, Group B *ts* mutants synthesize a normal amount of the λ2 protein, however, they are unable to condense outer capsid proteins onto the core particles to assemble complete particles (Morgan and Zweerink, 1974). Group C *ts* mutants (tsC447; S2 gene mutation) can produce empty outer capsid structures at nonpermissive temperature (Fields et al., 1971; Coombs et al., 1994). Group G *ts* mutants form core-like particles, indicating interactions between σ3 and μ1 are required for condensation of the outer capsid proteins onto nascent cores (Shing and Coombs, 1996). Mutant *ts*H11.2 produces ssRNA and normal levels of proteins at early time points, it is defective for dsRNA and viral proteins at later time points (Coombs, 1996). These *ts* mutants, having different characteristics in synthesis of their genome or proteins, show advantages for understanding the functions of reovirus genes/proteins on replication.

1.3.3.4. Reovirus inclusions

In the process of reovirus replication, especially late in infection, the majority of the cytoplasm of infected cells consists of specific structures (viral inclusions) filled with progeny virions (Fields et al., 1971). Reovirus inclusions have been studied by using a variety of microscopic techniques and first appear by phase-contrast microscopy as dense granules scattered throughout the cytoplasm. As infection progresses, these granules coalesce and localize about the nucleus, eventually forming pernuclear inclusions (Fields et al., 1971). The viral inclusion contains several types of filaments (Sharpe et al., 1982), dsRNA (Silverstein et al., 1970), viral proteins (Fields et al., 1971), and complete and incomplete viral particles (Fields et al., 1971). The "kinky" filaments are proposed to be intermediate filaments but not membrane-bound structures or ribosomes. The inclusions have a peculiarly dense consistency that distinguishes them from the adjacent cytoplasm and causes them to appear highly refractile by phase-contrast microscopy. Mammalian reovirus strains exhibit differences in viral inclusion morphology (Mbisa et al., 2000; Broering et al., 2002; Parker et al., 2002). Strain T1L forms filamentous inclusions, whereas strain T3D forms punctate or globular inclusions. This difference in inclusion morphology segregates with the M1 gene (encoding viral structural protein μ2) (Parker et al., 2002). The T1L μ2 protein associates with microtubules, but T3D μ2 does not have the function (Broering et al., 2002; Parker et al., 2002).

Antczak and Joklik (1992) used monoclonal antibodies directed against reovirus proteins to detect reovirus genome segment assortmant. Three reovirus proteins (nonstructural proteins σNS and μNS and structural protein σ3) were the earliest detectable viral protein-RNA complexes in early steps of viral genome replication in the infected cells. Immunofluorescence staining indicates that the inclusion structures contain both structural and non-structural proteins, the exact compositions of which change with time (Becker et al., 2001). Large cytoplasmic complexes of viral proteins can also be formed with some temperature-sensitive (*ts*) mutant viruses in the infected cells at non-permissive temperature (Mbisa et al., 2000; Becker et al., 2001). However, the inclusions of *ts* mutants are not functional and do not yield viable progeny because of alterations in the activity of the mutant viral proteins.

A single reovirus protein, μNS, is sufficient for forming phase-dense globular inclusions in the cytoplasm of transfected cells (Broering et al., 2002; Becker et al.,

2003). The inclusions formed by μNS in experiments are notably similar in appearance to globular reovirus factories formed in infected cells, as visualized by either phase-contrast or immunofluorescence (IF) microscopy (Broering et al., 2002; Parker et al., 2002; Becker et al., 2003), suggesting that μNS forms the matrix of the factories (Broering et al., 2002). Moreover, μNS can associate with several other reovirus proteins and recruit them to these inclusions in transfected cells. To date, the viral proteins that have been reported to be recruited to inclusions formed by reovirus μNS are microtubule-binding core protein μ2 (Broering et al., 2002,); nonstructural and ssRNA-binding protein σNS (Becker et al., 2003; Miller et al., 2003); and the core surface proteins λ1, λ2, and σ2 (Broering et al., 2004). However, the ssRNA-binding activity of σNS is more firmly established (Gillian and Nibert, 1998; Gillian et al., 2000; Gomatos et al., 1981; Richardson and Furuichi, 1985) and may play the more important role in ssRNA recruitment to or retention within the inclusions (Becker et al., 2003, Broering et al., 2004). The studies on the μNS homolog from avian orthoreoviruses have reached conclusions similar to these regarding the roles of MRV μNS in forming the factory matrix and recruiting other viral proteins to the factories (Tuoris-Otero et al., 2004a; 2004b). Recent research proved that carboxyl-terminal one-third of the 721-residue μNS protein is necessary and sufficient for forming viral inclusion (Broering et al., 2005); and deletion of as few as eight residues from the carboxyl terminus of μNS resulted in loss of inclusion formation. The region from 471 to 721 of μNS includes both of two predicted coiled-coil segments in μNS and these specific structures seem important for the function of μNS protein. Deletions of one amino acid in these regions would result in the loss of the function of the protein and mutation of either a His residue (His570) or a Cys residue (Cys572) within these sequences disrupted the phenotype. The His and Cys residues are part of a small consensus motif that is conserved in μNS homologs from avian orthoreoviruses and aquareoviruses, which suggests this motif of the protein may have a common function in these viruses. Therefore, research on the function and formation of the specific structure is still required in order to understand virus replication.

1.4. Cellular response

Reoviruses infect host cells and use the cell condition and material to replicate their progeny. Host cells have evolved strategies and methods to fight against viral invasion. There are many cellular pathways developed that perform differently according to the different cellular, environmental, and stress situations. Sometimes, the pathways may be combined to defend against viral invasion. Some of the pathways will be discussed as follows.

1.4.1. Interferon

Interferon (IFN) is an inducible cytokine, and reovirus infection of animal cells can lead to the induction of IFN. IFN was discovered as an antiviral agent during studies on virus interference (Isaacs and Lindenmann, 1957; Nagano and Kojima, 1958). There are several types of INF discovered and most types of virally infected cells are capable of synthesizing IFN-α/β in cell culture.

Reoviruses can induce infected cells to express interferon (Lai and Joklik, 1973; Henderson and Joklik, 1978; Sharpe and Fields, 1982; Sherry et al., 1998). The IFN inducing capacity of reovirus is dependent both upon the particular reovirus strain and the kind of cell or host animal infected. Reovirus T3D is a better IFN inducer than T1L in mouse fibroblasts (Sharpe and Fields, 1983). With *ts* mutants, IFN induction correlates with yields of infectious virus but not with amounts of viral dsRNA, ssRNA, or protein made in infected cells (Lai and Joklik, 1973). UV-treated reoviruses induce IFN more rapidly than occurs during productive infection. It is possible that UV-treatment destabilizes the viral core so that genomic dsRNA from input particles escapes into the cytoplasm (Henderson and Joklik, 1978). By using myocarditic and non-myocarditic reovirus strains, the levels of viral mRNA may determine the yields of IFN induction and the M1, S2, and L2 genome segments encoding core protein μ2, σ2, and λ2 are responsible for the INF induction (Sherry et al., 1998).

IFNs may inhibit reovirus replication by a variety of mechanisms. Commonly, PKR pathway is the major function. IFN-induced expression of elF-2α kinase, PKR can inhibit viral protein synthesis in some reovirus-infected cells (Gupta et al., 1982; Nilsen et al., 1982; Samual et al., 1984). Cells treated with IFN α/β induce Rnase L (2', 5'-oligo(A)-dependent endoribonuclease) that cleaves reovirus mRNA and performs antiviral function (Bagliomi et al., 1984; Miyamoto et al., 1980: 1983; Nilsen et al., 1982).

Reovirus has also evolved some mechanisms to escape the IFN anti-viral function, such as the σ3 protein can influence induction of IFN by binding viral dsRNA to compete with PKR for dsRNA activator (Imani and Jacobs, 1988; Schiff et al., 1988; Jacobs and Langland, 1998). There are also some reports about antiviral function of IFN on reovirus with different results. IFN-β response does not determine reovirus replication in skeletal muscle cells (Sherry et al., 1998). However, in cardiac fibroblasts and cardiac myocytes, IFN-β can inhibit reovirus replication, and in the absence of reovirus-induced IFN-β, reovirus replicates to higher titers in cardiac fibroblasts than in cardiac myocytes (Stewart et al., 2005). The other experiments (Smith et al., 2005) made by replication of reoviruses in cells lacking PKR and Rnase L indicate that reoviruses contribute to host shutoff; neither PKR nor RNase L exert an antiviral effect on reovirus growth; and some reovirus strains also replicate more efficiently in the presence of PKR and Rnase L than in their absence. There may still be some other pathways for the reaction between reovirus infection and IFN response worth determining.

1.4.2. Reovirus oncolysis

Reovirus oncolytic potential was originally shown back in 1970s, when Hashiro et al., (1977) found reoviruses replicated very well in transformed cells and "normal" non-transformed cells were resistant to virus infection. Later, Duncan et al ,(1978) reported that W1-38 cells not normally favorable to reovirus could be rendered infectable by transformation with the SV-40 large T-antigen. But the mechanism behind this selectivity was not elucidated. First proof –of principle studies for reovirus oncolytic therapy were carried out in human cancer cell lines and a murine tumor model in Dr. Patrick Lee's laboratory (Coffey et al., 1998). They found that reoviruses grew and killed over 80% of the transformed cell lines from different tumor types. The subsequent animal experiments in vivo also confirmed that ~ 80% of human glioma xenografts in severe immunodeficient (SCID) mice regressed after one intratumoral injection of living reovirus. They primarily proved that the reovirus killed tumor cells, but not killed nonproliferating normal cells; and the killing mechanism lies in reovirus usurping the specific signaling pathways (Ras) in tumor cells. Normal mouse fibroblasts (NIH 3T3 cells), resistant to reovirus infection, become susceptible to viral infection following transformation with activated Ras (Strong et al., 1998). Further research indicated that

reovirus replication in Ras-transformed cells depended primary on Ral/GEF signaling. However, the two most well known signaling pathways downstream of Ras, the Raf and the P13-kinase pathways, did not seem to be involved in this process. The cells with activated Ras and specific inhibition of the Ral or p38 pathways but not JNK pathway are resistant to reovirus infection (Norman et al., 2004).

To date several reovirus therapy for different cancers have shown satisfactory results. All studies demonstrate effective in vitro cell killing and/or cytopathic effects by reovirus in the majority of tumor cell lines tested, including colon, ovarian and breast cancer (Hirasawa et al., 2002; Norman et al., 2002). The mouse tumor models using tumor cell line implants in vivo have also been used to test the ability of reovirus oncolysis in vivo (Hirasawa et al., 2002). Wilcox et al demonstrated reovirus induced killing in 83% of malignant gloma cell lines studied and reovirus treatment of orthotopic, intracerebral tumors in nude mice led to significant increases in survival (Wilcox et al., 2001). These treatments also were effective against gliomas in competent immune mice (Yang et al., 2004). Correspondingly, pancreatic cancers are also sensitive to reovirus therapy in pre-clinical testing (Etoh et al., 2003) and also to lymphoid malignant diseases (Alain et al., 2002). Reoviruses are relatively non-toxic to humans and appear to be an ideal cancer killing agent as a novel potential cancer therapeutic, it is valuable to know whether the process of reovirus oncolysis is accompanied by cell apoptosis or necrosis.

1.4.3. Reovirus induced apoptosis

One of the most useful characterizations of reoviruses is the ability of the virus to induce cell apoptotic reaction in wide variety of cultured cells in vitro and in target tissues in vivo, including the heart and CNS (Clarke and Tyler, 2003). Reovirus infection has proven to be an excellent useful experimental model for studies of relation of viral infection and viral pathogenesis with apoptosis (Clarke et al., 2005).

Reovirus induced apoptosis requires participation of many cellular agents through several pathways. Reovirus infection transiently activates NF-κB (Nuclear factor κB) in variety of cells (L929, MDCK, and Hela cells). Inhibition of NK-κB can inhibit reovirus-induced apoptosis (Connolly et al., 2000). Reovirus infection can sensitize cells to TRAIL-mediated apoptosis by mechanism that results in an increase of activation of caspase 8 and 3 and is blocked by the caspase 8 inhibitor IETD-FMK (Clarke et al., 2000,;

2001a). Mitochondrial signaling systems play important roles in the reovirus-induced apoptotic pathways. Rodgers et al., (1997) observed that reovirus induced apoptosis was inhibited in MDCK cells that overexpressed Bcl-2. The cellular proteins in mitochondria such as Smac and cytochrome c are released from the mitochondria after reovirus infection (Kominsky et al., 2000b; 2002a). These mitochondria agents can activate caspase 9 and caspase 7, or caspase 3 to induce cell apoptosis. Reovirus infection results in a viral strain-specific pattern activation of the c-Jun N-terminal kinase (JNK) and the JNK-associated transcription factor c-Jun (Clarke et al., 2001b), and JNK and c-Jun may play important roles in reovirus induced apoptosis by mitochondrial pathways (Clarke et al., 2004). Global analyses of oligonucleotide microarrays were performed to detect the activation of cellular gene expression by reovirus infection (Debiasi et al., 2003; Poggioli et al., 2002). The expression of at least 24 celluler genes are related to apoptosis in cells infected with the apoptosis-inducing reovirus strain T3A (Table.**1.7**).

Reoviruses inducing cell apoptotic change differ in genetics of strain–specific characterization. T3 prototype strains Abney (T3A) and Dearing (T3D) can induce apoptosis more efficiently than the serotype 1 strain Lang (T1L) in cell culture (Tyler et al., 1995). By using reassortant viruses containing genes derived from T3D strain (apoptotic) and T1L strain (minimally apoptotic) to analyzes the function of the viral genes, reovirus S1 gene, that encodes the σ1 protein is responsible for virus to attach to cell receptor and activates cell receptors, is mainly responsible for virus gene inducing apoptosis in L929 (Tyler et al., 1995), MDCK (Rodgers et al., 1997) and Hela cells (Connolly et al., 2001). The M2 gene, that encodes reoviral major outer capsid protein μ1, is also a determinant of apoptosis in L929 and MDCK cells. Apoptosis can be inhibited by using monoclonal antibodies (MAbs) directed against either the μ1, σ1 or σ3 protein. Anti-μ1 and anti-σ3 MAbs, which do not inhibit viral attachment but do prevent virion uncoating, can inhibit apoptosis (Connolly et al., 2001; Rodgers et al., 1997; Tyler et al., 1995; 1996; Virgin et al., 1994).

Reovirus-induced apoptosis occurs in two processes: virus receptor engagement and onset of transcriptional activation. The experiments proved that UV-inactivated T3D viruses could induce apoptosis and inhibition of viral binding could also inhibit apoptosis (Tyler et al., 1995). Treatment with ammonium chloride (endosomal acidification

Table 1.7. Reovirus-induced alteration in expression of genes encoding proteins with known apoptotic involvement

Gene	GenBank accession	Change in expression (n=fold) T3A	T1L
Mitochondrial signaling			
Pin-2 proto-oncogene homolog	U77735	-2.2 +/- 0.1	
Mcl-1	L08246	2.0 +/- 0.0	2.2 +/- 0.0
BAC 15E1-cytochrome c-oxidase polypeptide	AL021546	2.1 +/- 0.0	
Par-4	U763809	2.1 +/- 0.0	
HSP-70 (heat shock protein 70 testis variant)	D85730	2.1 +/- 0.1	
BNIP-1 (Bcl-2 interacting protein)	U15172	2.3 +/- 0.1	
SMN/Btfp44/NAIP (survival motor neuron/ Neuronal apoptosis inhibitor protein)	U80017	2.5 +/- 0.1	
DRAK-2	AB011421	2.8 +/- 0.2	
SIP-1	AF027150	3.0 +/- 0.2	
DIP5	D833699	5.5 +/- 1.1	
Death receptor signaling			
Bcl-10	AJ006288	5.6 +/-1.1	
PML-2	M79463	3.4 +/- 0.3	
Ceramide plucosyltransferase	D50840	4.0 +/- 1.2	
Sp 100	M60618	6.1 +/- 0.5	
ER stress-induced signaling			
ORP150	U65785	-2.4 +/- 0.2	
GADD34	U83981	3.7 +/- 0.2	2.9 +/- 0.2
GADD45	M60974	4.9 +/- 0.1	4.4 +/- 0.1
Proteases			
Calpain	X04366	-2.6 +/- 0.1	
Beta-4 adducin	U43959	-2.1 +/- 0.1	
Caspase 7	U67319	2.6 +/- 0.2	
Caspase 3	U13737	3.2 +/- 0.2	2.8 +/- 0.1
Undefined			
Frizzled related protein	AF056087	-25 +/- 0.1	-3.3 +/- 0.5
TCBP (T cluster binding protein)	D74015	3.3 +/- 0.2	
Cug-BP/hAb50 (RNA binding protein)	U63289	6.6 +/- 1.1	

GenBank accession number corresponds to the sequence from which the Affymetrix microarray U95A probe set was designed. Data are means +/- standard errors of the means.

Copied from (Clarke et al., 2005)

inhibitor) or the protease inhibitor E64 can inhibit reovirus induced apoptosis; and ISVPs retained the capacity to induce apoptosis; which identified that the reovirus-induced apoptosis was also triggered by intracellular process during reovirus replication cycle (Connolly et al., 2002). Treatment of Hela cells with ribavirin, a guanosine nucleoside analog inhibiting the reovirus dsRNA-dependent RNA polymerase, blocked the synthesis of viral single-stranded (ss) and dsRNA (ds), but did not inhibit apoptosis (Connolly et al., 2002). Different temperature sensitive (*ts*) mutants of T3D such as *ts*C447 (core assembly), *ts*B352, *ts*G453 (outer capsid assembly), *ts*D357 and *ts*E320 (dsRNA synthesis), and empty virus component (no dsRNA genome) had the capacity to induce apoptosis in both L929 and Hela cells at non-permissive temperature (Connolly et al., 2002). These experiments showed that reovirus induced apoptosis occurred at the early stage of virus replication prior to viral transcription and not related to virus replication.

Reovirus-induced apoptosis is not limited to the virus-infected cells in vitro, but reovirus also triggers apoptotic reaction in experimental models of reovirus-induced encephalitis and myocarditis. Reovirus-induced apoptosis in vivo is the major mechanism of reovirus-induced tissue injury of the central nervous system (Obenhaus et al., 1998; Richardon-Burns et al., 2002; 2004; 2005) and heart (Sherry, 1998). Reovirus strain T3D infection induced apoptosis in the brains of newborn mice and the cell DNA fragmentation was characterized by oligonucleosomal length ladders of apoptosis, positive staining for TUNEL and activated caspase 3; accompanied by high yields of virus and histopathologically detected areas of viral injury (Obenhaus et al., 1998; Richardon-Burns et al., 2002; 2004). Double staining of tissue sections for both viral antigen and apoptosis showed that apoptosis occurred both in productively infected cells (direct apoptosis) and in uninfected cells near the infected cells (bystander apoptosis) (Obenhaus et al., 1997; 1998). The antibiotic minocycline, known to be neuroprotective in several models of neurodegenerative disease, was used to treat the T3D-injected (IC) mice; the treated groups survived longer, accompanied by reduction and delay in onset of the extent of CNS viral injury in thalamus, hippocampus, and cingulated gyrus as well as decrease in the number of apoptotic neurons in these brain regions (Richardson-Burns et al., 2005). A reovirus reassortant strain isolated in vivo from reovirus strains T1L and T3D (called 8B) was injected into neonatal mice (IM) and efficiently caused extensive

cardiac tissue injury even at low doses (Sherry, 1998). The myocardial injury induced by 8B was the result of apoptosis. The injured areas showed extensive TUNEL-positive nuclei and activated caspase 3 staining. A variety of apoptosis inhibitors such as caspase inhibitors have been shown to reduce reovirus-induced apoptosis in vitro (DeBiasi et al., 2001; 2004). Further experiment of transgenic caspase 3-deficient mice infected with reovirus variant 8B showed reduced cardiac lesion (58% reduction in lesion area comparing to the wild mice infected), less apoptosis and more long-term survivors (DeBiasi et al.. 2004).

The reovirus-induced apoptosis seems to be very complex process. There are still many puzzles in the process; such as how many cellular pathways join in and which pathway plays the most important roles in apoptosis. The regulation and interaction of cellular pathways of the reovirus-induced apoptosis is much less understood, and also why some apoptosis signaling events are cell type specific.

1.5. The tumor suppressor p53:

P53, also known as tumor suppressor protein, is a transcription factor that regulates the cell cycle and hence functions as a tumor suppressor. It is very important for cells in multi-cellular organisms to suppress cancer and maintain the cell stability. Normally,p53 does not express (Ko and Prive,1996), and the level and activity of p53 is very low and cannot be detected in the normal cell without any stress. However, after stresses, such as UV radiation, oncogenes, DNA damaging drugs and some virus infections, p53 is induced and becomes able to bind to specific target gene DNA sequences, then functions as a multi-way regulator through a series of post-translational modifications. The p53 recognition sequence is very loose and has been found on several hundred genes that differentially respond (induced or repressed) depending on the cell type, the nature and extent of stress and the extent of DNA damage. At low cellular levels, p53 modulates only a subset of the genes regulated at higher levels. The kinetics of target gene modulation may also vary (Vousden and Lu, 2002).

P53 belongs to a small family of related proteins that includes two other members-p63 and p73 (Melino et al., 2002). Although structurally and functionally related, p63 and p73 have clear roles in normal development (Blandino and Dobbelstein, 2004); p53 seems to

have evolved in higher organisms to maintain cell stability to prevent tumor development (Vousden and Lu, 2002). Human p53 is 383 amino acids long and has three domains: (1) an N-terminal transcription-activation domain (TAD), which activates transcription factors; (2) A central DNA-binding core domain (DBD) contains two copies of the 10-mer (5'-RRRCA/TT/AGYYY-3'), as well as zinc molecules and Arginine amino acid residues (Thomas et al., 1999); and (3) a C-terminal homo-oligomerisation domain (OD). Tetramerization greatly increases the activity of p53 in vivo (Weinberg et al., 2004). Mutations that deactivate p53 in cancer usually occur in the DBD. Most of these mutations destroy the ability of the protein to bind to its target DNA sequences, and thus prevents transcriptional activation of these genes (Laptenko and Prives, 2006).

Posttranslational modification of p53 is a major mechanism regulating protein function. It may be phosphorylated and dephosphorylated, cis/trans isomerized, acetylated and deacetylated, ubiquitinated and de-ubiquitinated, methylated and demethylated, sumoylated, neddylated, glycosylated or ADP-ribosylated at different sites. These biochemical modifications can determine or reflect p53 biological importance. Multisite modifications of p53 molecule are also possible. P53 exhibits cell and tissue specificity and depends on the cell cycle. It is governed by is a very complex regulatory program that fluctuates in response to cellular signaling triggered by DNA damage, proliferation and senescence, and thus appears as a dynamic 'molecular barcodes' (Yang, 2005).

P53 plays multiple roles in cells. Expression of high levels of wild-type (but not mutant) p53 has two major outcomes: cell cycle arrest and apoptosis, as well as the other functions such as joining damaged DNA repair, angiogenesis and senescence. Modulation of cell cycle-related genes by activated p53 may mediate arrest of cells at one of two major cell-cycle checkpoints, in G1 near the border of S-phase (key role played by p21) (Ko and Prives, 1996) or in G2 before mitosis (important roles for GADD45α and 14-3-3s) (Yu et al., 1999). The transcriptional program responsible for p53-mediated apoptosis is much less clearly defined, but p53 may modulate the expression of genes associated with either the extrinsic or the intrinsic apoptotic pathways. The extrinsic pathway (such as TNFRSF10A, TNFRSF10B, FAS, PERP, LRDD) involves engagement of particular 'death' receptors (Miled et al., 2005; Vousden and Lu, 2002). The intrinsic pathway is triggered in response to DNA damage and is associated with mitochondrial

depolarization and release of cytochrome c from the mitochondrial inter-membrane space into the cytoplasm (Miled et al., 2005; Vousden and Lu, 2002). Some genes associated with this pathway are APAF1, BAK1, BCL2 (repressed), FDXR, PMAIP1, and BBC3. Both pathways lead to a cascade of activation of caspases, ultimately causing apoptosis (Miled et al., 2005; Vousden and Lu, 2002). p53 also appears to be able to act directly at the mitochondria. It can interact with BCL2 family members, such as anti-apoptotic BCL2 and BCL-XL, or pro-apoptotic BAK, BAX, thereby resulting in mitochondrial outer membrane permeabilization and apoptosis (Schuler and Green, 2005).

The central core region of p53 is of key importance in regulating apoptotic function, either transcription-dependent or -independent, as supported by the number of mutations affecting this region in apoptosis-deficient p53 cells. In addition to inducing genes that drive apoptosis, p53 can also activate the expression of genes that inhibit survival signalling (such as PTEN) or inhibit inhibitors of apoptosis (such as BIRC5) (Nakamura, 2004; Vousden and Lu, 2002; Yu and Zhang, 2005). Besides the central core, the proline-rich domain has been specifically associated with the apoptotic activity of p53 (Walker and Levine, 1996).

p53 mutations are the most frequent genetic events in human cancer. They have been found in most types of tumors, with frequencies ranging from 5% (cervix) to 50% (lung). Between 20 and 35% of breast tumors have been shown to express a mutant p53. However, most of the information on p53 mutations is derived from sequence analysis that included only exons 5–8 (residues 126–306) within the p53 sequence, and examination of the whole p53 coding sequence is beginning to reveal an increasing number of mutations in the N- and C-termini of the protein (Vousden and Lu, 2002). Nevertheless, the majority of p53 mutations appear to be localized in the DNA-binding domain, which is the central part of p53. Notably, this domain is the binding site for ASPP1 and ASPP2, important cofactors in the trans- activity of p53 in relation to apoptotic genes. Most of the hot-spot p53 mutations render the protein unable to interact with ASPP1 (Vousden and Lu, 2002).

The great majority of mutant p53s are defective in trans-activation and may exert a dominant negative effect by preventing wild-type p53 from binding to the promoter of its target genes (Willis, et al., 2004). However, it must be noted that not all p53 mutations

are inactivating. For instance, some mutant p53s display only partial loss of their DNA binding activity, allowing the mutant to bind only to a subset of p53 response elements (Friedlander et al., 1996; Rowan et al., 1996).

1.6. Hypothesis and objectives of study.

During the process of reovirus inducing cell response (apoptosis or cell arrest), it has been shown that some cellular pathways such as NF-κB pathway (Connolly, et al. 2000), death receptor signaling system (Clarke et al., 2000; Kominsky et al., 2002), mitochondrial signaling pathway (Rodgers et al., 1997; Verhagen et al., 2000) and JNK and the transcription factor c-Jun activation (Clarke et al., 2001) play important roles in the processes of reovirus inducing apoptosis of some cell lines. Reoviruses induce oncolysis in a wide variety of cancer cells and tumors by the presence of an activated Ras signaling pathway of Ras/RalGEF/p38 activation (Norman et al., 2004). However, there are still many puzzles existing in reovirus-induced cell responses that need to be monitored. For example, these cellular signaling pathways are known to play a role in reovirus infection, but the regulation of these pathways, and the way in which these pathways interact, are less understood. The p53 protein and p53 related proteins are key cellular regulators that mediate several functions such as cell cycle arrest, apoptosis, DNA repair or senescence (Braon and Pagano, 1997). **The underlying hypothesis of this study is p53 or p53 related proteins would participate in reovirus induced cell responses and play some important roles in reovirus infection.**

The goals of this Ph.D study were to:

(1) Evaluate the response of cellular p53 and p53 related proteins in reovirus-induced apoptosis:

The tumor suppressor p53 regulates cellular responses to stress by serving in the nucleus to regulate transcription of genes involved in processes including cell cycle arrest, DNA repair, and apoptosis (Baptiste and Prive, 2003). The p53 pathway activated with some virus infection results in cell arrest or apoptosis. The hypothesis is that reovirus infection can activate p53 protein or p53 related proteins, and that reovirus cannot grow in p53-null cells. To test the hypothesis, the objectives of this research work include:

(1) determine if reovirus induces apoptosis or other cell responses under normal infections;

(2) detect the reaction of p53 protein and p53 related protein in reovirus induced apoptosis;

(3) monitor the relation of the detected p53 or p53 related protein with other cellular pathways in reovirus-induded apoptosis.

During the course of this major study, as a result of some data obtained, I also examined:

(2) Test the hypothesis that siRNA interfere with reovirus replication specifically:

How would the infected cells respond on the first part of reovirus life cycle such as virus entering cells and uncoating if the replication of reovirus in cells is inhibited? RNA interference (siRNA) is a powerful tool used for analysis of gene function. There are several reports that siRNA can specifically inhibit the replication of some human viruses including HIV-1, hepatitis B virus, influenza virus and rotavirus. **The hypothesis is that siRNA can also interfere with mammalian reovirus replication.** To test the hypothesis, the objectives of this research include:

(1) inhibit the reovirus replication by synthesizing some sets of reoviru siRNA;

(2) examine the function of full-length viral dsRNA on reovirus replication by using complete reovirus dsRNA genomes.

(3) Test the hypothesis that transfection can improve reovirus core infection:

The reovirus core is transcriptionally active, but, by itself, poorly infectious, presumably because the core lacks entry signals present in outer capsid proteins $\sigma 1$ and $\mu 1$ (Dryden et al., 1993; Coombs, 2002). Transfection of DNA into cells using different methods has been widely used in molecular biologic research with great success (Sambrook, et al. 1996). There are a few reports that viruses such as poliovirus (Wilson et al., 1977) and HIV (Hodgson, et al. 1996) can be transfected into cells with liposomes to reproduce new daughter virions. Therefore, **I hypothesize that transfection can enhance reovirus core replication**. To investigate this hypothesis, the objectives of this research include:

(1) monitor the ability of reovirus core replication;

(2) determine if lipofectamine reagent transfection can improve reovirus core replication.

(3) compare the efficiency of different transfection methods on reovirus core replication.

(4) Determine that reovirus T2W is a new type 2 reovirus by sequencing viral S genome segments.

In tissue culture, the type 2 reoviruses grow much slower than type 1 and type 3, and are not widely used to infect cell for testing the cell responses. A potentially new reovirus strain was isolated from the central nervous system of an 8-week-old female infant with a history of active varicella, oral thrush, hypoalbuminemia, intermittent fevers, diarrhea and feeding. This reovirus strain was tentatively identified as a member of the serotype 2 group by virus neutralization and RNA-gel electrophoresis studies and has been named type 2 Winnipeg (T2W) (Hermann et al., 2004). The reovirus T2W does not replicate to very high titers either. For this study **I hypothesized that T2W is a novel reovirus type 2 strain** and tested this hypothesis by determining the nucleotide sequences of the T2W S1, S2, S3 and S4 genome segments to allow molecular comparison with other reoviruses.

Chapter 2. *MATERIALS AND METHODS.*

2.1. Stock cell lines and viruses.

2.1.1. Stock cell lines. (1) L929 cells: Spinner-adapted mouse fibroblast L929 cells (L929) were grown in suspension culture at a density of 4.5 – 5.0 x 10^5 cells/ml in Joklik's modified minimal essential medium (J-MEM) (Gibco, Grand Island, NY) supplemented with 2.5% fetal calf serum (FCS) (Intergen, Purchase, NY), 2.5% agammaglubulin-neonate bovine viable serum protein (VSP) (Biocell, Carson, CA) and 2mM L-glutamine. The L929 cells generally grow to ~1x10^6/ml each day and are passaged every day. (2) Vero cells: Africa green monkey kidney cells (Vero cells) were grown as monolayer in T75 Corning culture dish with D-MEM (Gibco, Grand Island, NY) with 10% FCS and 2mM L-glutamine, 1mM glucose. (3) H1299 cells: Human lung carcinoma cells (H1299, a p53-null cell) and H1299 ($p53^+$) cells (transfected with p53 plasmid to express wild –type p53 protein transiently) were cultured with D-MEM (Gibco, Grand Island, NY) with 10% FCS and 2mM L-glutamine and H1299 ($p53^+$) cells were also cultured with 1μg/ml of Tetracycline.

2.1.2. Reovirus strains. Reovirus strains T1L, T2J, and T3D are laboratory stocks. Virus 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 the media described above plus 100U/ml of penicillin, 100μg/ml streptomycin sulfate and 100μg/ml amphotericin-B as previously described (Coombs, et al. 1994).

2.2. Virus passaging and plaque assay.

2.2.1. Virus passaging. 2 x 10^6 L929 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 to allow attachment, the cells were infected with reovirus strains with 0.5ml of plaque-purified P_0 stock. The virus was allowed to adsorb to the cell monolayer for 1 hour at room temperature with regular rocking every 10 – 12 minutes. After adsorption, the infected cells which were added with 4.5ml of MEM supplemented with antibiotics (**2.1.2**) incubated at 37°C. The 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 then

the P_1 cell lysates were collected. The viruses for P_2 and P_3 stocks were cultured as above for P_0-P_1 passage by using different sizes of flasks.

2.2.2. 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. The cell pellet was re-suspended to a final concentration of 1 – 2 x 10^7cells/ml with culture media and infected with virus from a P_2 or P_3 stock at a MOI of 3 – 5 PFU/cell. After adsorption for 1 hour at room temperature with periodic swirling every 10 minutes, the infected cells were diluted to 6.5 x 10^5 cells/ml with 75% fresh prepared MEM containing antibiotics (**2.1.2**) and 25% pre-adapted media. The suspension of infected cells was incubated at 33.5°C for ~ 65 hours for most strains. Some slower growing viruses required longer incubation times. In these cases, the infected cells were fed fresh prepared media (**2.1.2**) at 48 hours. Cells were harvested when there was 60% CPE determined by Trypan Blue exclusion using a hemocytometer. 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 treatment.

2.2.3. Plaque Assay. On day 0, ~1.2 x 10^6 L929 cells were plated onto Corning Costar 6-well or 0.5 x 10^6 L929 cells for 12-well dishes (Fisher, Nepean, ON) and incubated overnight at 37°C with 5% CO_2 to form monolayers.

On day 1, serial 1:10 dilutions of virus stocks 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). The cell monolayers were infected in duplicate with 100µl/6-well (50µl/12-well of each dilution) and viruses allowed to attach with cells for 1 hour with periodic rocking every 10-12 minutes, each well was overlaid with 3ml/6-well (1ml/12-well) 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 and 5% CO_2 (If the cell monolayers were prepared the same day as the virus infection, the

numbers of cells were doubled to add into the wells and cultured for 3-4 hours). On day 3, cells were fed with 2ml/6-well (0.8ml/12-well) fresh agar/M199 (prepared as above). Cells were stained with a 0.04% neutral red solution (neutral red added to a 1:1 ratio of 2x PBS to 2% agar) on day 6 or day 7 (depending upon the plaque sizes). Viral plaques were counted 15-18 hours later and titers calculated.

2.2.4. Plaque Purification: To replenish viral stocks, viral plaques were isolated and picked 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 for P_{0-1} stock. The stock was used 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/thaw three times (described in **2.2.1**). To ensure picked plaques were identical to the initial isolate, growth characteristics and sequence data were compared with the original isolate.

2.3. Preparation of reovirus cores.

2.3.1. Purification of reovirus particles. Reovirus infected cells in HO buffer (**2.2.2**) were kept on ice and sonicated for 10 seconds to disrupt cell membranes and any cell clumps. Cell membranes were further disrupted with the addition of $1/50^{th}$ the sample volume of 10% desoxycholate (DOC). Samples were vortexed and incubated on ice for 30 minutes.

Virus particles were obtained by organic solvent extraction, by adding Vertrel® XF (Vertrel) (1,1,1,2,3,4,4,5,5,5-decafluoropentane) (DuPont Chemicals, Wilmington, DW) at a volume of solvent equal to $2/5^{th}$ of the sample volume, 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 $2/5^{th}$ 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. The top aqueous layer was transferred to a fresh 30ml COREX tube. A second extraction was completed by addition of $9/10^{th}$ volume of the solvent to the sample and re-emulsified and re-centrifuged as above. If the collected aqueous phase remained hazy a third solvent extraction was performed. The resultant aqueous phase was layered onto a pre-formed 1.2 – 1.5g/ml

cesium chloride (CsCl) gradient made in a SW-28 polypropylene ultraclear tube (Beckman Instruments Inc., Palo Alto, CA) and ultracentrifuged for a minimum of 5 hours in a Beckman SW-28 rotor at 25,000rpm at 4°C.

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) at least three times 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 $OD_{260} = 2.1 \times 10^{12}$ particles/ml (Smith et al. 1969).

2.3.2. Purification of core particles. Purified virus was concentrated to 6.5×10^{13} particles/ml and α-chymotrypsin was added to a concentration of 200 μg/ml. The digestion was incubated at 37°C for 3 hours with vortexing every hour. The digested core particles were then loaded onto 1.2-1.5 g/ml cesium chloride (CsCl) density gradients and centrifuged at 28,000 rpm for 6 hours or overnight in a Beckman SW41 rotor as previously described (Yin, et al. 1996). Purified cores were harvested and dialyzed against core dialysis buffer (1M NaCl, 25mM HEPES, 100 mM $MgCl_2$, pH 8.0) overnight and dissociation was checked by electron microscopy (**2.13**) and characteristic protein profiles in SDS-PAGE (**2.9.2**) (Yin, et al. 1996).

2.4. Preparation of RNA.

2.4.1. Preparation of reovirus dsRNAs. Reovirus-infected cells were transfered to centrifuge tubes and pelleted by centrifugation at 500Xg for 10 minutes. The cell pellets were resuspended in 450μl NP-40 buffer (140mM NaCl, 1.5mM $MgCl_2$, 10mM Tris, pH 7.4) and dissolved by adding 50μl 5% NP-40, then incubated for 30 minutes on ice. Cellular nuclei and organelles were spun down and supernatants were removed to microfuge tubes (Fisher, Nepean, ON). The viral dsRNA genomes were purified by Phenol/Chloroform extraction and precipitated with 1/10 volume 3M NaOAc and 2.5X volume ice-cold 100% EtOH. The precipitated dsRNA genomes were pelleted in a Biofuge A at 12,500 rpm for 30 minutes. The dsRNA pellets were lyophilized for 30

minutes, then resuspended in electrophoresis sample buffer and resolved by 10% SDS-PAGE (**2.9.2**).

2.4.2. Total cellular RNA preparation. Suspension cells (L929), or the monolayer cells (L929, Vero, or H1299) that had been harvested with trypsin were pelleted by centrifugation. The pelleted cells were lysed in TRIZOL Reagent (Invitrogen, CA) by repetitive pipetting at the ratio of 1ml of the reagent per 5-10 × 10^6 cells.

The homogenized samples were incubated for 5 minutes at room temperature (RT) (20 to 25°C) to completely dissociate nucleoprotein complexes, chloroform was added at 0.2 ml of chloroform/per 1ml of TRIZOL Reagent. The tubes were shaken vigorously by hand for 15 seconds and incubated at RT for 2 to 3 minutes. The samples were spun at 12,000rpm for 15 minutes at 4°C. The upper aqueous phase (containing RNA exclusively) was transfered to a fresh tube. The RNA was precipitated by mixing with isopropyl alcohol at 0.5ml of isopropyl alcohol/per 1ml of TRIZOL Reagent used for the initial homogenization, incubated for 30 minutes at RT, and spun at 12,000rpm for 10 minutes at 4°C. The RNA pellets were washed once with 75% ethanol, briefly air-dried or vacuum-dried for about 5 minutes (RNA was not dried by centrifugation under vacuum). RNA was dissolved in 100% formamide (deionized) or RNase-free water and stored at -70°C for use.

2.5. RNA protection assays (RPAs).

2.5.1. Synthesis of RNA probes. Each probe was labeled as described by manufacturer (BD). Briefly, 1μl Rnasin, 1μl NTP mixture (GACU pool), 2μl DTT, 4μl 5X transcription buffer, 1μl Multi-Probe Template Set (h-Hstress-1 or h-APO3), 10μl [α-^{32}P] UTP and 1μl T7 RNA polymerase (kept at –20°C until use) were mixed gently, quick-spun, and incubated at 37°C for 1 hour. The mixture was supplemented with 2μl of Dnase and incubated at 37°C for 30 minutes to terminate reaction. The synthesized probe was purified by adding the following reagents in order: 26μl 20mM EDTA, 25μl Tris-saturated phenol (pH7.5-7.8) (Invitrogen, CA), 25μl chloroform: isoamyl alcohol mixture (50:1), and 2μl yeast tRNA and spun down. The upper aqueous phase was transferred to a new 1.5 ml Eppendorf tube, and treated with 50μl chloroform/isoamyl alcohol (50:1) once. The upper aqueous phase was precipitated with 50 μl 4 M ammonium acetate and

250µl ice-cold 100% ethanol for 40 minutes at –70°C, and spun at 12,500rpm for 30 minutes at 4°C. The probe pellet was washed with ice-cold 90% ethanol, air-dried about 8 minutes (not in a vacuum evaporator centrifuge), and dissolved with 50µl of hybridization buffer. 1µl of duplicate samples were measured in a scintillation counter (expecting a maximum yield of –3 x10^6 Cherenkov counts/µl without the presence of scintillation fluid), and stored at –20°C for use.

2.5.2. RNA hybridization: 8µl of each target RNA sample (generally 10 µg or more RNA) in a 1.5ml tube, was mixed with 2µl of the synthesized probe (diluted to an appropriate concentration with hybridization buffer), spun after gently mixed. A drop of mineral oil was added to each tube for reaction. (RNA samples had been stored in water, the RNA samples were precipitated with 1/10 volume 4 M ammonium acetate and 2.5X volume ice-cold 100% ethanol, and the pellet was dissolved with hybridization buffer). The samples were hybridized in a heat block by re-warming to 90°C, immediate reduction of the temperature to 56°C and incubated at 56°C for 12 – 16 hours, then cooled (about 15 minutes) to room temperature. The hybridized RNA samples were digested with 100 µl of RNase cocktail (RNase cocktail: 2.5ml of RNase buffer with 6µl RNase A+T1 mix/per 20 samples) for 45 minutes at 30°C, then transfered (avoiding transfer of the oil) to fresh tubes containing 18µl aliquots of proteinase K cocktail (390µl Proteinase K buffer, 30µl proteinase K (10mg/ml) and 30µl yeast tRNA for 20 samples) to stop Rnase activation for 15 minites at 37°C. The hybridized RNA samples were extracted with Phenol/Chloroform, and pelleted with 1/10 volume 4M ammonium acetate and 2.5 volume ice-cold 100% EtOH. The pellets were dissolved with 5 µl of electrophoresis loading buffer for PAGE.

2.5.3. Gel resolution of protected probes: 4.75% acrylamide (19:1 acryl/bis)- 2M Urea gel (>40cm in length) was prepared and pre-electrophoresed at 40W constant power for 45 min. The hybridized dsRNA samples and controls (including ^{32}P-labeled probe, diluted to 1000-2000 cpm in 10µl loading buffer) were run in the gel at 55 watts constant power with 0.5X TBE until the leading edge of the bromophenol blue reached 30 cm from the bottom of the well. The gel was dried and exposed to X-ray film at –70°C.

Using the undigested probes as markers, a standard curve of migration distance *vs* log

nucleotide length was plotted. The curve was used to establish the identity of "RNase-protected" bands in the experimental samples.

2.6. Examination of apoptosis.

2.6.1. Cell viability assay by trypan blue exclusion: At various time points post infection, or mock), all floating and live cells infected with viruses were collected and concentrated, then stained with trypan blue (Sigma) for 15 minutes. A total of 200 cells were counted in triplicate in a hemocytometer for live (unstained) and dead (stained). The percentage of dead cells was calculated and used as an index for the degree of cell death (Martin and Lenardo, 1998).

$$\% \text{ apoptotic cells} = \frac{\text{(total no. of cells counted)- (total no of live cells)}}{\text{total no of cells counted}} \times 100$$

2.6.2. Apoptotic staining with acridine orange and ethidium bromide: Cells were stained with acridine orange for determination of nuclear morphology and with ethidium bromide stain to determine cell viability, at a final concentration of 1μg/ml for both. 1μl of the dye was placed in the bottom of a 12X 75-mm glass tube, 25μl of the cell suspension was added and mixed gently by hand. 10μl of stained cells were placed on a microscope slide covered with a coverslip and the numbers of different fluorescent cells counted by epifluorescence microscopy (Martin and Lenardo, 1998). The percentage of cells containing nuclei and/or marginated chromatin in a population of 200 cells was determined.

2.6.3. Flow cytometry: Cells were measured by flow cytometry to determine the binding of Annexin V to phosphatidylserine and were simultaneously measured for propidium iodide incorporation. Annexin V-PE (BD Bioscience, Mississauga. ON) and 7-AAD (7-aminopanctinomycin) (Sigma, ON) were used to detect the apoptotic cells. The cells were washed twice with cold PBS and then re-suspended in 1X binding buffer (0.01M Hepes/NaOH, pH7.4, 0.14M NaCl, 2.5mM $CaCl_2$) at a concentration of about 10^6 cells/ml. 100μl of cells was transfered to a 5ml culture tube, 5μl of Annexin V-PE and 5μl of 7-ADD were added, incubated for 15 minutes at RT (25°C) in the dark, 400μl of

binding buffer was added to each tube, and the stained cells were analyzed by flow cytometry as soon thereafter as possible.

PI straining of DNA for DNA cycle or apoptosis. The cells were washed with cold PBS, the cold cell suspension was rapidly pipetted into the cold EtOH for least 15 minutes or kept in 4°C for later use, then washed once with cold PBS and as much liquid as possible removed. The cells were added to 125μl of RNase solution (500units/ml) incubated for 15 minutes in a 37°C water bath, removed from water bath and 125μl PI stain solution added. After standing at room temperature for at least 30 minutes, the stained cells were measured with flow cytometry.

2.7. Transfection

2.7.1. Lipofectamine transfection. The cell monolayer (for a 35mm dish) was prepared one day early and grown to about 70% confluency before transfection. After gentle mixing 10μl Lipofectamine™ 2000 (Invitrogen) was diluted in 250μl Opti-MEM® I medium (Invitrogen, CA) without serum in a separate vessel and incubated for 5 minutes at room temperature. Various amounts of siRNA, or dsRNA, or virus, or core particles calibrated to 1 or 100 particles/per cell (counted as in **2.3.1** and **2.3.2**), were diluted in 250μl Opti-MEM® I medium without serum for 5 minute incubation. The two mixtures were combined together and incubated for another 20 minutes at room temperature. After cell monolayers were washed 2-3 times with Opti-MEM® I medium (no serum and antibiotics), 500μl complete growth medium was added (no antibiotics and sera) to cell dishes, then 500μl of the transfected mixture was added and the dishes gently rocked. After incubating the cells at 37°C in a CO_2 incubator for 4-5 hours, 1000μl of new complete growth media (containing 2X sera and antibiotics) was added and incubation continued at 37°C until ready to harvest cells or perform further assays.

2.7.2. Transfection with calcium phosphate. Aliquots of virus and viral core particles, pre-calculated to represent treatment ratios of 1 or 100 particles per cell, were gently mixed with 10μl of 2M $CaCl_2$, and slowly added into 2X Hepes buffer saline (HBS). After 30 minute incubation at room temperature, the precipitated solution was added drop-wise to cells in 35mm dishes and incubated at 37°C for various periods of time

before the total amount of progeny virus produced was determined by plaque assay (**2.2.2**).

2.8. Small RNA interference (siRNA).

2.8.1. Design of siRNA fragments. Two sets of siRNA fragments were designed, (1): RVL1-1 (sense): 5' aacaugguacuuggcagcggcg**att** 3' corresponding to a region of the L1 gene (encoding reovirus RdRp), and the antisense (RVL1-2): 3` **tt**uuguaccaugaaccgucgccgcu 5'. (2): RVM2-1(sense): 5' aauaagacugacccugagaugaa**tt** 3', corresponding to M2 gene (encoding major outer capsid protein μ1), and the antisense (RVM2-2): 3`**tt**uuauucugacugggacucuacuu 5'. The '**tt**' in these strands was not part of the reovirus genes, but were added for requirement of siRNA function. All of these siRNA were synthesized by Boehringer Mannheim Corp (Lavel, Quebec). The GADD45 siRNAs (human or mouse) used in assays were from Santa Cruz Bio (CA).

2.8.2. Assays of siRNA. The Reovirus siRNA were transfected with oligolipofectamine reagent (Invitrogen, CA), and the GADD45 siRNA were transfected with transfection reagent (Santa Cruz Bio, CA). Transfection methods were described earlier (**2.7.1**). 24 hours after transfection, the transfected cells were infected with reoviruses T1L or T3D at required doses. The titers of virus replication were measured by plaque forming assay (**2.2.2**). The expression of the target genes was verified by Western Blotting (**2.9**).

2.9. Western blotting.

2.9.1. Preparation of cell lysate. The cell monolayers in 100mm plates were washed with cold PBS twice, and scraped from the plates. The cells were pelleted and dissolved in electrophoresis sample buffer (0.24M Tris, pH6.8, 1.5% dithiothreitol (DTT), 1% SDS) and frozen at –20°C for use.

2.9.2. SDS-PAGE. All RNA or protein sample to be resolved by SDS-PAGE were dissolved with 1X electrophoresis sample buffer (ESB) (240mM Tris-HCl, pH 6.8, 1.5% dithiothreitol (DTT), 1% SDS), heated for 5 minutes (65°C for RNA, 95°C for protein) before loading. For protein analysis 10% linear or 5-15% gradient SDS gels (16w X 12h X 0.1t cm or Mini-Protean® II system 8.3w X 6.2h X 0.075t cm) were poured and allowed to polymerize for 1 hour for linear (10%). Resolution was at 180V for 1hour for

protein separation in Mini-Protean® II system or at 500mA for about 3 hours for separation of structural proteins using 16w X 12h X 0.1t cm gels. Resolution of RNA samples was at 18mA and 250V for 45 hours.

For direct observation of SDS-PAGE results, protein gels were first fixed in an 10% isopropyl alcohol, 30% acetic acid solution, than stained with Coomassie Brilliant Blue (0.5% Coomassie Brilliant Blue solution) and destained in 15% methanol, 7.5% acetic acid solution. All of these steps were performed either using conventional staining protocol or MAPS (microwave assisted protein staining) method (Nesatyy et al., 2002). For all steps in the MAPS method, the gel was placed in a glass (or plastic) dish and covered with a minimum of 100ml of the appropriate solution and than incubated in the microwave oven at 700W for 1min respectively. Protein bands were visualized by a Gel Doc 2000 (Bio Rad) apparatus and subsequently gels were dried between layers of cellophane for preservation. The RNA gels were stained with ethidium bromide (3μg/ml) and RNA gene segments visualized with UV irradiation by a Gel Doc 2000 (BioRad) apparatus. For Western Blotting, The protein gels were transblotted to membranes and detected with specific antibodies (see **2.9.3**).

2.9.3. Western Blotting analysis. The gels were soaked in TransBlot buffer (48mM Tris, 39mM Glycine, 0.0375% SDS and 20% MeOH) for 5 minutes after SDS-PAGE. At the same time, the transfer membranes (Immobilon^{TM}, Millipore, Bedford, MA) were first wet with 100% MeOH for 1minute, then ddH_2O for 1minute, and finally soaked in TransBlot buffer for 5 minutes. Two pieces of filter paper for each membrane were also soaked in TransBlot buffer. Transferring sandwich consisted of the following order: one piece filter paper, gel, membrane, and another piece filter paper (from Black to white in gel transfer cassette) was placed into a TRANS-BLOT SD. The transfer of proteins from SDS-PAGE to membrane was performed at 0.9A for 45 minute to 1hour. After transfer, membranes were washed once with ddH_2O, then stained with 2% Ponceau-S (3-hydroxy-4-[2-sulfo-4-(4-sulfophenylazo0-phenylazo]-2, 7-naphthalenedisulfonic acid (Sigma, Oakville, Ontario). Membranes were rinsed with dH_2O three times until protein bands became visible, and soaked in blocking buffer (5% Skim milk in TBST buffer (20mM Tris-base, 100mM NaCl and 0.05% Tween 20) for 2-3 hours. Various monoclonal or polyclonal antibodies at suitable dilution (anti- whole virus, anti-hGADD45, anti-p65,

anti-p50, anti-actin) were used to incubate different membranes for 3-4 hours at room temperature or overnight (4°C). The membranes were washed three times with TBST for 15 minutes to remove non-specifically bound primary antibodies. Then secondary antibodies (1: 1,500 –1: 2,000), goat anti-rabbit IgG or goat anti-mouse IgG coupled to alkaline phosphatase (Sigma, Oakville, Ontario) or to Horseradish peroxidase, were added to membranes and incubated for 90 -120 minutes. The membranes were washed with TBST three times for 15 minutes. Then the specific bands were developed; for Alkaline phosphatase the substrate 5-bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) was used (Sambrook, et al 1989), and for Horseradish peroxidase, ECL solution (Amersham Bio. UK) was used to develop and exposed with X-ray film for seconds to minutes to a desired intensity.

2.10. Sequencing of reovirus T2W S-genes.

2.10.1. Design of the sequencing primers. In order to sequence all S-genes in both directions, internal gene specific primers were designed to generate overlapping fragments of each S gene. To initially design these primers, the S-genes of reoviruses T1L, T2J and T3D were aligned and the highest homologous regions were selected for primer construction (designated "U" and "L" in Table **2.1**). All of the primers were synthesized by Invitrogen Corp.

2.10.2. Viral RNA preparation and 3' end ligation. Purified T2W virions were mixed with 5x protein electrophoresis sample buffer and denatured at 65°C for 5min. Viral genomic RNA was separated on a 1.2% agarose gel and the viral S RNA fragments were collected and purified with Qiagen columns. 3' end ligation was performed as previously reported (Lambden et al., 1992; Yin et al., 2004). Briefly, the oligonucleotide 3'L1 (Table **2.1**) was ligated to the 3' ends of reovirus S genes with T4 RNA ligase at 37°C overnight according to the manufacturer's directions (Roche). The ligated genes were repurified with agarose gel and Qiagen columns to remove the unincorporated 3'L1 oligonucleotide. The ligated genes were precipitated and dissolved in 4μl of 90% DMSO.

2.10.3. Reverse transcriptase polymerase chain reaction (RT-PCR). The purified 3'ligated viral dsRNA were denatured for 45 minutes at 50°C. Reverse-transcription and polymerase chain reaction (RT-PCR) was performed with primer 3'L2 (Table**2.1**) and

appropriate U and L primers (described in **2.10.1**). The viral dsRNA template was converted to ssDNA by reverse transcriptase (RT). A 1µl volume of purified dsRNA template (prepared as in section **2.4**) was heated for 45 minutes at 50°C to denature the strands. A "master mix" was prepared which contained 16U (0.4µl of 40Uµl) of RNAse inhibitor (Gibco-BRL), 0.1µM (0.5µl of 10µM) each of the appropriate upstream and downstream primers (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), 4µl 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, and the final volume was brought up to 20µl with ddH_2O and then incubated at 42°C for 2– 3 hours. Samples were used for amplification immediately as ssDNA is relatively unstable.

The cDNA produced by RT above was amplified with the Expand High Fidelity PCR System with Expand HF Buffer (Roche Applied Science). To amplify one strand, 2µl of cDNA was diluted to a final volume of 50µl containing 0.1 µM (0.5µl of 100 µM) of each terminal primer, 0.2mM each of dATP, dCTP, dGTP, dTTP (1µl of dNTP mixture with 10mM of each nucleotide), 1X Expand HF Buffer, 1.5mM $MgCl_2$ (5µl of 10X concentration of Expand HF Buffer with 15mM $MgCl_2$) and 1.5U Expand High Fidelity PCR System enzyme mixture. The cycling reaction was performed on a Flexigene (Techne Limited, Duxford, UK) for 94°C denature for 5 minutes, 30-35 reaction 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) with a final elongation step at 68°C for 10 minutes. Samples were then cooled to 4°C for analysis or stored at -20°C for later checking.

To confirm the amplified cDNA product, 3-5µl of the products were diluted in DNA electrophoresis sample buffer and run on a 0.9% horizontal agarose gel at 100V for ~1.0 hour 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 1kilobase (kb) DNA ladder (Boehringer Manheim) was used as markers. Next, a large volume of cDNA product was run on a larger 0.9% agarose gel as described above. The

Table 2.1. Primers used for RT-PCR and Sequencing of T2W S1, S2, S3 and S4 genes:

Name	Sequence	Location	Direction
3'L1:	5'-CCCCAACCCACTTTTTCCATTACGCCCCTTTCCCCC-3'		
3'L2:	5'-GGGGGAAAGGGGCGTAATGGAAAAAGTGGGTTGGGG-3'		
3'L3:	5'-GGGGGAAAGGGGCGTAAT-5'		
T2W-S1:			
S1-U:	5'-GCTATTCGCGCCTATGGATG-3'	1-20(T1L)	5'-3'
S1-L:	5'-GATGATTGACCCCTTGTGCC-3'	1458-1438(T1L)	3'-5'
S1-1:	5'-GCTGATGTCCTGGATGGAA-3'	765-746(T2W)	3'-5'
S1-2:	5'-TAACAGCAGTGGTCAAAT-3'	702-720(T2W)	5'-3'
S1-3:	5'-TCCAGATTCTCAACGGGCGC-3'	1094-1114(T2W)	5'-3'
S1-4:	5'-AACCAGTCTGCCACCCTGAA-3'	1182-1162(T2W)	3'-5'
T2W-S2:			
S2-U:	5'-GCTATTCGCTGGTCAGTTATGGC-3'	1-23(T1L)	5'-3'
S2-L:	5'-GATGAATGTGTGGTCAGTC-3'	1331-1312(T1L)	3'-5'
S2-1:	5'-GGGGTGATGACTCAAGCTCAG-3'	1056-1077(T2W)	5'-3'
S2-2:	5'-CGCAAGGAAGGGATAATC-3'	420-402(T2W)	3'-5'
S2-3:	5'-CTGTGATGATTATCCCTTCC-3'	395-415(T2W)	5'-3'
T2W-S3:			
S3-U:	5'-GCTAAAGTCACGCCTGTTGTC-3'	1-23(T1L)	5'-3'
S3-L:	5'-GATGATTAGGCGCCTCCCACC-3'	1198-1177(T1L)	3'-5'
S3-1:	5'-GAAAGTTGATATGCGAGAG-3'	971-990(T2W)	5'-3'
S3-2:	5'-CCAGCGAGACGGGCGAAT-3'	442-424(T2W)	3'-5'
S3-3:	5'-CTCGCTGGCTAGGCTTTT-3'	435-453(T2W)	5'-3'
T2W-S4:			
S4-U:	5'-GCTATTTTTGCCTCTTCCC-3'	1-19(T1L)	5'-3'
S4-L:	5'-GATGAATGGAGCCTGTCCCA-3'	1196-1176(T1L)	3'-5'
S4-1:	5'-ATTAGGTATGCGATTGGG-3'	963-981(T2W)	5'-3'
S4-2:	5'-AACAAATGAGAGCATCCC-3'	342-324(T2W)	3'-5'
S4-3:	5'-CGATCCGGGGTCAATGTT-3'	440-458(T2W)	5'-3'

3'L1, used for 3' ligation of dsRNA. 3'L2 primer and -U or –L primer of each reovirus S gene used for RT-PCR and cDNA sequencing, and 3'L3 and –1, to –4 primers used for cDNA sequencing.

resultant bands were 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.

2.10.4. Sequencing of the viral S1, S2, S3 and S4 genes: The RT-PCR products were purified (**2.10.3**). Sequencing primers were 3'L3, -U and –L gene specific primers (Table **2.1**), and additional primers as needed (Table **2.11.1**). 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 all T2W cDNA strands: 5pmol (0.35μM) primer, 20-50ng cDNA template, 6μl BigDye™ Terminator Ready Reaction Mix and ddH_2O to a final volume of 15μl. Cycle sequencing was performed 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 immediately "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.

2.10.5. Analysis of gene sequence. DNA sequences were analyzed by EDIT SEQ and SEQMAN programs (Lasergene, DNASTAR) and also by BLAST (Altschul et al., 1990), and CLUSTAL-W (Thompson et al., 1994). Phylogenetic analyses were performed with the neighbor-joining (NJ) methods and PHYLIP software package version 3.5C (Felsenstein, 1993). The nucleotide sequence data reported here have been deposited in the GenBank database and assigned accession numbers for each gene are shown in Table **2.2**, along with all other reovirus S1, S2, S3 and S4 genes used for comparative purposes.

2.11. Reovirus gene cloning. The cDNA of reoviral genome S genes (T1L dsRNA as the template) was prepared by RT-PCR from reovirus dsRNA fragments (described in **2.10.3**), and excised from agarose gels with the QIAquick™ gel extraction kit (Qiagen,

Germany). The purified cDNAs were directly ligated into the TOPO-vector according to supplied protocols (Invitrogen, CA). The positive colonies were picked and the size of the cDNA fragments were checked with specific restriction enzymes and sequenced for correct orientation of the cloned gene.

2.12. Matrix-assisted laser desorption/ionization source coupled to a tandem quadrupole/time-of-flight (MALDI-QqTOF) mass spectrometry.

2.12.1. Sample preparation. The methods were essentially as previously published (Swenson et al., 2002). Reovirus peptides were separated by SDS-PAGE (**2.9.2**). The σ1, σ2 and σ3 protein bands were separately collected and destained by repeated washing with alternating 100mM NH_4NCO_3, a 2:3 (v/v) mixture of Acetonitrile: 100mM NH_4NCO_3 and 100% Acetonitrile solutions. After complete destaining, the gel pieces were swollen with 2-3 gel volumes of 100mM DTT in 100mM NH_4NCO_3 for 45 minutes at 57.5°C. The gel pieces were digested with 50-100µl of trypsin (5ng/µl, Sigma, sequencing grade) in 50mM NH_4NCO_3 at 37°C overnight. The digestion mixture was inactivated with 10µl 2% TFA and the resultant fragments were extracted by 30min of sonication (on ice) in 2-3 gel volumes of 0.01% TFA in 50% Acetonitrile and shrunk with 2-3 gel volumes of Acetonitrile. The whole extraction was repeated three times. All solutions for each protein were collected in one tube and dried in a SpeedVac. The samples were resuspended in 2µl 0.01%TFA and left at -80°C until ready to use.

2.12.2. Zip-Tip to remove gel pieces. For optimal results, Zip-Tip treatment of the samples were performed. The samples were diluted with 1%TFA to yield a final concentration of 0.5% TFA. The Zip-Tip was equilibrated for sample binding by first aspirating 10µl of 50% ACN, dispensing to waste and repeated three times. The Zip-Tip was then balanced by aspirating 10µl of 0.1% TFA three times. Then the Zip-Tip was combined with peptides by aspirating and dispensing samples through 10 cycles, washed and dispensed to waste by pipitting 3 cycles of 0.1%TFA. The peptides were eluted by dispensing 1-4 µl of 50%ACN, 1%TFA into a clean vial, and aspirated and dispensed this elution through the Zip-Tip at least 5 times without introducing air. Finally the eluted solutions were spotted directly onto MALDI target.

2.12.3. Mass spectrometry analysis. Samples were analyzed on a prototype MALDI-

Table 2.2. Reovirus strains used for sequence analysis

Abbreviation	Virus strain	GenBank accession No.			
		S1	S2	S3	S4
T2W:	T2/Human/Canada/T2W/97	DQ220017	DQ220020	DQ220018	DQ220019
T1L:	T1/Human/Ohio/Lang/53	M10260	L19774	M18389	X61586
T2J.	T2/Human/Ohio/Jones/55	M10261	L129775	M18390	X60066
T3D:	T3/Human/Ohio/Dearing/55	M17871	M25780	M01627	K02739
T2N84:	T2/Human/Netherlands/1984	AY862138		U35347	
T2N73:	T2/Human/ Netherlands/1973	AY862137		U35350	
T1N84:	T1/Human/Netherlands/1984	AY862136		U24348	
T1N83:	T1/Human/Netherlands/1983	AY862135		U35346	
T1C23:	T1/Bovine/Maryland/Clone23/59	AY862134			U15075
T1C50:	T1/Bovine/Maryland/Clone50/60	AY862133			U15079
T3AV1:	T3/AV1 isolate	AY86061			
T3C96:	T3/Human/Colorado/1996	AY302467			
T3C18:	T3/Maryland/Clone18/61	L37684	L19770	U35358	U15074
T3C31:	T3/Bovine/Maryland/Clone31/59	L37683	L19771	U35357	U15076
T3C43:	T3/Bovine/Maryland/Clone43/59	L37682			
T3C44:	T3/Bovine/Maryland/Clone44/60	L37681			U15077
T3C45:	T3/Bovine/Maryland/Clone45/60	L37680			U15078
T3C8:	T3/Human/Tahiti/Clone8/60	L37679	L19768	U35355	U15082
T3C84:	T3/Human/Wash.D.C./Clone84/57	L37678	L19772	U35354	U15080
T3C87:	T3/Human/Wasg\h.D.C./Clone87/57	L347677	L19773		U15081
T3C9:	T3/Murine/France/Clone9/61	L37676	L19769	U35352	U15084
T3C93:	T3/Human/Wash.D.C./Clone93/57	L37675			U15083
T1C62:	T1/Human/Wash.D.C./Clone62/57		L19767	U35356	
T1C49:	T1/Bovine/Maryland/Clone49/60		L19766		
T1C11:	T1/Human/Wash.D.C./Clone11/59		L19765	U35359	
T2To	T2/Human/Tokyo/90			U35360	
T3N83	T3/Human/Netherlands/83			U35349	
T3Co	T3/California/Cornell/68			U35362	
T2SV59	T2/Maryland/SV59/68			U35361	
T3A	T3/Wash.D.C./Abney/55			U35353	
T1N67	T1/Human/Netherlands/ 67			U35351	
T1C15	T1/Human/Wash.D.C.?Clone15/58				U15073
T3C100	T3/Bovine/Maryland/Clone100/60				U15072

The strains are named using the following scheme: serotype/species of origin/place of origin/strain designation/year of isolation by the description of GenBank. All are from GenBank data.

QqTOF mass spectrometer (Figure **2.1**) built at the University of Manitoba in collaboration with MDS Sciex (Concord, ON). It consists of matrix-assisted laser desorption/ionization (MALDI) source coupled to a tandem quadrupole/time-of-flight (QqTOF) mass spectrometer by means of a collisional damping interface (Figure **2.1**) (Shevchenko et al., 1997; Chernushevich et al.,1999). The target (with peptides) was placed about 4mm from the entrance of the quadrupole ion guide q0, where they were aerosolized and given an ionic charge by exposure to a nitrogen laser beam (Laser Science Inc., model 337ND) operating at a repetition rate of 10 Hz for single MS. Ionized peptides are then carried through an electromagnetic field towards a time of flight (TOF) analyzer. The TOF analyzer detects how long it takes for a given ion to travel a given distance. A peptide ion's flight is proportional to its mass/charge ratio (m/z). The instrument is extremely sensitive and has been measured to have an accuracy of <0.1 Da within a range of 500 to 4500Da (Loboda et al., 2000). For each cleaved protein, a spectrum of peptide m/z ratios is produced in approximately 1 minute. Argon was used as the cooling gas in Q_0. Mass spectra data were acquired, recorded and analyzed using *Tofma* software developed in-house. Calibration of the mass spectrometer was based on the quadratic equation and was calculated using two known mass peaks. Mass spectra were analyzed using M/Z and *Knexus* proteomics programs (Genomic Solutions, Boston, MA). The peptides were identified by searching on-line databases against monoisotopic masses using *ProFound* (http://hs1.proteome.ca/prowl/knexus.html) or using the *Mascot* search engine (Matrix Sciences, Ltd., London, UK) on the Internet.

2.12.4. MS/MS analysis. To identify peptide molecular weights from spectra that were not matched to reovirus peptides using *ProFound* or *Mascot* the tandem mass spectra (MS/MS) of unidentified peptides were acquired using the same MALDI-QqTOF instrument. The further fragmentation of the peptide of interest can also be performed to confirm the amino acid sequence. The unmatched peptides were further fragmented using the same laser but for MS/MS operating at 20 Hz. As peptides are flying through the electromagnetic quadrupoles, the TOF analyzer detects these smaller peptide fragments, the size of which differs by a single amino acid sequence and in that way amino acid sequence ladder is produced. Tandem mass spectra were accumulated for several minutes as opposed to just one minute in single MS spectra. For tandem mass spectrometry,

Figure 2.1.

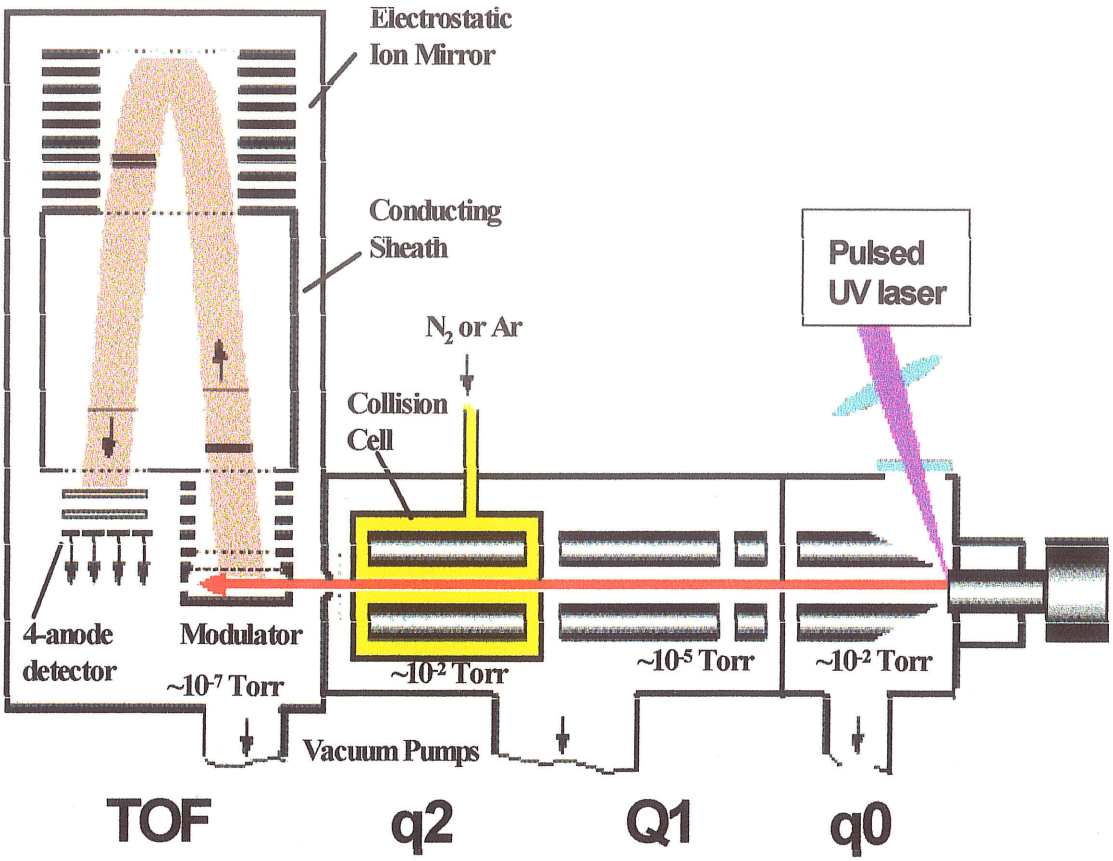


Figure 2.1. The MALDI QqTOF mass spectrometer. The proteolytically cleaved protein sample is spotted onto target and exposed to a laser beam. The peptides are accelerated and cooled through an electromagnetic field (q0, Q1 and q2) and enter a time of flight (TOF) analyzer. The laser is pulsed at a repetition rate of 10Hz for single MS and 20Hz for tandem MS. The length of time required for a peptide fragment to reach the TOF detector is proportional to its mass and charge. During tandem MS specified peptide is isolated and further fragmented in the q2 quadropole of mass spectrometer. This allows for the analysis of the peptide amino acid sequence. The computer programs used to analyze the spectra produced by the MALDI QqTOF were M/Z, Knexus, *Profound* and *Sonar ms/ms* (Proteomics, New York, USA). This figure was provided by Drs. Werner Ens and Ken Standing (University of Manitoba, Department of Physics and Astronomy).

selected ions were fragmented in a collision cell that contained argon. The collision energy was adjusted manually between 32 and 183 eV to obtain optimum fragmentation of the parent ions by applying the accelerating voltage to the rule 0.5V/Da. The produced fragmented peptide m/z ratios were analyzed using M/Z and *Knexus* suite of proteomics programs and matched against the NCBInr virus database using *Sonar ms/ms* (http://hs1.proteome.ca/prowl/knexus.html) search engine on the Internet to determine the identity or homology of the protein.

2.13. Electron microscopy of reovirus particles. For observation by electron microscopy (EM), gradient purified virus or core 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, viral particles 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 50,000X or 100,000X. Electron micrographs were photographed onto Kodak 5303 direct positive film and printed onto Kodak Polycontrast III paper.

2.14. Statistics analysis of data. Density data of the samples on Western gels were selected to analyze the statistical differences for different time points of post-infection and different doses or different virus strains by TWO-WAY ANOVA with Statistics program SPSS 13.0. P values of less than 0.05 were considered statistically significant.

Chapter 3. *REOVIRUS INDUCES APOPTOSIS OR CELL ARREST AND CELLULAR RESPONSE.*

3.1. Introduction.

Apoptosis is an active process of cell death that occurs in response to various stimuli, including viral infection (Roulston et al., 1999). It involves a number of distinct morphological and biochemical features such as cell shrinkage, translocation of phosphatidylserine from the inner to the outer surface of the cell membrane, plasma membrane blebbing, chromatin condensation, loss of the inner mitochondrial transmembrane potential, and internucleosomal DNA cleavage (Earnshaw et al., 1999). In some cases, apoptosis triggered by virus infection may serve as a host defense mechanism to limit virus replication (O'Brien, 1998). In others, apoptosis may increase virus infection by facilitating virus spread or allowing the virus to evade host inflammatory or immune response (Cohen, 1991; Teodoro and Branton, 1997). Reovirus induces programmed cell death (apoptosis) in a wide variety of cultured cells in vitro (Tyler et al., 1995; Clarke and Tyler, 2005). Reoviruses that induce apoptosis differ in genetics of strain–specific characteristics. T3 prototype strains Abney (T3A) and Dearing (T3D) can induce apoptosis more efficiently than the serotype 1 strain Lang (T1L) in cell culture (Tyler et al., 1995).

The UV inactivated reoviruses or some reovirus *ts* mutant strains are also good apoptosis inducers (Tyler et al., 1995). Analysis of reassortant viruses that contain mixtures of gene segments from T1L and T3D indicate that the viral S1 gene is the primary determinant of differences in the efficiency of apoptosis induction exhibited by these strains in L929 (Tyler et al., 1995), MDCK (Rodgers et al., 1997) and HeLa cells (Connolly et al., 2000). The M2 gene that encodes μ1/μ1C (a virus major outer capsid protein) also functions on the efficiency of apoptosis induction (Tyler et al., 1995; Rodgers et al., 1997). Further studies indicate that reovirus induced apoptosis is determined by receptor utilization, and binding of reovirus attachment protein σ1 to both cell surface sialic acid and Junction adhesion molecule (JAM) is required to achieve maximal levels of apoptosis after reovirus infection (Barton et al., 2001; Connolly et al., 2001).

During the process of reovirus induced apoptosis, some cellular pathways such as

NF-κB pathway (Connolly et al., 2000), death receptor signaling system (Clarke et al., 2000; Kominsky et al., 2002), mitochondrial signaling pathway (Rodgers, et al 1997; Verhagen et al., 2000) and JNK and the transcription factor c-Jun activation (Clarke et al., 2001) play important roles in some cell lines. However, there are still many puzzles that exist in reovirus-induced cell responses. The tumor suppressor protein p53 is an essential component of an emergency stress response preventing the growth and survival of damaged or abnormal cells (Fridman and Lowe, 2003) and would be expected to play some roles in reovirus infection and also in the regulation of these pathways. In addition, the ways in which these pathways interact are not well understood. Determination of apoptotic functions in primary cells and infected tissue is critical for developing drugs to inhibit or enhance the apoptotic action, and would benefit human health.

3.2. Results.

3.2.1 Detection of apoptosis induced by reovirus infection.

3.2.1.1. Observation of the cell lines on reovirus infection. Three cell lines (Mouse L929 cells, Vero cells and H1299 cells) were used to examine reovirus induced apoptosis. The L929 cells and Vero cells can express p53, but H1299 cells are p53-null. In initial experiments, mouse L929 cells were infected with reoviruses T1L or T3D at multiplicity of infection (MOI) of 100 PFU/cell, the same dose routinely used by others (Tyler et al. 1995). The cells showed CPE at 24 hours post-infection (PI) (Figure **3.1**) and reached more than 99% of CPE less than 72 hours PI. The characteristic CPE was the cells becoming bright, round and smaller, and dislodged from the wall of culture plates (Figure **3.1B, C** and **3.2A**); these results were same as in previous reports (Tyler et al., 1995).

Under natural conditions, reovirus infects human or animals at much lower MOI. Thus, a physiologically relevant question is if less reovirus was used to infect, how would the infected cells react and would they still show CPE? To test this, 2 other doses (MOI=10, or 0.5 PFU/cell) of reoviruses T1L and T3D were used to infect L929 cells. Cells infected with dose of MOI=10 PFU/cell began to show little CPE at 24 hours PI, and over 95% of cells had CPE at 72 hours. However, cells infected with doses MOI=0.5 PFU/cell showed CPE at 48 hours and about 25% of CPE at 72 hours (Figure **3.2A**), and over 95% of CPE happened near 96 hours PI with dose of MOI=0.5 PFU/cell. The infected cell numbers

Figure 3.1.

A. (Mock) **B. (T1L)** **C. (T3D)**

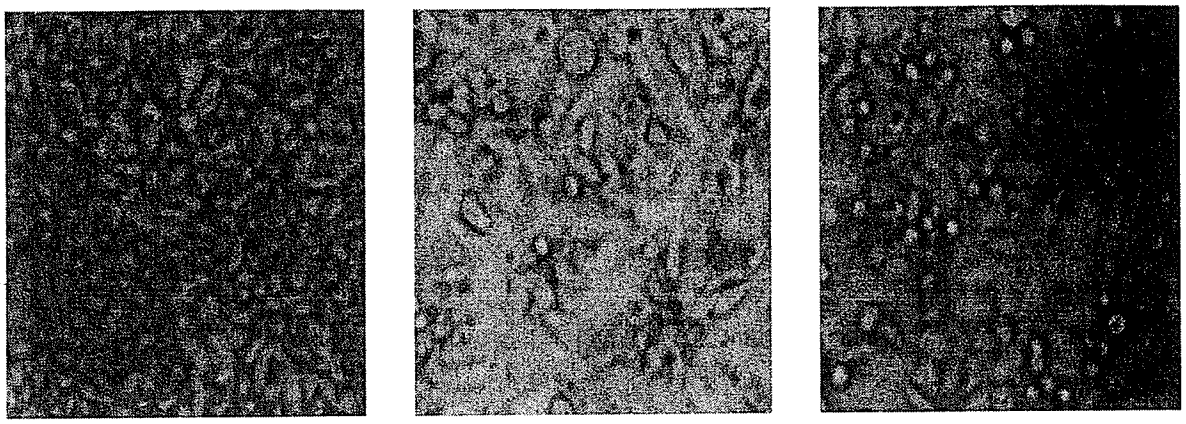


Figure 3.1. L929 cells infected with reoviruses T1L or T3D. L929 cells were infected with mammalian reoviruses T1L or T3D at dose MOI=100 PFU/cell and showed CPE at 24 hours post infection (PI). A. Uninfected control cells; B. Cells infected with T1L; and C. Cells infected with T3D.

Figure 3.2.

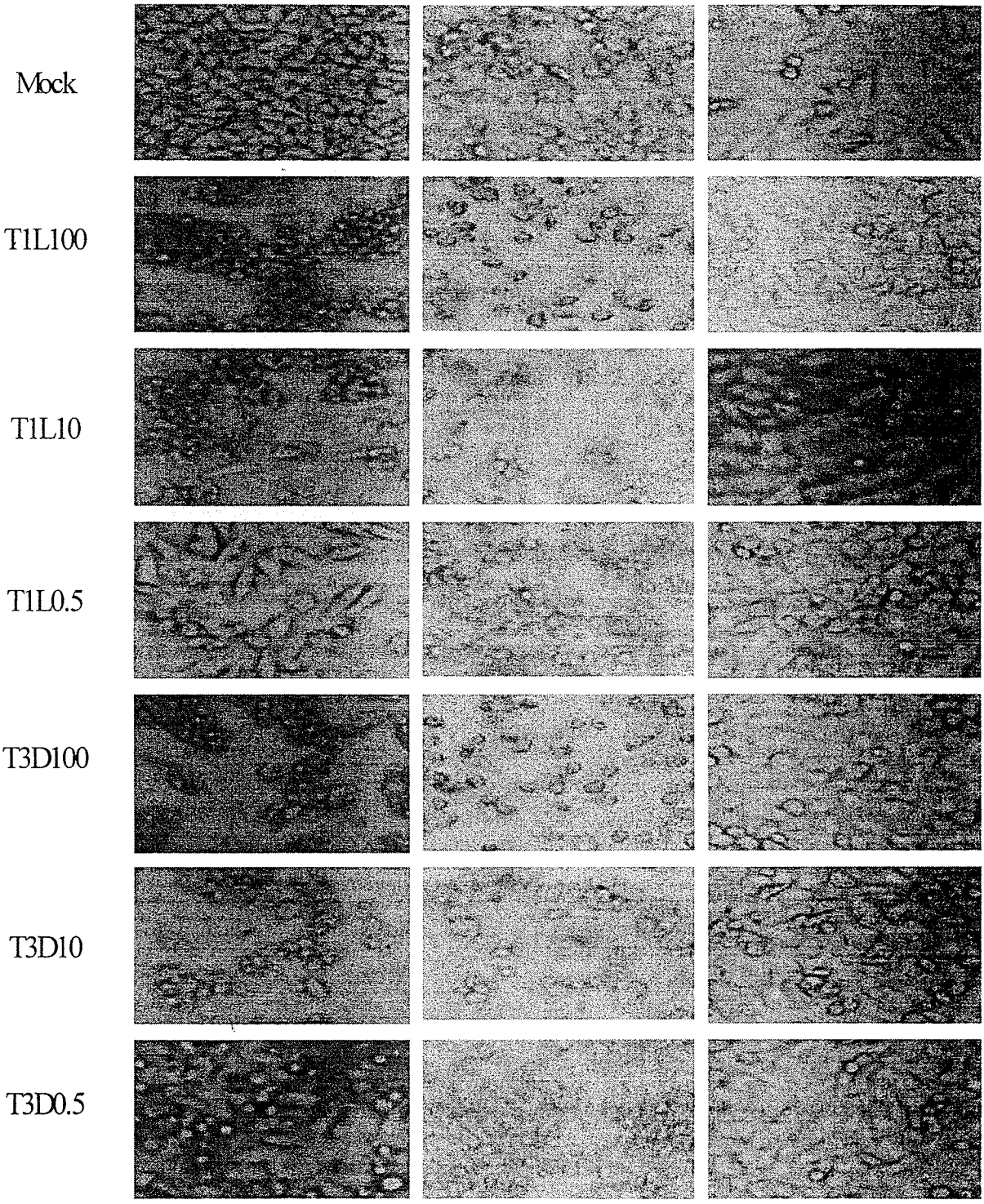

Mock
T1L100
T1L10
T1L0.5
T3D100
T3D10
T3D0.5
A. L929 cells
B. Vero cells
C. H1299 cells

Figure 3.2. Three cells infected with reoviruses T1L or T3D at different doses. L929, Vero, and H1299 cells were infected with mammalian reoviruses T1L or T3D at different doses (MOI=100, 10 or 0.5 PFU/cell) and showed different reaction at 72 hours PI. A. L929 cells infected with reovirus; B. Vero cells infected with reovirus; C. H1299 cells infected with reovirus.

were not changed compared to the mock-infected cell controls at different time points post-infection (Figure **3.5A**).

H1299 cells, a human lung carcinoma cell line, are p53-null (Chen et al., 1996). The p53 protein is a tumor suppressor that controls cellular responses by serving in the nucleus to regulate transcription of genes involved in processes including cell cycle arrest, DNA repair, and apoptosis (Baptiste and Prives, 2003). Under normal conditions, p53 gene in mammalian cells remains silent. When the cells are affected by some stresses, such as UV, or drugs that damage DNA, p53 gene will be activated and transcribed. The expression of p53 activates different cellular signaling pathways to induce the expression of different series of target genes (cell cycling regulated proteins) that perform regulation functions (Baptiste and Prives, 2003). I used the H1299 to test whether and how p53 functions on reovirus replication. Based on the L929 cell results, H1299 cells were also infected with reoviruses T1L or T3D at 3 doses (MOI=100, 10, 0.5 PFU/cell). The H1299 cells reacted completely different with L929 cells after infection. H1299 cells did not show CPE up to 7 days PI no matter what doses of reoviruses were used. Instead, the cells did not grow, but became larger (Figure **3.2C**). The infected H1299 cell numbers declined significantly compared to the mock control at the time points counted (Figure **3.3C**). These data indicated that reovirus infection could stop H1299 cell replication.

Vero cells (Africa green monkey kidney cells) have also been used to culture reoviruses (Keirstead and Coombs, 1998; Butler et al., 2000). Vero cells were also infected with reoviruses at doses of MOI=100, 10 or 0.5 PFU/cell. The cells showed nearly similar characteristics as L929 cells. The infected Vero cells showed CPE after reovirus infection (Figure **3. 2B**) and the numbers of cells were not significantly changed compared to uninfected controls (Figure **3.3B**)

3.2.1.2. Reovirus replication in 3 cell lines. Reovirus infection caused measurably different amounts of CPE in the above cells. I then examined whether the different cellular changes affected virus replication. Virus cultures of L929 cells, H1299 cells and Vero cells were monitored for virus replication by plaque assays after cells were harvested at different time points. Reoviruses replicated in all 3 cell lines, and reached very high titers that were not significantly different in the three cell lines (Figure **3.3-III**). Similar replication patterns were also observed in the 3 cell lines; that is a 24 hour 'lag'

Figure 3.3.

A. L929 cells B. Vero cells C. H1299 cells

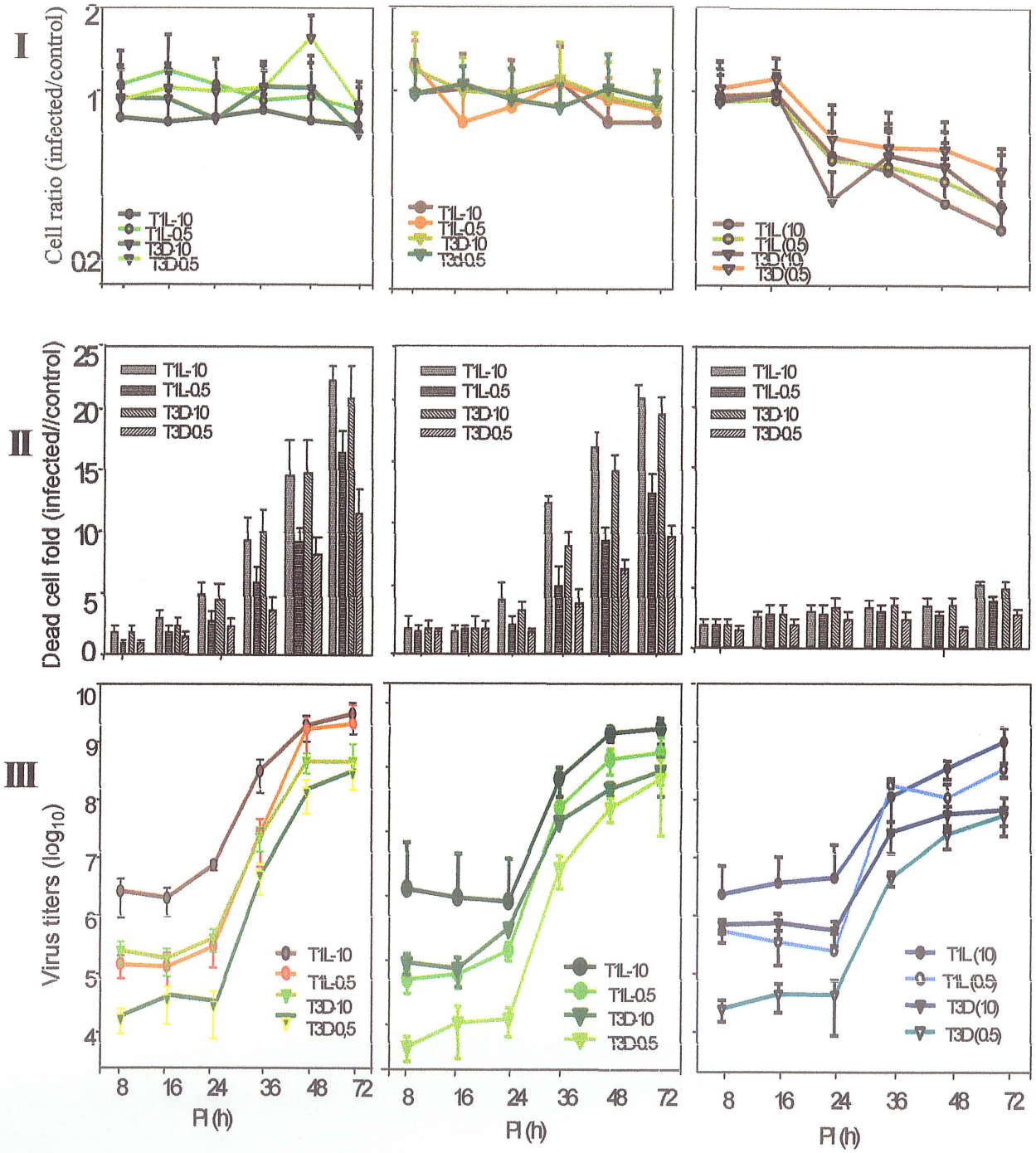


Figure 3.3 Growth curves of cell lines, dead cell ratio and virus growth in 3 cells infected with reoviruses . Three cell lines infected with reoviruses T1L or T3D at doses of MOI=10 or 0.5 PFU/cell were incubated and harvested at different time points. **(I) Growth curves of cells:** The total numbers of cells were counted with a hemocytometer. The results were expressed as the mean ratios of total cells of the infected groups compared with the mock groups. **(II) Dead cell ratio:** The cell numbers were counted by a hemocytometer. The results were expressed as the mean folds of dead cells of the infected cells compared with the mock cells. **(III) Growth curves of reovirus:** The virus titers were measured by plaque assays and the results were expressed as the mean virus titers. **A.** L929 cells; **B.** Vero cells; and **C.** H1299 cells. The data were from three independent experiments (n=3). Error bars indicate standard deviation of the means.

was followed by a 100-1000 fold amplification within the next 48-72 hour PI (Figure **3.3-III**). Reovirus T1L also generally replicated in the 3 cells 3-10 fold higher than reovirus T3D did (Figure **3.3-III**). When using lower doses of reovirus infection (MOI=0.5 PFU/ml), the virus replication curves showed a little later for the virus reaching to the highest titer level (Figure **3.3-III**).

3.2.1.3. Apoptotic cells measure. There are numerous reports confirming the function of reovirus induced cell apoptosis and experimental reovirus infection has been considered one of the most thoroughly investigated viral models of apoptosis (Barton et al., 2001; Clarke and Tyler, 2003; Clarke et al., 2005a; 2005b; Forrest et al,. 2003; O'Donnell et al., 2003; Tyler et al., 2001). Reovirus type 3 strains (T3D, T3A) can more strongly induce cell apoptosis than type 1 strain T1L (Tyler et al., 1995).

In order to know the functions of cellular pathways on reovirus induced apoptosis, I first should perform some experiments for reovirus induced apoptosis in our conditions. The L929 cells were infected with reoviruses T1L or T3D at the dose of MOI=100 PFU/cell, and the infected L929 cells were examined for apoptotic change by staining with Annexin-V and 7-aminoactinomycin (7-AAD) and cells counted by FACS (Figure 3.4). The results showed that reovirus induced cell apoptosis beginning at 12 hours PI with about 5% apoptotic cells, over 50% apoptotic cells at 36 hours and 100% cell apoptosis at 72 hours when infected with the dose of MOI=100 PFU/cell (Table **3. 1**). The 2 reovirus strains T1L and T3D showed almost similar capacity to induce cell apoptosis in L929 cells (Table **3.1; 3.2**). The double positive staining of Annexin-V and 7-AAD of the cells were also considered as the apoptotic change (it is a characteristic of later cell apoptosis). An additional three methods: (1) Annexin-V and 7-AAD stain combined with FACS; (2) acridine orange and propidium iodide staining with Fluorescent microscope (Figure **3.5**); and (3) trypan blue were compared to count apoptotic cells. Trypan blue staining is simple and easy to perform under different time points. Therefore, since results were not different between these methods, most experimented data were from trypan blue stain method.

In L929 cells infected with reoviruses, there were 5 fold more dead cells when cells were infected with T1L or T3D at dose of MOI=10 or 0.5 PFU/cell than the mock control at 36 hours PI. At 48 hours PI, the dead cells increased significantly and were 10-fold

Figure 3.4.

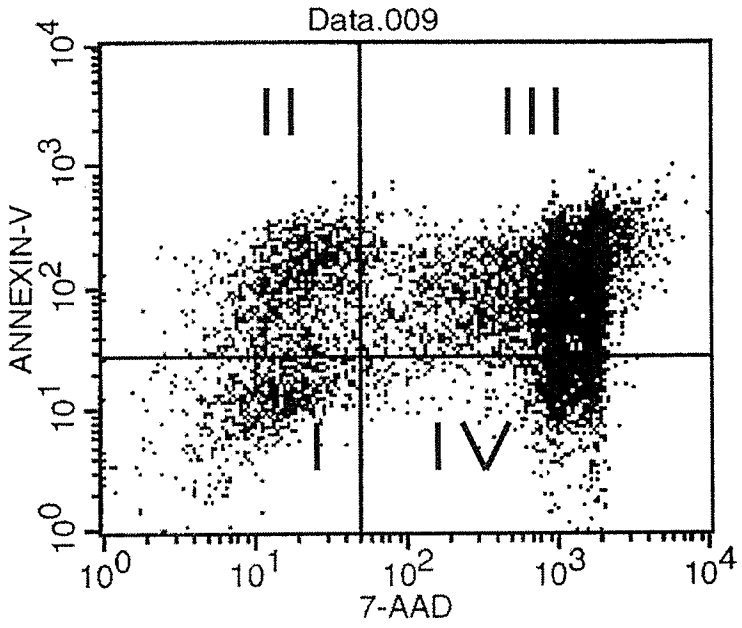


Figure 3.4. Apoptotic cells measured by FACS. A flow cytometry profile showing cell apoptotic change distribution of L929 cells infected with reovirus T1L at MOI=100 PFU/cell for 48 hours and stained with Annexin-V and 7-ADD. **1**. alive cells; **II.** apoptotic cells; **III**. later apoptotic cells or necrotic cells; and **IV**. necrotic cells.

Table 3.1. Results of apoptotic cells infected with reoviruses at dose of MOI=100 PFU/cell detected and measured with FACS.

Virus	Time (hours PI)	Apoptosis (%)	Living (%)	Later apoptosis or necrosis (%)
T1L	**12**	**2.98**	**83.16**	**12.88**
	36	**12.63**	**43.11**	**38.38**
T3D	**12**	**2.72**	**84.43**	**12.05**
	36	**23.22**	**38.84**	**29.79**

Table 3.2. Results of apoptotic cells infected with reoviruses at different doses were harvested at 48 hours PI and detected with FACS.

Virus	MOI (PFU/cell)	Apoptosis (%)	Living (%)	Later apoptosis or necrosis (%)
T1L	**100**	**10.20**	**4.99**	**82.32**
	10	**9.58**	**9.99**	**78.83**
	0.5	**4.31**	**74.85**	**19.95**
T3D	**100**	**25.16**	**3.24**	**71.11**
	10	**17.47**	**16.46**	**61.48**
	0.5	**9.32**	**74.85**	**13.56**

Figure 3.5.

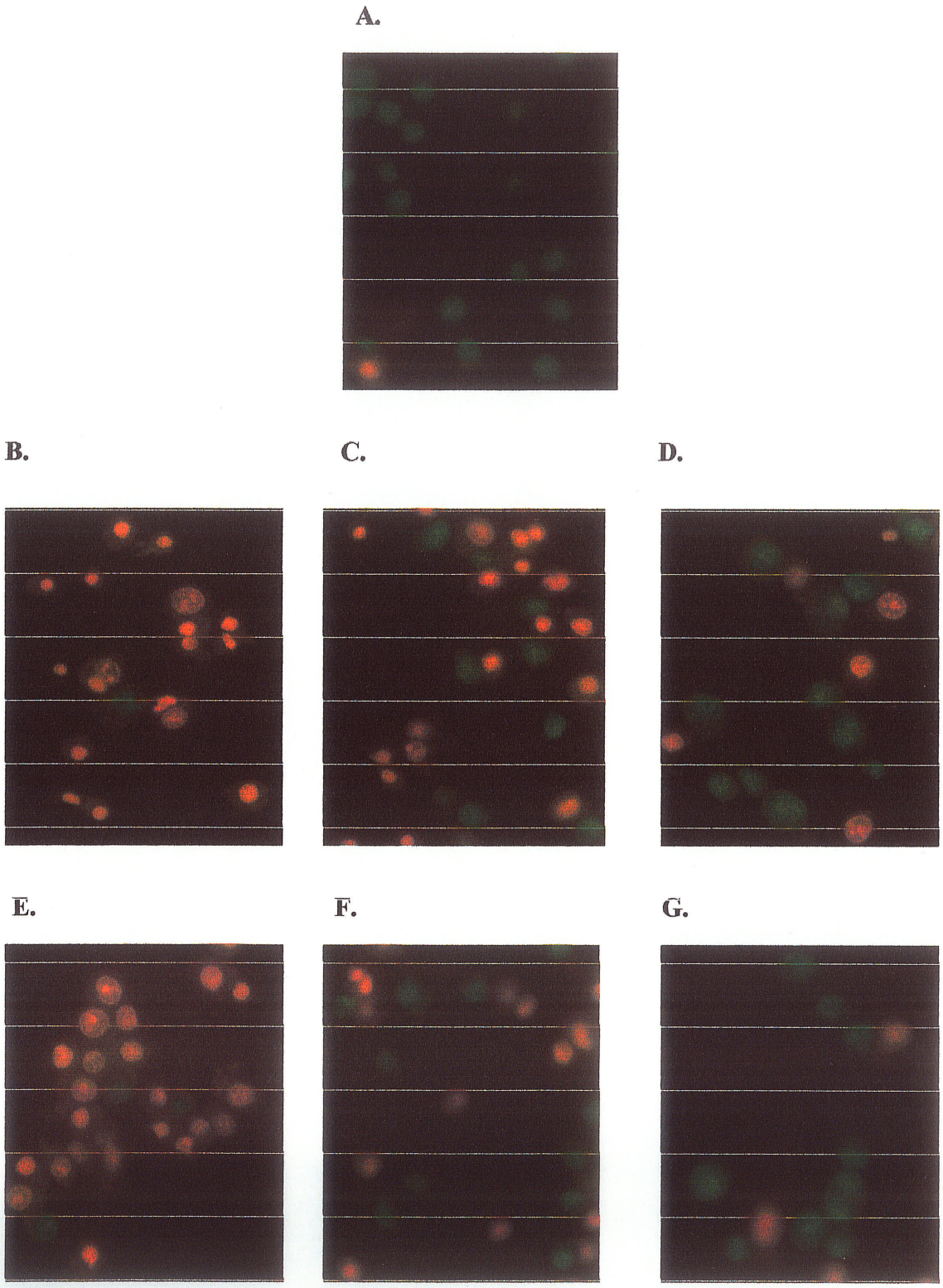


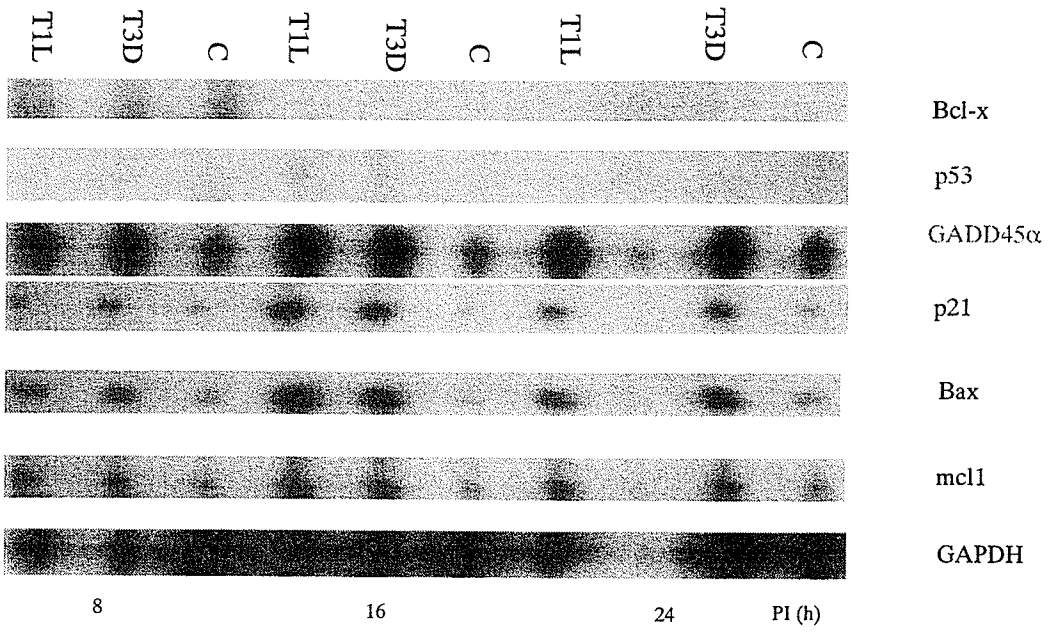
Figure 3.5. Apoptosis detection of L929 cells infected with reoviruses by epi-illumination fluorescence microscopy (FM). L929 cells infected with reoviruses T1L or T3D at three doses (MOI=100, 10, or 0.5 PFU/cell) were harvested at 72 hours PI. The cells were stained with acridine orange staining and detected with an epi-illumination fluorescence microscopy (FM). **A**. Uninfected control cells; **B**. **C**. **D**. Cells infected with T1L at dose of MOI=100, 10, or 0.5 PFU/cell individually; **E**. **F**. **G**. The cells infected with T3D at dose of MOI=100, 10, or 0.5 PFU/cell individually.

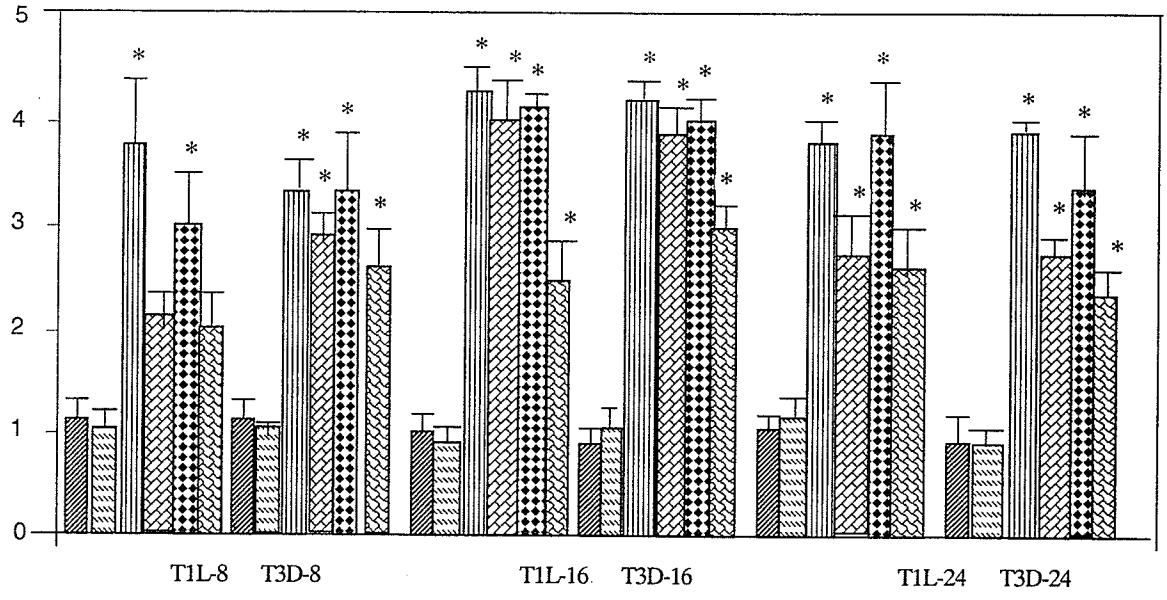
higher in T1L infection at MOI=10 or 0.5 PFU/cell and T3D at dose of MOI=10 PFU/cell than the control, and about 8-fold more in T3D at dose of MOI=0.5 PFU/cell than the control. By 72 hours PI, almost all cells had died (very few living cells were counted) when cells were infected with T1L at doses of MOI=10 and T3D at dose of MOI=10 PFU/cell (Figure **3.3.II A**). The cell counting results matched infected cell CPE. In Vero cells, dead cells were increased as the period of infection increased. At 36 hours PI, the dead cells began to increase. The cells infected with T1L or T3D at doses of MOI=10 or 100 PFU/cell were almost dead to the same extent as L929 cells at 72 hours PI (Figure **3.3.II B**). H1299 cells, infected with reoviruses T1L or T3D at same doses, remained alive up to 96 hours PI (Figure **3.2.C**); the dead cells did not significantly increase and did not show much difference no matter the infection doses (Figure **3.3.II C**).

3.2.2. Detection of cellular gene response. Cells respond to reovirus induced apoptosis with various signaling pathways. These include the TRAIL signaling (Clarke et al., 2000). Reovirus-induced apoptosis is inhibited in MDCK cells with over-expressed Bcl-2 (Rodgers et al., 1997). Reovirus infection transiently activates NF-κB in a variety of cell types (Connolly et al., 2000) and reovirus infection results in viral strain-specific pattern activation of JNK and c-Jun (Clarke et al., 2001b). It is not clear whether cellular p53 and p53-related genes respond to reovirus-induced apoptosis. Therefore, a human stress multi-probe RNA protection assay (RPA) from BD Biosciences (hStress-1) including p53 and some p53-regulated genes: GADD45α (Kastan et al., 1992), p21 (Harper et al., 1993), bax (Myeshta and Reed, 1993) was used to detect whether these genes respond to reovirus infection in L929 cells (mouse fibroblast cells) and H1299 cells (p53-null human lung carcinoma cell line) with RNA protection assays (RPA). The infected L929 cells showed that GADD45α, p21, Bax, and mcl1 gene RNAs were induced 2-4 fold higher in T1L and T3D infected cells than the uninfected control from 8 to 24 hours PI and p53 was not induced to express (Figure **3.6A**). When H1299 cells were infected with reoviruses, GADD45α, p21, bax, and mcl1 RNA in the two virus infected groups increased much more than the mock control from 8 to 36 hours PI. In particular, GADD45α RNA was constantly induced more than 4 fold in two infected cells and p53 RNA also did not appear to be induced (Figure **3.6B**). p21, bax, or mcl-1 mRNA was variably transcribed with the different hours PI in the cells. However, there also appeared

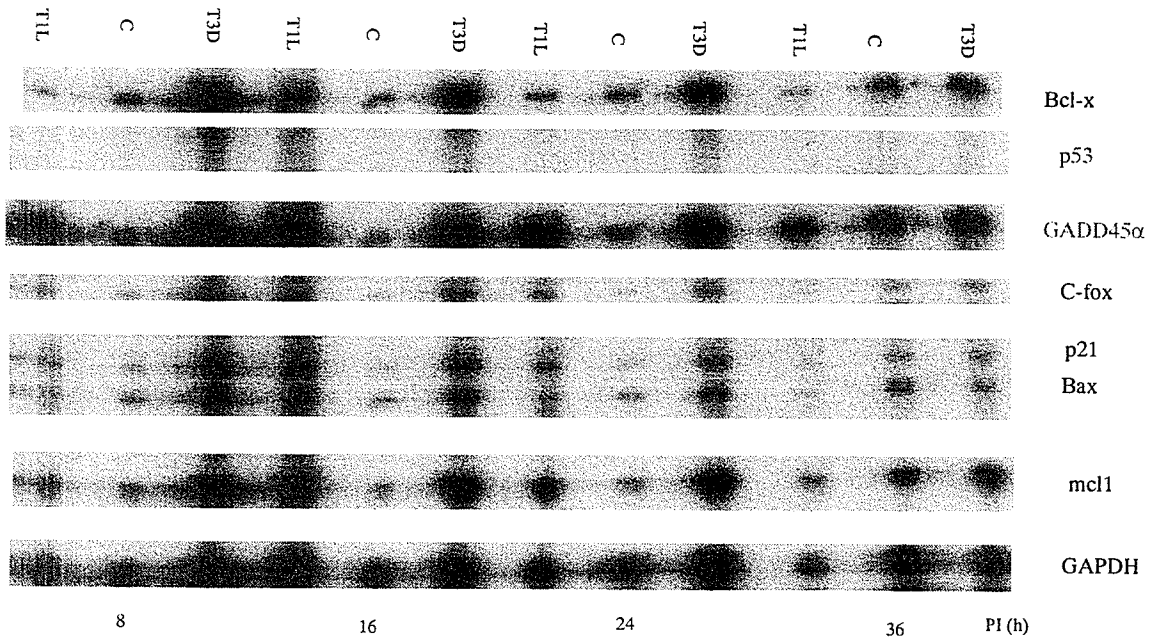
Figure 3.6.

A (L929).





B (H1299).


T1L
C
T3D
T1L
C
T3D
T1L
C
T3D
T1L
C
T3D
Bcl-x
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GADD45α
C-fox
p21
Bax
mcl1
GAPDH
8
16
24
36
PI (h)

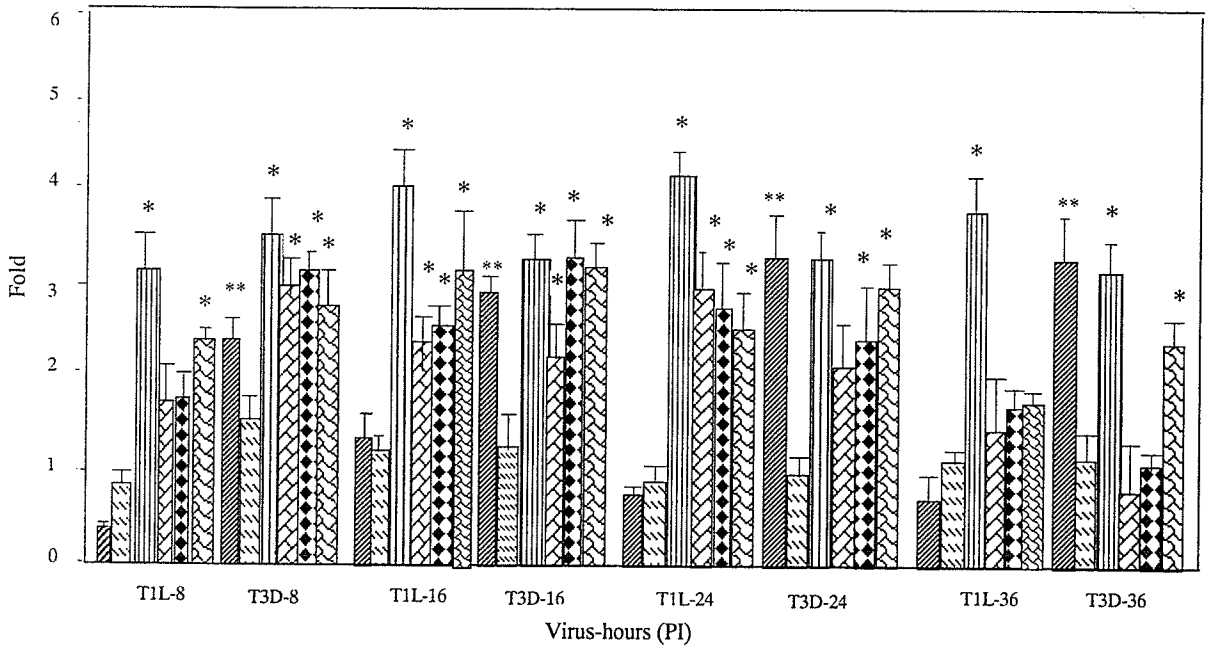

Fold
0
1
2
3
4
5
6
T1L-8
T3D-8
T1L-16
T3D-16
T1L-24
T3D-24
T1L-36
T3D-36
Virus-hours (PI)

Figure 3.6. Detection of hStress gene mRNAs of the reovirus infected cells by RPA. The total RNA of the cells infected with reoviruses at dose of MOI=100 PFU/cell were prepared and the mRNAs of human hStress genes were detected with hStress multi-probe template set by RPA. The mRNA density levels of the genes transcribed were analyzed with 'Scion Image' program; and the results were expressed as the mean fold of mRNA in the infected cells compared to the mock cells from three independent experiments (n=3). Error bars indicate standard deviation of the means. **A**. L929 cells; and **B**. H1299 cells. Bcl-x; p53; GADD45α; p21; bax; and Mcl1. Single asterisks ($P < 0.05$) indicate asignificant difference of these gene mRNA in cells infected with reovirusT1L or T3D compared to that in the mock control at different time points. Double asterisks ($P < 0.05$) indicate significant difference of the Bcl-x mRNA level in the cells infected with reovirus T3D compared to that in the cells infected with T1L (Comparison of P values by ANOVA's *F*- test and Q-test).

to be virus type-3 specific responses in H1299 cells. The bcl- x mRNA of the cells infected with reovirus T1L was inhibited to transcribe. However, the bcl-x RNA in the cells infected with T3D was induced to increase 3-5 fold higher from 24 to 36 hours PI; thus, there was a sighnificant difference in the mRNA levels of Bcl-x in the cells infected with reovirus T1L compared to T3D (Figure **3.6B**). However, bcl-x gene mRNA did not respond in the L929 cells infected with reovirus (Figure **3.6A**)

3.2.3. p53 function on reovirus infection. The p53 protein is a tumor suppressor that controls cellular responses by functioning in the nucleus to regulate transcription of genes involved in processes including cell cycle arrest, DNA repair, and apoptosis (Baptiste and Prives, 2003). In the previous experiments, the p53 gene did not respond to reovirus infection, and no p53 mRNA was detected in infected L929 cells and H1299 cells (Figure **3.6**). Reoviruses also replicated well in H1299 cells (Figure **3.3**). In order to determine the function of p53 on reovirus infection, a tetracyclin inducible transcient expression of wild –type p53 gene H1299 cell line (gotten from Dr. Chen's lab) (Chen et al., 1996) was infected with reoviruses. Reovirus strains T1L and T3D could replicate in the H1299 cells containing wild-type p53 gene very well. The titers of both reoviruses were almost the same in the production and replication curves in the p53-positive and p53-null H1299 cells (p53-null) (Figure **3.7**). However, p53 RNA was induced to express heavily in p53-transfected H1299 cells (Figure **3.8**). This further indicates that p53 did not function in reovirus infection.

3.2.4. Detection of more genes that responded in reovirus infection. Another human apoptosis multi-probe template set, the hAPO-3 (BD Biosciences) including TRAIL (Clarke et al., 2000; 2001) and caspase-8 (Kominsky, et al. 2002) that were induced to respond in reovirus induced apoptosis, was also used to examine other cellular RNA reaction in the L929 cells and H1299 cells infected with reoviruses at dose of MOI=100 PFU/cell. The RNA of Caspase-8, FASL, DR3, TRAIL, TRADD and RIP of the infected L929 cells harvested at 8 and 16 hours PI did not increase much compared with the mock infection control cells (Figure **3.9A**). The same situation was also seen in the H1299 cells between the infected and mock control cells (Figure **3.9B**).

3.2.5. Functions of cellular GADD45α in reovirus infection.

3.2.5.1. GADD45α RNA response at the different doses of reovirus infection. The

Figure 3.7.

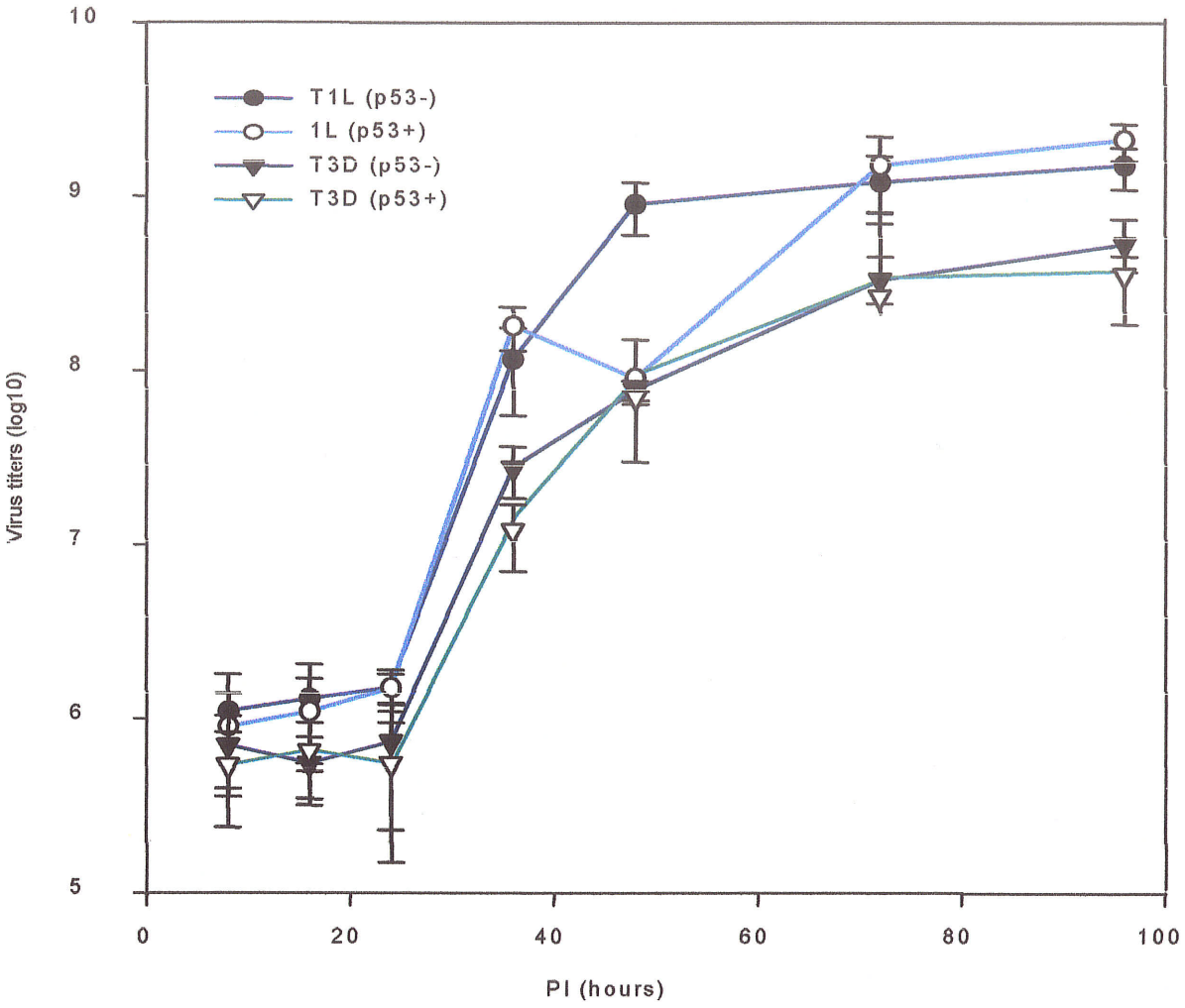


Figure 3.7. Function of p53 on reovirus replication. H1299 cells transfected with human wild type p53 were controlled by tetracycline. The cells were induced to express p53 and infected with reoviruses T1L or T3D at dose of MOI=100 PFU/cell. The virus cultures were harvested at different time points and the titers were measured with plaque assays. $p53^-$ + T1L (—●—) ; $p53^+$ + T1L (—○—); $p53^-$ + T3D (—▼—); and $p53^+$ + T3D (—▽—). The data represents the average of three experiments (n=3) and the error bars represent one standard deviation.

Figure 3.8.

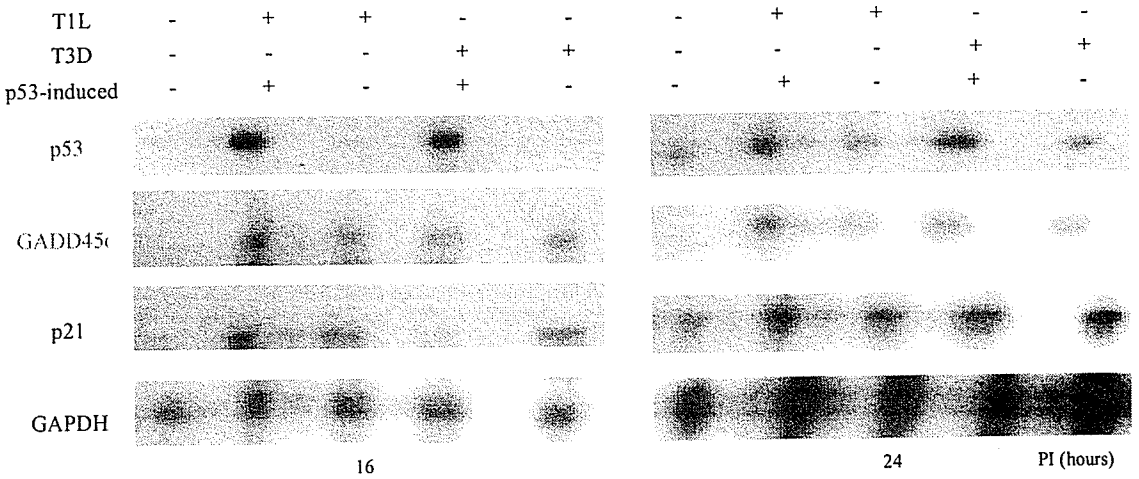
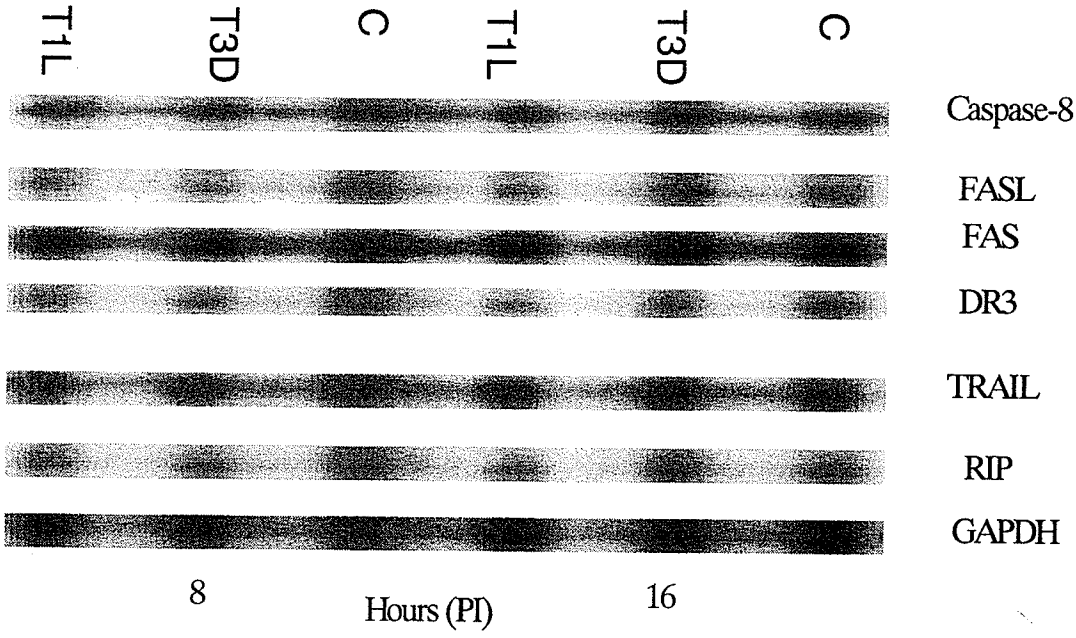
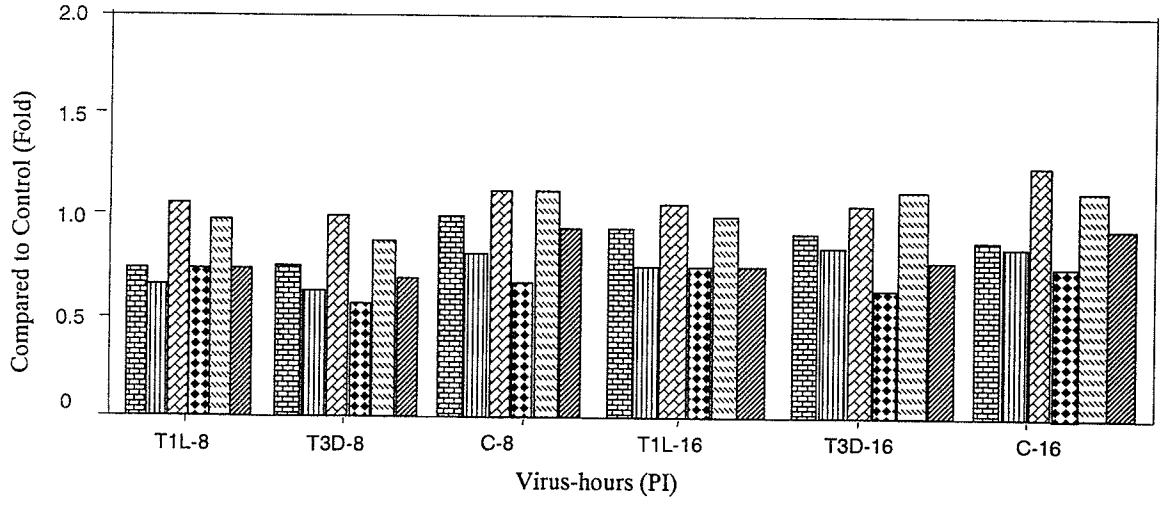


Figure 3. 8. Detection of the mRNAs of p53 and some p53-related genes in the p53 inducible cells infected with reoviruses. The p53 inducible cell lines were cultured and induced to express p53 by withdrawing tetracycline and infected with reoviruses T1L or T3D at dose of MOI=100 PFU/cell at same time. After 16 or 24 hours, the total RNA was purified from the cells and the p53 and p53 related genes were analyzed with the h-Stress multi-probe template set by RPA. The gels were dried and exposed to X-ray film. The p53, p21, GADD45 and GAPDH (the sample control) are indicated.

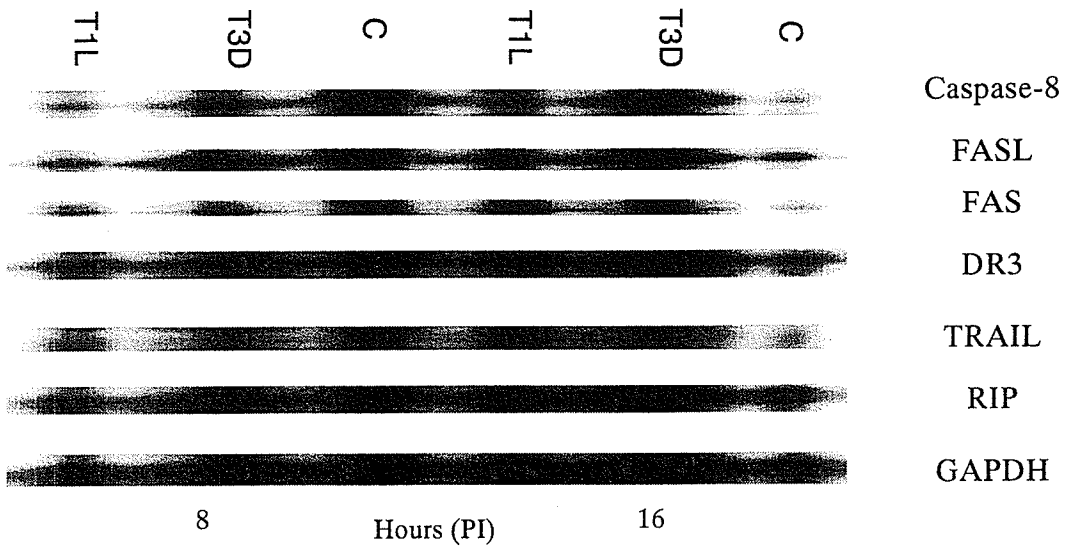
Figure 3.9.

A (L929).





B (H1299).



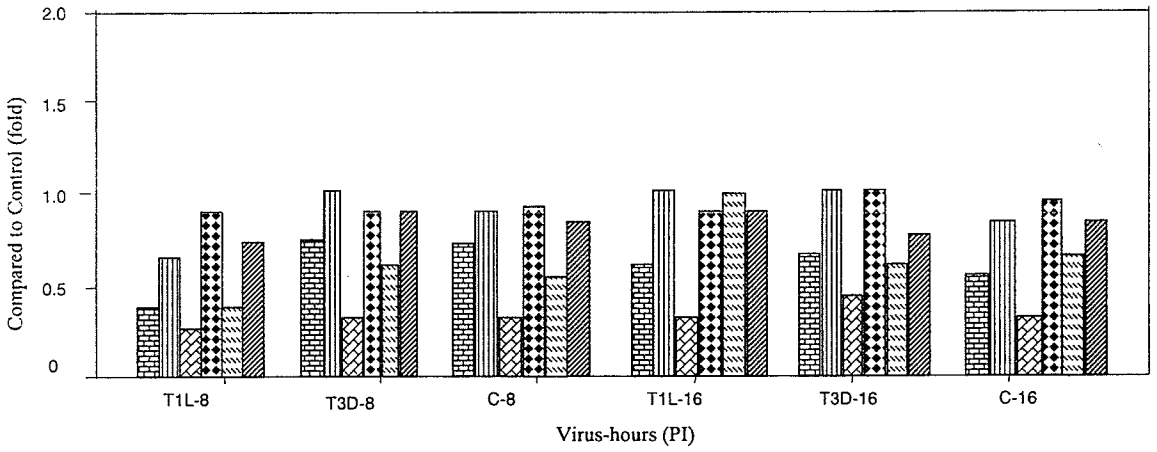


Figure 3.9. Detection of human hAOP-3 related gene mRNA by RPA. The total RNA of the cells infected with reoviruses at dose of MOI=100 PFU/cell were prepared and the mRNAs of human hAOP-3 genes were detected with hAOP-3 multi-probe template set by RPA. The mRNA levels of the genes transcribed were detected and the gene density on the X-ray films was analyzed with 'Scion Image' program. **A.** L929 cells; and **B.** H1299 cells. Caspase-8; FASL; FAS; DR3; TRAIL; and RIP.

growth arrest- and DNA-damage-inducible protein 45 (GADD45α) is a cell cycle regulator. It was shown that GADD45α interacts with and inhibits the kinase activity of the cdk1/cyclin B1 complex (Zhan et al., 1999). This function of GADD45α has been implicated in activating a G_2/M checkpoint in response to certain genotoxic stress agents, such as UV radiation (Wang, et al. 1999). In the experiments reported in this thesis, GADD45α mRNA of the L929 cells and H1299 cells infected with reoviruses at dose of MOI=100 PFU/cell were positively induced to transcribe over 3- 4 fold more than the mock control. The transcription of GADD45α mRNA was also stable with two virus strains and persisted for a long period of time (Figures **3.6**). Therefore, I concentrated this research on the reaction of GADD45α gene to reovirus infection and tried to find the function of the GADD45α protein in the cells infected with reovirus. In order to monitor the expression of GADD45 in reovirus infection, first, the L929 cells and H1299 cells were infected with reoviruses at different doses of MOI=100, 10 or 0.5 PFU/cell, and GADD45α mRNA was detected with RPA. The results showed that the induction of GADD45α was dependent on the doses of reovirus infection. If the cells were infected with large amounts of reovirus (MOI=100 or 10 PFU/cell), the GADD45α mRNA was induced to transcribe in the infected cells. The RNA levels increased a little more in the cells infected with reovirus at dose of MOI 100 PFU/cell; however, there was not much difference between the cells infected with MOI=100 PFU/cell or MOI=10 PFU/cell (Figure **3.10A**). Therefore, the GADD45α mRNA of the cells infected with reoviruses at doses of MOI=100, or 10 PFU/cell was transcribed 4-5 fold more than that of the cells infected with the viruses at dose of MOI=0.5 PFU/cel (Figure **3.10A**). The GADD45α was also induced in H1299 cells infected with reovirus and showed the similar expression pattern as in the infected L929 cells (Figure **3.10 B**). There was also no obvious transcription of GADD45α mRNA in the 2 cells infected with reovirus at dose of MOI=0.5 PFU/cell; and there was not much difference in the induction of GADD45α in these cells with reovirus strains T1L or T3D (Figure **3.9**).

3.2.5.2. Expression of GADD45α protein in the cells infected with reovirus. Next, I investigated whether the GADD45α protein was expressed and how much expression compared to GADD45α mRNA transcription in these cells infected with reoviruses.

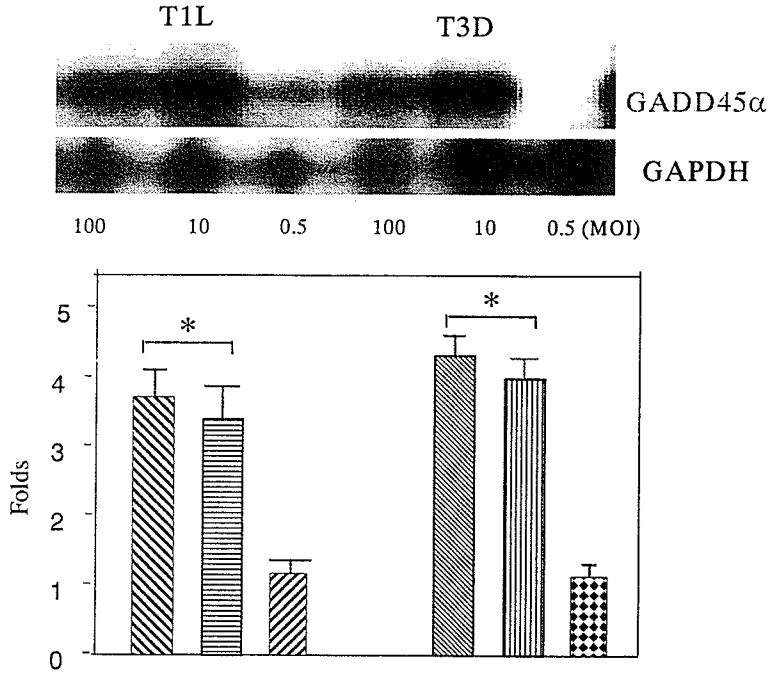
Western blotting with specific antibodies for GADD45α was used to detect the levels of GADD45α protein in the infected cells. As in Figure **3.11**, the expression of GADD45α protein was increased and can be detected in the L929 cells infected with reovirus at doses of MOI=100 PFU/cell and MOI=10 PFU/cell from 24 hours PI. GADD45α reached the highest levels by 48 and 72 hours PI. The expression of GADD45α appeared more in the infected cells with the larger dose of reoviruses (MOI=100 PFU/cell) than in the cell with dose of MOI=10 PFU/cell, but the levels of their expression were not significantly different. The cells did not express the GADD45α protein when infected with reoviruses at dose of MOI=0.5 PFU/cell during the whole periods of virus replication. The patterns of GADD45α expression appeared the same in the cells infected with reovirus strain T1L or T3D (Figure **3.11**), and also expressed in H1299 cells and Vero cells infected with reoviruses at larger doses. The expression patterns of GADD45α protein in the three cell lines were very similar after reovirus infection (Figure **3.11**).

3.2.5.3. Detection of GADD45α protein in cells infected with dead reoviruses. UV-inactivated reoviruses can induce cell apoptotic change (Tyler et al.,.1995). We wondered whether the expression of GADD45α protein could be induced in cells infected with dead reoviruses. Purified reovirus particles were inactivated with UV radiation, no living virus was detected by plaque assays after inactivation. L929 cells were infected with the inactivated T1L or T3D virus particles at the same particle doses corresponding to living reoviruses T1L or T3D at MOI=100, 10 or 0.5 PFU/cell. The L929 cells infected with inactivated reoviruses did not induce the expression of GADD45α protein (Figure **3.12**). This indicates the expression of GADD45α protein was only induced by living reoviruses at doses of MOI=100 or 10 PFU/cell.

3.2.5.4. Inhibition of GADDD45α protein expression by siRNA effects reovirus replication. Previous results showed that reovirus infection could induce the transcription and expression of cellular GADD45α gene. Next, siRNA inhibition of cellular GADD45α was performed to determine whether the specific inhibition affected reovirus replication. Two sets of GADD45α siRNA fragments "human GADD45α siRNA (h): sc-35440 and mouse GADD45α siRNA (m): sc-35439" (Santa Cruz Bio. Santa Cruz, CA) were transfected into L929 cells or H1299 cells as described in **2.7.1** and

Figure 3.10.

A (L929).



B (H1299).

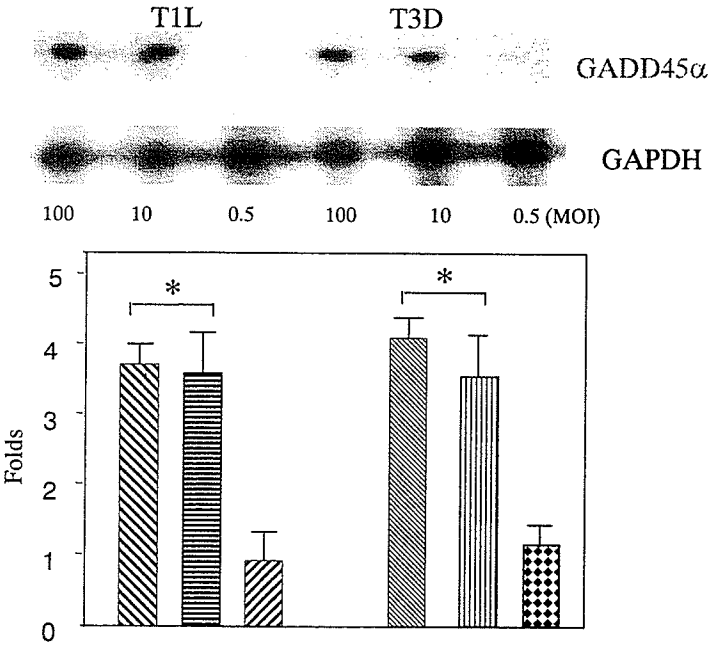


Figure 3.10. Detection of GADD45α mRNA levels in the cells infected with reoviruses at three doses. L929 cells and H1299 cells were infected with reoviruses T1L or T3D at three doses and collected at 16 hours PI. The total RNA of the cells were prepared and detected by RPA. The gene density on the X-ray films was analyzed with 'Scion Image' program and the results were expressed as the mean fold of mRNA of the infected cells compared with the mock cells from three independent experiments (n=3). Error bars indicate standard deviation of the means. **A**. L929 cells; and **B**. H1299 cells. T1L=100 PFU/cell; T1L=10 PFU/cell; T1L=0.5 PFU/cell; T3D=100 PFU/cell; T3D=10 PFU/cell; and T3D=0.5 PFU/cell. Asterisks ($P < 0.05$) indicate significant difference in comparison of GADD45α mRNA in cells infected with T1L or T3D at the doses (MOI=100 and 10 PFU/cell) to that at the dose of MOI=0.5 PFU/cell (Comparison of P values by ANOVA's *F*- test and Q-test).

Figure 3.11.

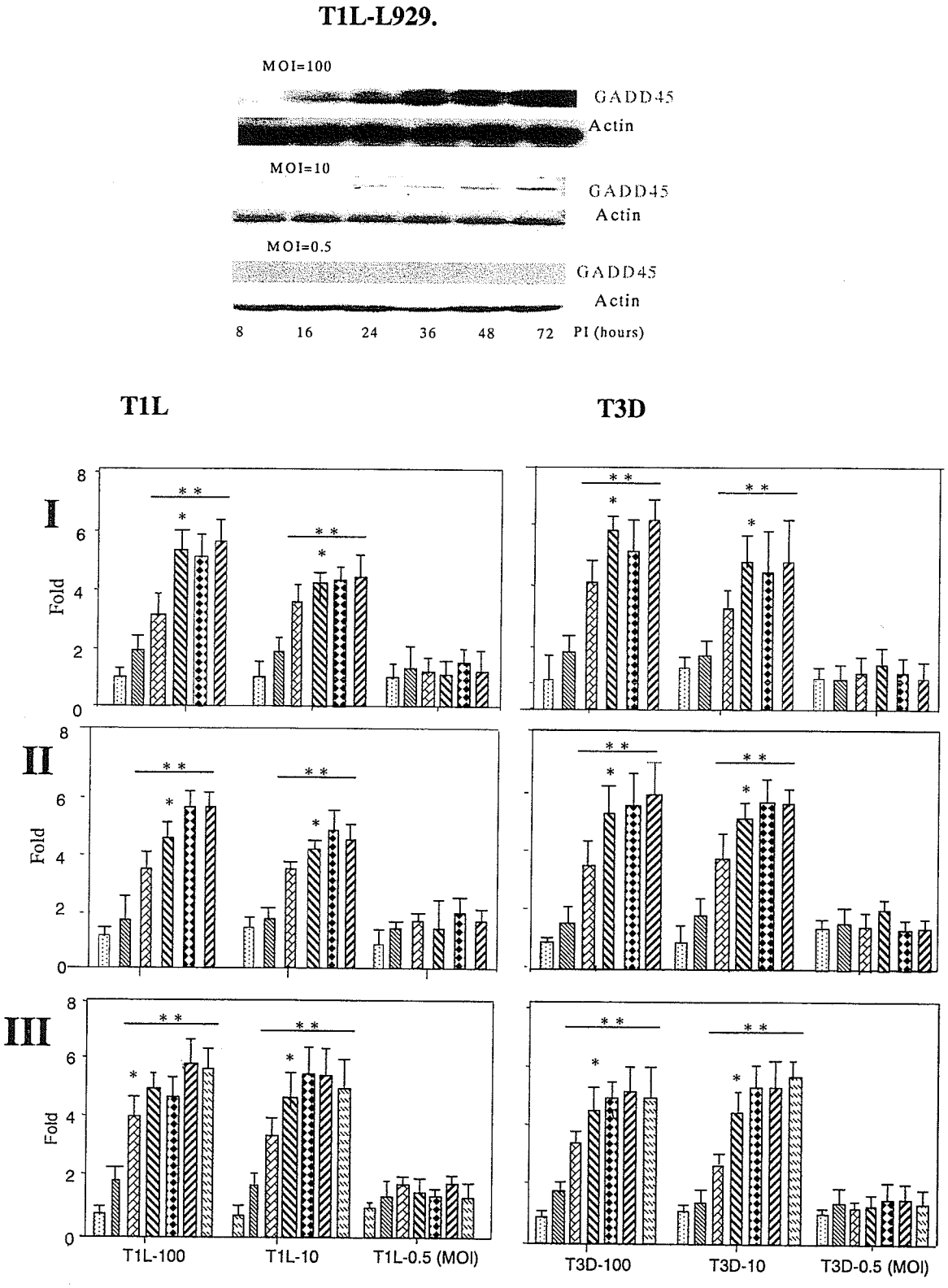
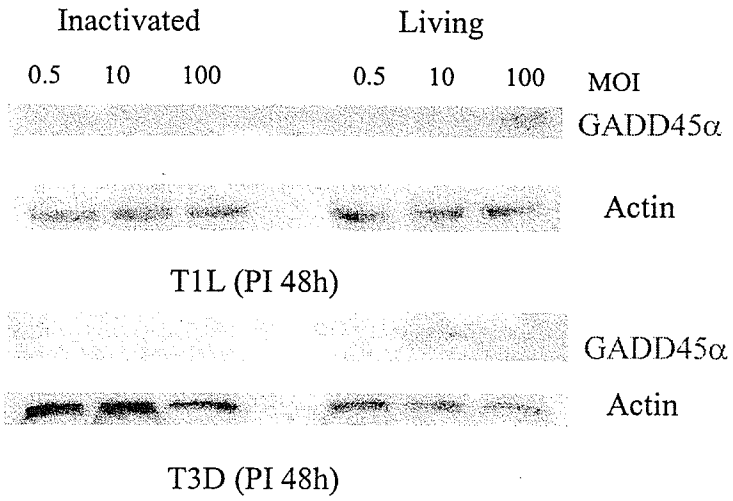

T1L-L929.
MOI=100
GADD45
Actin
MOI=10
GADD45
Actin
MOI=0.5
GADD45
Actin
8 16 24 36 48 72 PI (hours)
T1L
T3D
I
II
III
Fold
T1L-100
T1L-10
T1L-0.5 (MOI)
T3D-100
T3D-10
T3D-0.5 (MOI)

Figure 3.11. Detection of GADD45α protein by Western blotting. The cell lysates of three cells infected with reoviruses T1L or T3D and harvested at indicated time points were analyzed by Western blotting to detect the expressed levels of GADD45α protein, with actin as control. The protein density on the X-ray film was analyzed with 'Scion Image' program and the results were expressed as the mean folds of GADD45α protein of the infected cells divided by the control of actin level from three independent experiments (n=3). Error bars indicate standard deviation of the means. (**I**) L929 cells; (**II**) Vero cells, and (**III**) H1299 cells. 8hours PI; 16hours PI; 24hours PI; 36hours; 48hours PI, 72hours PI and 96hours PI. Single asterisks ($P < 0.05$) indicate significant difference of GADD45α protein level in cells infected with reovirus compared to the preceding times during the different times PI; and double asterisks ($P < 0.05$) indicate significant difference of GADD45α protein level in cells infected with reovirus at doses (MOI=100 or 10 PFU/cell) compared to these at dose of MOI=0.5 PFU/cell (Comparison of P values by ANOVA's *F*- test and Q-test).

Figure 3.12.

A (L929).



B (H1299).

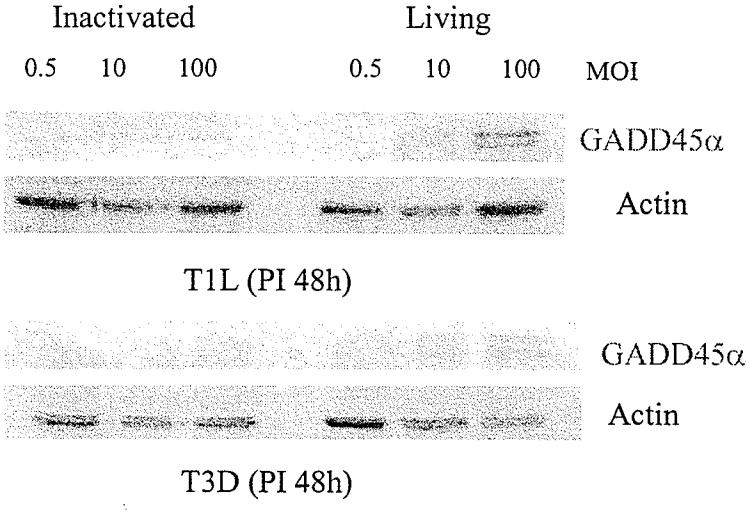


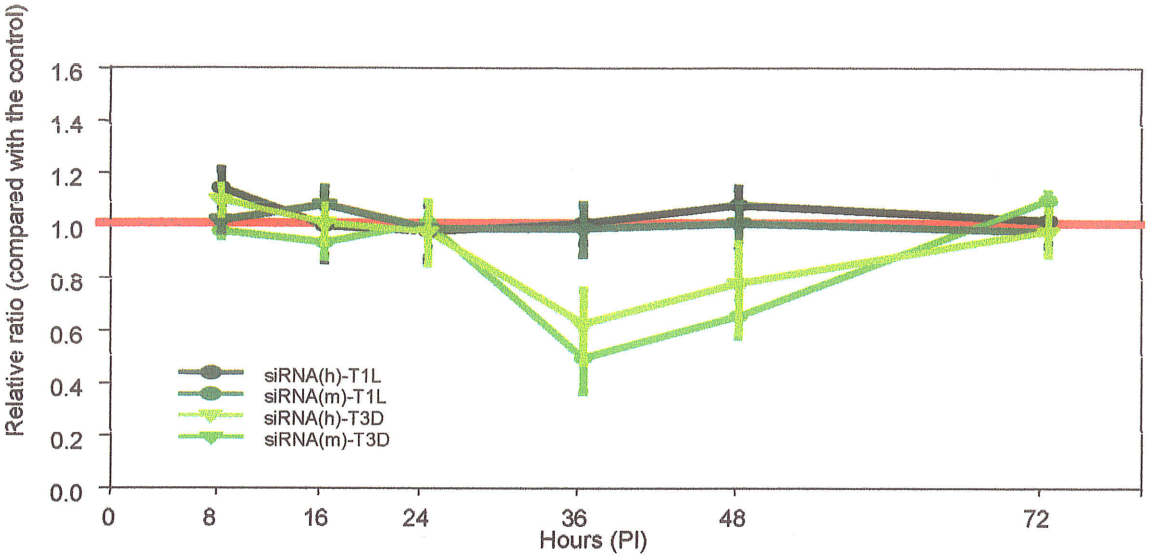
Figure 3.12. Comparison of GADD45α protein induced by different treatment of reovirus. The cells were "infected" with UV-killed reoviruses or live viruses at doses of MOI=100, 10 or 0.5 PFU/cell and harvested at 48 hours PI. The cell lysates were measured for the GADD45α protein by Western blotting. The experiments were repeated three times. **A.** L929 cells; and **B.** H1299 cells.

infected with reoviruses T1L or T3D at dose of MOI=10 PFU/cell at 24 hours post-transfection. The virus titers of the cell cultures were collected at various time points and measured. The results showed that virus replication could be inhibited at 36 and 48 hours PI in the cells transfected with same source of GADD45α-siRNA (Figure **3.13**). The virus titers of the L929 cells transfected with mouse GADD45α-siRNA (m) were about 2 fold less than the control or when a different source of GADD45α-siRNA (h) was used and transfected at 36 or 48 hours PI (Figure **3.13A**). The virus titers of the H1299 cells transfected with human GADD45α-siRNA (h) were about 2-4 fold less than the control or when the different source of GADD45α-siRNA (m) was used and transfected at 36 or 48 hours PI (Figure **3.13B**). However, there was not much difference of the virus titers in these two cells transfected with GADD45 α-siRNA and infected with reoviruses at time points 8, 16, 24, or 72 hours PI (Figure **3.13**). To detect whether the expression of GADD45α protein was affected by GADD45α siRNA, additional L929 and H1299 cells were transfected with these sets of siRNA. For L929 cells, the expression of GADD45α protein of the cells transfected with mouse GADD45α-siRNA and then infected with reovirus T1L was inhibited and the protein expression level was about 2 fold less than the control or other treated groups, and there was no difference in all the cells with different treatments (Figure **3.14A**); and for H1299 cells the expression of GADD45α protein of the cells transfected with human GADD45α-siRNA and infected with reoviruses T1L or T3D was inhibited about 2-3 fold less than the control in the cells and other treatments (Figure **3.14B**).

3.2.5.5. Relation of NF-κB and GADD45α for reovirus infection. Up to now, it has been known that the expression of GADD45α is regulated by two pathways in cells: one is the p53 regulation pathway (Wang et al., 1999), and the other is the NF-κB signaling pathway (Zerbini et al., 2004). My results show that p53 is not induced by reovirus infection, and indeed its presence or absence (as shown by results in the $p53^+$ H1299 cells or $p53^-$ H1299 cells) is not required. Therefore, the p53 regulation pathway of GADD45α is not needed in reovirus infection. The expression of NF-κB gene induced by reovirus has been proven in reovirus induced apoptosis (Connolly et al., 2000). We wondered whether the expression of GADD45α induced by reovirus infection at larger doses is

Figure 3-13.

A (L929).



B (H1299).

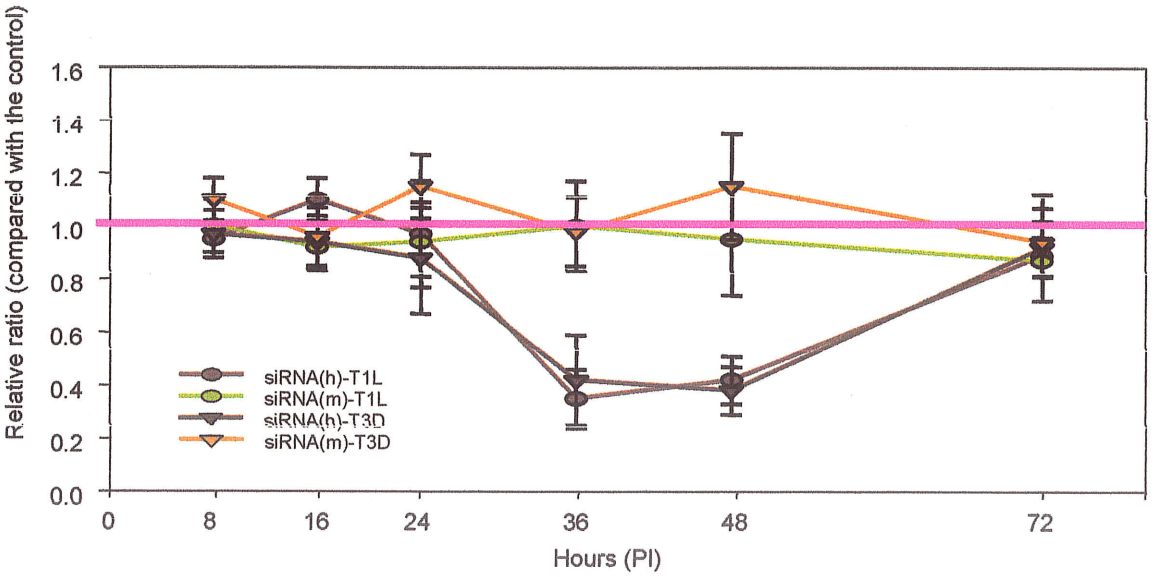
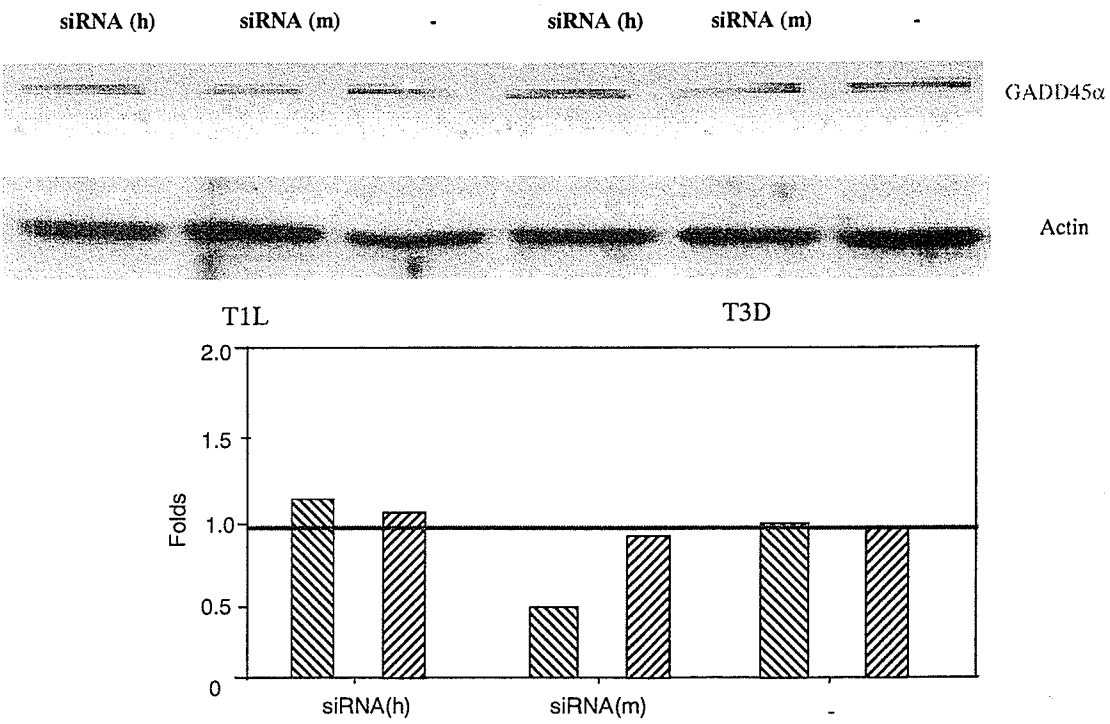


Figure 3.13. Virus titers of the cells transfected with GADD45α siRNA and infected with reoviruses. The cells were transfected with GADD45α siRNA and then infected with reoviruses, and harvested at the indicated time points and measured for virus titers by plaque assay. The results were expressed as the mean ratio of virus titers compared to the control from three independent experiments (n=3). Error bars indicate standard deviation of the means. (siRNAh means siRNA for human GADD45 and siRNAm siRNA for mouse GADD45).

Figure 3.14.

A (L929).



B(H1299).

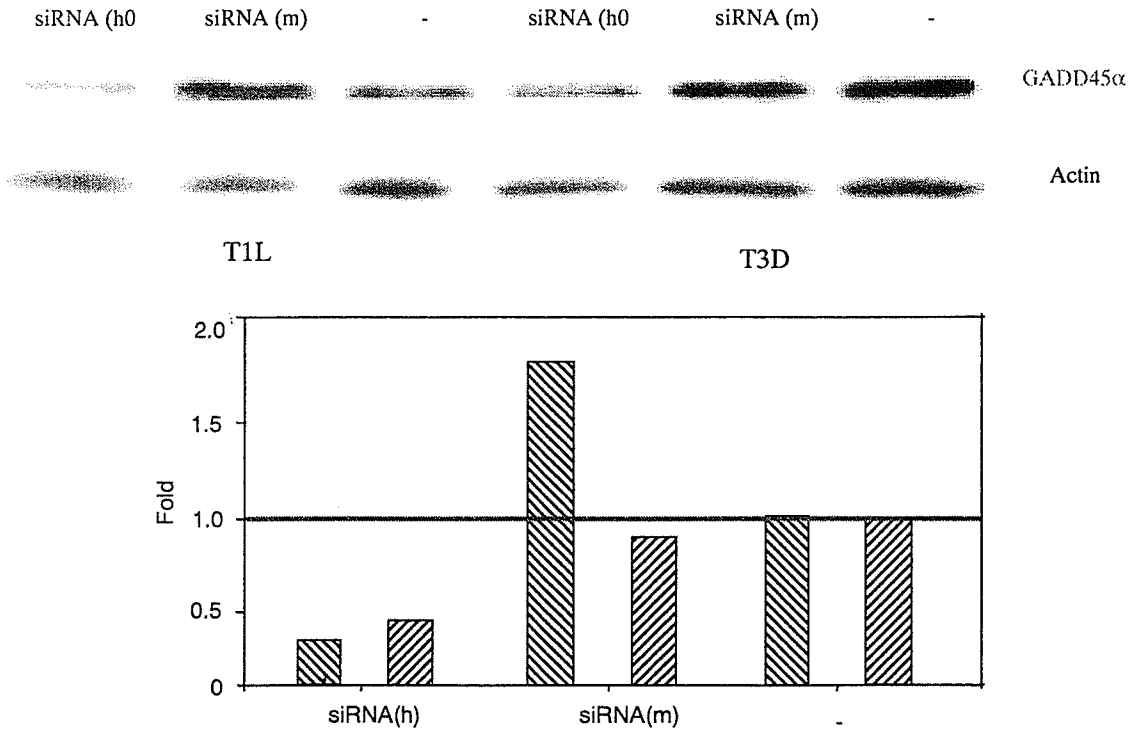


Figure 3.14. GADD45α protein level of the cells transfected with GADD45α siRNA and infected with reoviruses. The cells were transfected with GADD45α siRNA and then infected with reoviruses. The cell cultures were harvested at 48 hours PI and GADD45α protein levels were measured by Western blotting. The protein density on the X-ray films was analyzed with the program 'Scion Image'. **A**. L929 cells, and **B**. H1299 cells.  T1L; and T3D. (siRNAh means siRNA for human GADD45 and siRNAm siRNA for mouse siGADD45)

Figure 3. 15.

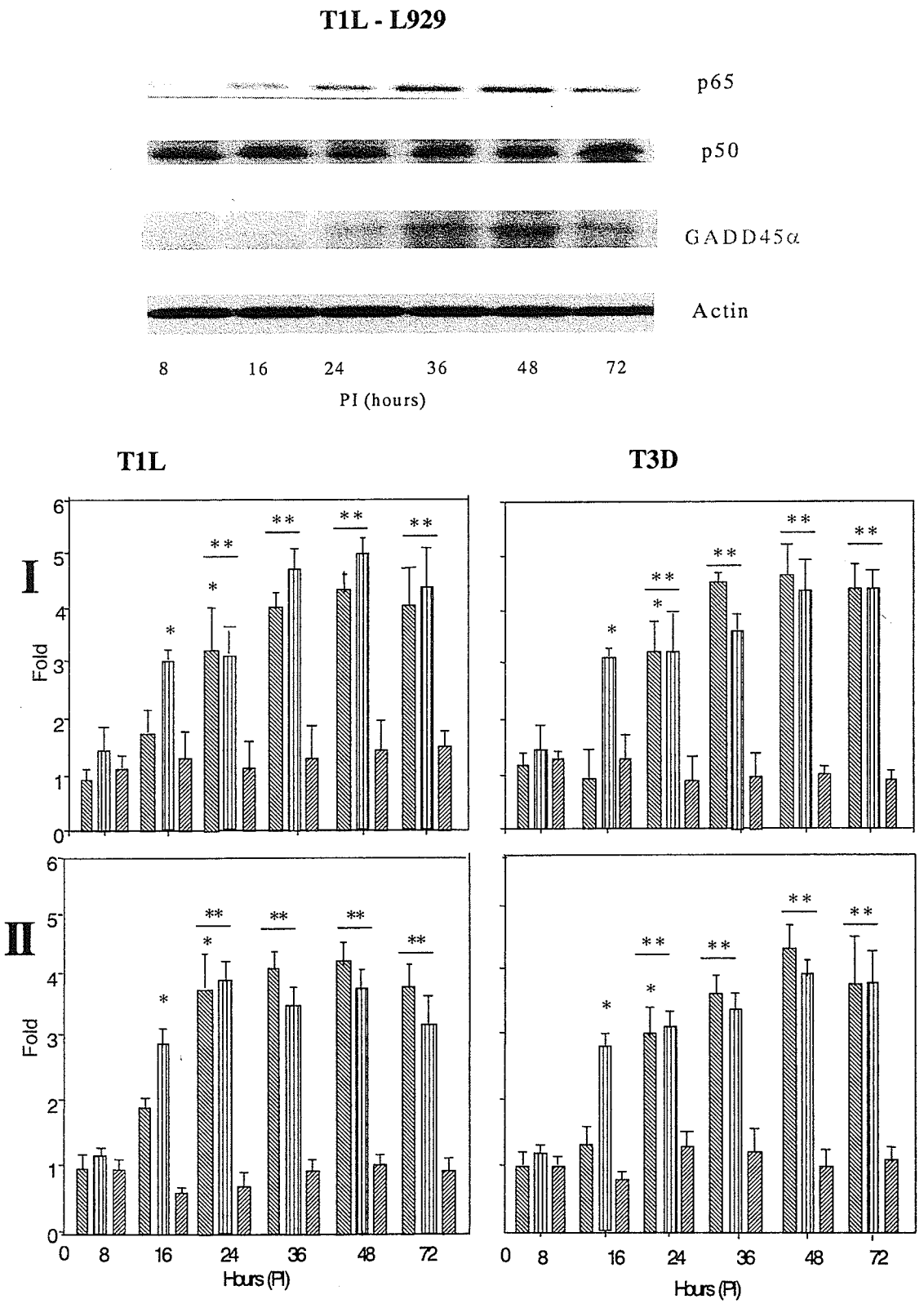

T1L - L929
p65
p50
GADD45α
Actin
8
16
24
36
48
72
PI (hours)
T1L
T3D
I
II
Fold
Hours (PI)

Figure 3.15. Examination of GADD45α and NF-κB proteins in cells infected with reoviruses by Western blotting. The cell lysates of two cells infected with reoviruses T1L or T3D and harvested at the indicated time points were analyzed by Western blotting to detect the expressed levels of NF-κB p65, p50 and GADD45α proteins. Actin was the loading control. The protein density on the X-ray films was analyzed with 'Scion Image' program and the results were expressed as the mean fold of proteins of the infected cells divided by the control of actin level from three independent experiments (n=3). Error bars indicate standard deviation of the means. (**I**) L929 cells, and (**II**) H1299 cells. ▨ GADD45α; ▥ p65; and ▩ p50. Single asterisks ($P < 0.05$) indicate significant difference of GADD45α protein or NF-κB protein levels in cells infected with reovirus and harvested at the times PI compared to these of the preceding times PI during the infection periods. Double asterisks ($P < 0.05$) indicate significant difference of GADD45α protein and NF-κB subunit p65 protein levels compared to NF-κB subunit p50 protein level in the cells infected with reovirus (Comparison of P values by ANOVA's *F*- test and Q-test).

related to NF-κB signaling pathway induced by reoviruses. The cell lysates of L929 cells that were infected with reoviruses T1L and T3D (proven previously to have the induced expression of GADD45α) were examined with the anti- NF-κB p65 or p50 antibodies from Santa Cruz Bio Corp (CA). The NF-κB p65 subunit was positively induced to express beginning at about 16 hours PI and reached the highest level at 36-48 hours PI in L929 cells infected with reoviruses at dose of MOI=10 PFU/cell (Figure **3.15A, B**); and there was no difference of expressed levels between reovirus strains T1L and T3D (Figure **3.15A, B**). The expression of p65 was generally detected earlier (16 hours PI) than the expression of GADD45α that could commonly be detected at 24 hours PI (Figure **3.15A, B**). However, the p50 protein levels at the different times PI were not obviously changed (Figure **3.15A, B**). The positive expression of NF-κB p65 was also observed in the H1299 cells infected with reoviruses T1L and T3D at the dose of MOI=10 PFU/cell and the expression pattern was similar to that in the infected L929 cells (Figure **3.15C, D**). The results indicated that cellular protein NF-κB p65 was induced to express in reovirus-induced apoptosis, and probably regulates the expression of GADD45α in reovirus-induced apoptosis.

Full discussion for the experiments and results is in Chapter 7. Here I can make the following conclusions.

3.3. Conclusion.

1. Reovirus infection can induce different cellular responses that depend on the cell types. In L929 cells and Vero cells reovirus induces apoptosis; however, cell growth arrest in H1299 cells. Reovirus induced cell apoptosis or arrest also depends upon the infection doses of reovirus; that is the infectious doses must be larger than MOI=10 PFU/cell. The cell response is not induced by reovirus infection at dose of MOI=0.5 PFU/cell.
2. Reovirus induced cell response is unrelated to the p53 pathway. Reovirus infection does not induce the transcription of p53; and the expression of p53 in cells also does not affect virus replication.

3. The expression of cellular genes GADD45α, p21, mcl1, bax are induced by the infection of reovirus strains T1L and T3D. The other genes such as p53, TRAIL, DR3, caspase-8, RIP, FAS, FASL do not respond to reovirus infection. The expression of gene Bcl-x depends upon the reovirus strain: reovirus strain T3D can positively induce the expression of Bcl-x and reovirus strain T1L may inhibit the expression of Bcl-x in H1299 cells.

4. The expression of cellular gene GADD45α is stably induced by reovirus infection. This expression corresponds with the cell responses to reovirus infection, and also is decided by the infectious doses of reovirus (MOI ≥10 PFU/cell). UV-inactivated reovirus particles do not induce the expression of GADD45α.

5. The expression of GADD45α is regulated by NF-κB pathway in reovirus infection. However, there are still other pathways to regulate the expression GADD45α because the dead reovirus does not induce the reaction. Inhibition of GADD45α expression by siRNA can affect the reovirus replication and decrease the virus titers at 36 and 48 hours PI.

6. The expression of GADD45α can inhibit the cell cycle of replication that makes some suitable conditions for reovirus growth and replication. So GADD45α protein plays important roles in reovirus infection.

Chapter 4. *FUNCTION OF siRNA ON REOVIRUS REPLICATION.*

4.1. Introduction.

RNA interference (RNAi) pathway was first characterized in the nematode worm *Caenorheditis elegans* by Fire and colleagues (Fire et al., 1998), who found the double-stranded RNA (dsRNA) induced a more potent sequence-specific silencing response than single-stranded antisense RNA alone, which was customarily used for this purpose. In the past 5 years, an intensive research effort has facilitated the rapid movement of RNAi from a relatively obscure biological phenomenon to available tool used to silence target gene expression and perform large-scale functional genomic screens (Tomari and Zamore, 2006).

RNAi is carried out in two distinct steps. In the first step, long dsRNA are processed into short 21- to 23-nucleotide-long effector dsRNA called small interfering RNA (siRNAs) (Elbashir et al., 2001; Hamilton et al., 2002; Zamore et al., 2000). In the second step, the siRNAs are assembled into RNA-induced silencing complex (RISCs), which direct the specific cleavage of the target mRNAs. In these complexes, the short dsRNA duplex is unwound, generating active RISCs containing single siRNA strands (Nykanen et al., 2001). In principle, either of the two siRNA strands can be incorporated into RISCs. In practice, however, there appear to be rules that govern the selection or stability, resulting in a bias of one strand chosen over the other (Khvorova et al., 2003). siRNA pairs with its cognate mRNA, leading to degradation of target mRNA and amplification of gene-specific silencing signals.

Long dsRNA cleaved into siRNA is mediated by a multidomain RNase III family enzyme (Dicer) (Bernstein et al., 2001). The enzyme is relatively well conserved in eukoryotes and has been found both in the nucleus and in the cytoplasm (Billy et al., 2001). Dicer is also essential for mouse (Bernstein et al., 2003) and zebrafish (Wienholds et al., 2003) development. Recently, some investigators have indicated that an RNAi-based approach can be utilized to inhibit replication of a number of viruses, including HIV-1 (Novina et al., 2002), HCV (Kapadia et al., 2003), HBV (Konishi et al., 2003), HDV (Chang and Taylor, 2003), Influenza virus (Ge et al., 2004), rotavirus (Dector et al., 2002), and SARS (severe acute respiratory syndrome) (Wang et al., 2004). Here, I tried to test whether siRNAs inhibit reovirus replication. These experiments could explain how

and when reoviruses can function on the expression of GADD45α protein in the process of the viral replication (determined in previous chapter).

4.2. Results.

4.2.1. Effect of synthesized siRNA on reovirus replication. To test the ability of siRNAs to function in repressing gene expression, two siRNAs, one copied from MRV genomic L1 (RNA-dependent RNA polymerase) and another from M2 (major outer capsid protein), were made (as described in **2.8.1**). Transfection of both siRNAs was carried out by oligofectamine reagent at doses of 40ng or 80ng per well in 24-well plates, then the transfected cells were infected with reoviruses at dose of MOI=5 PFU/cell. The titers of the viruses harvested at different time points (Days 1, 2, or 3 PI) were measured by plaque assays. The results showed that the two siRNAs did not inhibit reovirus replication, and the titers of reovirus strains T1L and T3D were not much different compared to the non-transfected L929 cells (Figure **4.1**). The same transfection was carried out on H1299 cells and the siRNAs did not inhibit reovirus T1L or T3D growth either (Figure **4.2**).

4.2.2. Effect of reoviral genome dsRNA on reovirus replication. Long dsRNAs cleaved into siRNA is mediated by a multidomain RNase III family enzyme (Dicer) (Bernstein et al., 2001). The enzyme Dicer is also present in mammalian cells and found to be essential for mouse development (Bernstein et al., 2003). This importance of Dicer is also proven in zebrafish development (Wienholds et al., 2003). Long dsRNA triggers sequence-specific mRNA degradation in mouse oocytes and early mouse embryos (Svoboda, et al. 2000; Wianny, et al. 2000), and several cell lines in vitro such as undifferentiated mouse embryonal stem cells (Yang et al., 2001), undifferentiated embryonal carcinoma cells (Billy, et al. 2001), differentiated mouse neuroblastoma cells (Gan, et al. 2002), human peripheral blood mononuclear cells (Bhargava, et al. 2004), rat hypothalamas (Yi, et al. 2003) and muscle cells (Kong, et al. 2004). The reovirus genome consists of double-stranded RNA (dsRNA) segments. Because the short dsRNA segments (siRNA) were expected to specifically interfere with the expression of the target genes, and the results of two siRNAs showed they did not (Figures **4.1** and **4.2** above), we wondered whether transfected full-length reovirus genomic dsRNAs would inhibit virus replication. The reovirus dsRNAs were prepared and purified from batch viral infections

Figure 4.1.

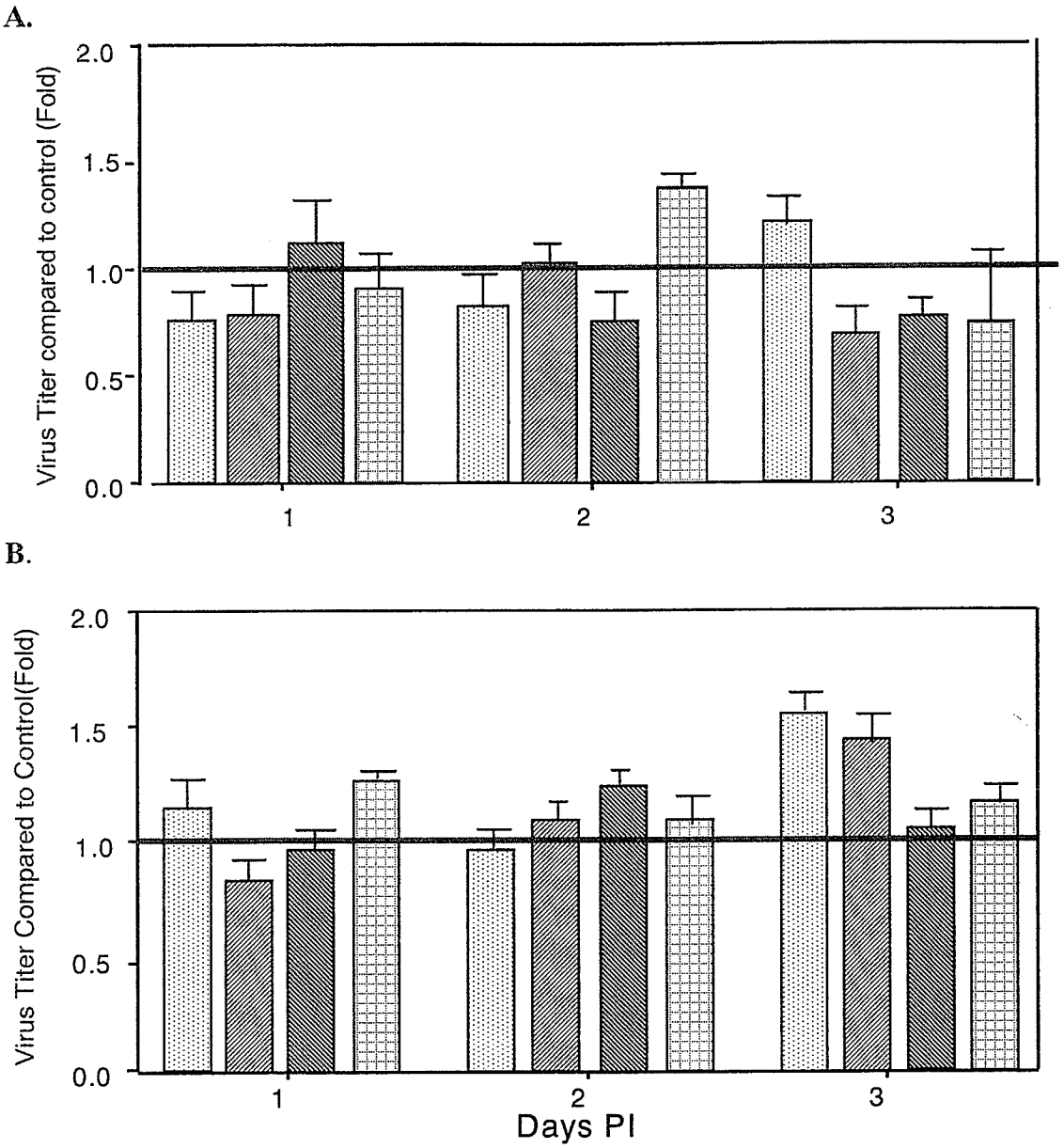


Figure 4.1. Reovirus replication in L929 cells transfected with siRNA. Mouse L929 cells were transfected with siRNAs at doses of 40 or 80 ng/well, and one day later, the transfected cells were infected with reovirus T1L or T3D at dose of MOI= 5 PFU/cell and harvested on different days PI. The virus titers of the transfected cells were divided by the non-transfected cells infected with reovirus at same dose. The data were from three independent experiments (n=3). Error bars indicate standard deviation of the means. **A.** reovirus T1L, and **B.** reovirus T3D. siRNA-L at 40ng/well; siRNA-L at 80ng/well; siRNA-M at 40ng/well; and siRNA-M at 80ng/well.

Figure 4.2.

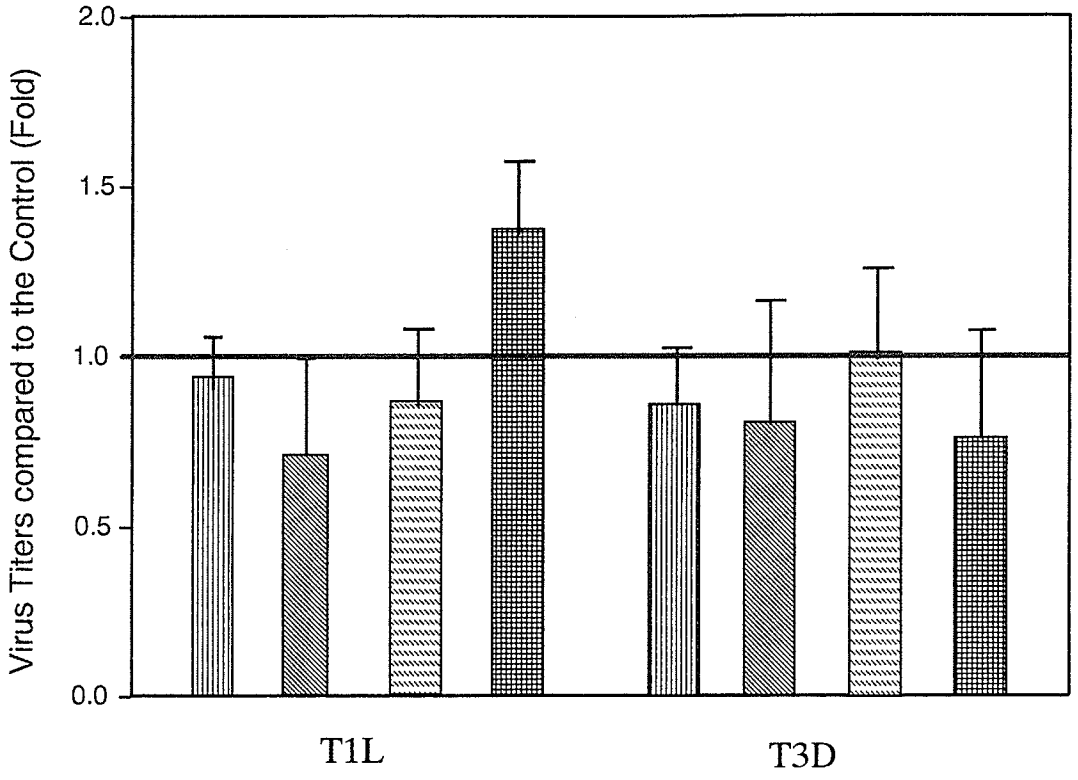


Figure 4.2. Reovirus replication in H1299 cells tranfected with siRNA. Human lung carcinoma H1299 cells were transfected with siRNAs at doses of 40 or 80 ng/well, and one day later, the transfected cells were infected with reovirus T1L or T3D at dose of MOI= 5 PFU/cell and harvested on day 3 PI. The virus titers of the transfected cells were divided by the non-transfected cells infected with reovirus at same dose. The data were from three independent experiments (n=3). Error bars indicate standard deviation of the means. siRNA-L 40ng; siRNA-L 80ng; siRNA-M 40ng; and siRNA-M 80ng.

as described in **2.4.** The purified reoviral dsRNAs were directly transfected into L929 cells at doses of 5μg dsRNA/well in 24 well plates. 24 hours later, the transfected cells were infected with reoviruses T1L or T3D at MOI = 5PFU/cell. When harvested at 72 hours PI, the titers of reovirus in the reoviral dsRNA transfected L929 cells were only ~20-40% of the titers in the infected/non-transfected control cells (Figure **4. 3**). Thus, reovirus replication in the transfected cells was inhibited. However, virus replication in cells to which genomic dsRNAs was directly added (mock transfection) were not affected, and the virus titers in this group were almost identical to control groups (Figure **4.3**). This inhibition of dsRNA on reovirus replication was also present in the dsRNA transfected H1299 cells (Figure **4.4**).

4.2.3. Further examination of the inhibition of long dsRNAs on reovirus replication. In order to further characterize long dsRNA inhibition of reovirus replication, three different doses of dsRNAs (1μg, 5μg, or 10μg/well in 24 well plate) were transfected into L929 cells and infected at dose of MOI= 5 PFU/cell. The results showed that the replication of reoviruses T1L or T3D were inhibited similarly, no matter the amount of transfected dsRNAs. The virus titers in the cells transfected with dsRNAs at 3 doses (1μg, 5μg, and 10μg) were reduced about 3-5 fold compared to controls (Figure **4.5**).

We know that the dsRNA genome of reovirus is present as L-, M- and S-size segments. I then tested whether the specific size classes would effect reovirus replication. In order to separately test the three dsRNA classes on reovirus replication, the L-, M- or S-genes were separated in agarose gels, and purified and transfected at dose of 50ng/well (full-length dsRNA was not purified from gel and used with more amount in transfection) into L929 cells. All dsRNA sizes inhibit the replication of reovirus T1L or T3D (Figure **4.6**), and the virus titers in the cells transfected with three different parts of dsRNAs were not different from the titers of cells transfected with the whole complement of reoviral dsRNA (Figure **4.6**).

The inhibition of full length dsRNAs on reovirus replication occurred in mammalian cells. Next, the effect of transfected dsRNAs was measured when cells were infected with different doses of reovirus. When the dsRNA transfected cells were infected with reovirus at MOI= 100 PFU/cell, reovirus replication was also inhibited; however, the inhibition was not as strong as when the infectious dose was MOI=5 PFU/cell. The virus

Figure 4.3.

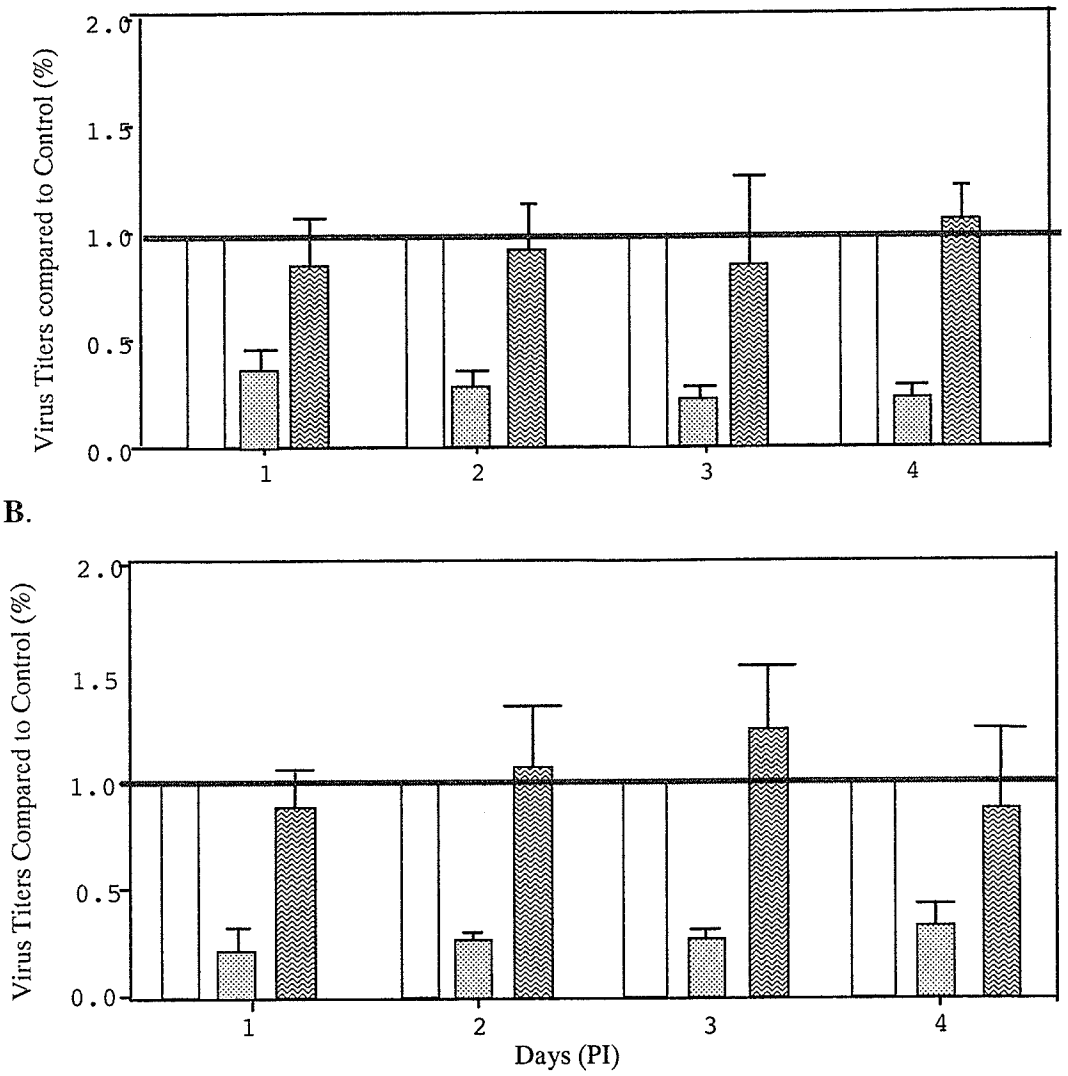


Figure 4.3. Inhibition of transfected long reovirus dsRNAs on reovirus replication in L929 cells. Mouse L929 cells were transfected with reovirus full-length genomic dsRNA at 5μg/well, and one day later, the transfected cells were infected with reovirus T1L or T3D at dose of MOI= 5 PFU/cell and harvested on different days PI. The virus titers of the transfected cells were divided by the non-transfected cells infected with reovirus at same dose. The data were from three independent experiments (n=3). Error bars indicate standard deviation of the means. **A.** reovirus T1L, and **B.** reovirus T3D. ☐ Infected only with no dsRNA added as control; ▒ dsRNA transfected; and ▒ dsRNA added directly (without transfection reagents).

Figure 4.4.

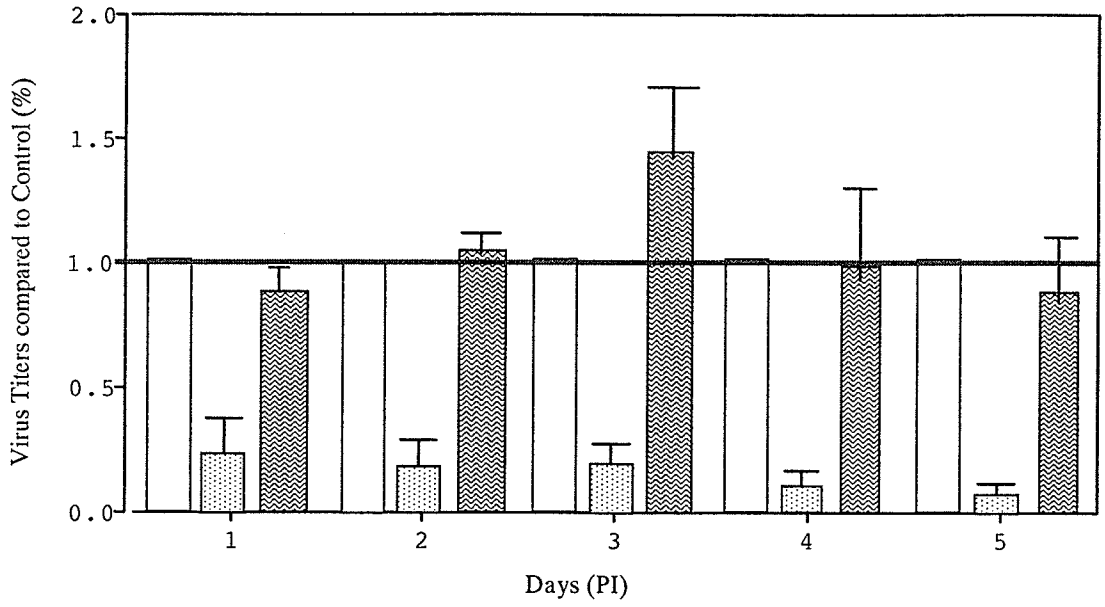
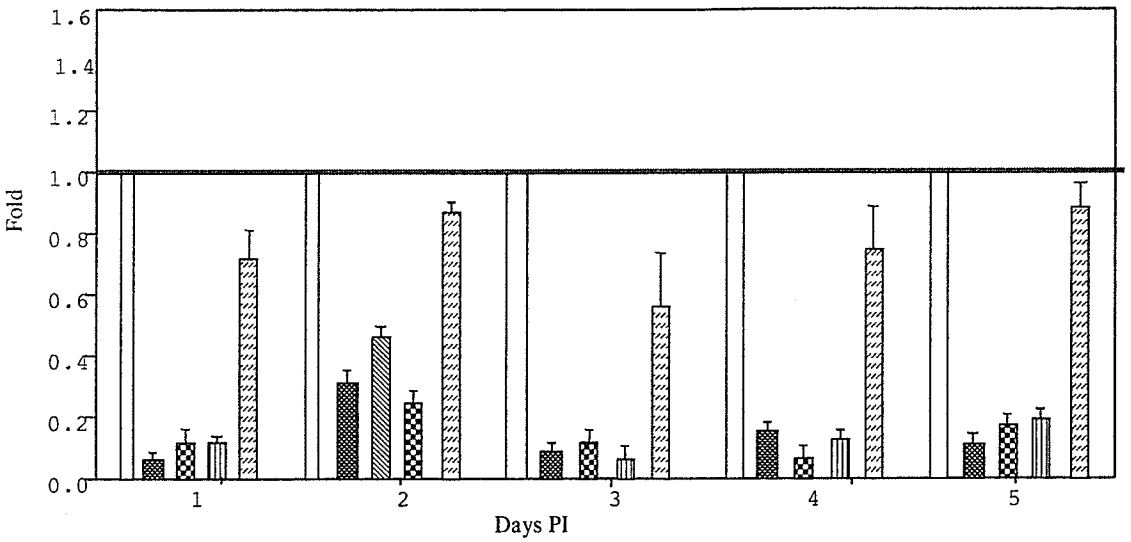


Figure 4.4. Inhibition of transfected long reovirus dsRNAs on reovirus replication in H1299 cells. Human lung carcinoma H1299 cells were transfected with reovirus full-length reovirus genomic dsRNA at 5μg/well, and one day later, the transfected cells were infected with reovirus T1L at dose of MOI= 5 PFU/cell and harvested on different days PI. The virus titers of the transfected cells were divided by the non-transfected cells infected with reovirus at same dose. The data were from three independent experiments (n=3). Error bars indicate standard deviation of the means. ☐ Infected only with no dsRNA added as control ▨ dsRNA transfected; and ▨ dsRNA added directly (without transfection reagents).

Figure 4.5.

A.



B.

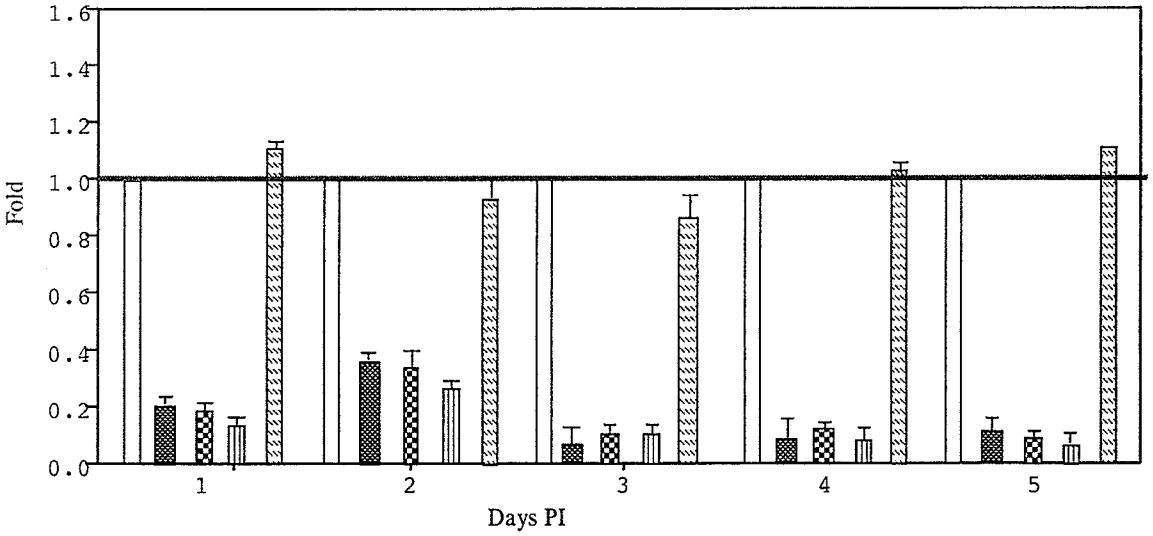


Figure 4.5. Inhibition of reovirus dsRNAs transfected at different doses on reovirus replication. Mouse L929 cells were transfected with reovirus long dsRNA at 1, 5 or 10μg/well. One day later cells were infected with reovirus at dose of MOI= 5 PFU/cell and harvested on different days PI. The virus titers of the transfected cells were divided by the control. The data were from three independent experiments (n=3). Error bars indicate standard deviation of the means. **A.** T1L, and **B.** T3D. ▭ Infected only with no dsRNA added as control; ▩ dsRNA –transfected (1μg/well); ▩ dsRNA –transfected (5μg/well); ▥ dsRNA –transfected (10μg/well); ▨ dsRNA-added directly (10μg/well).

Figure 4.6.

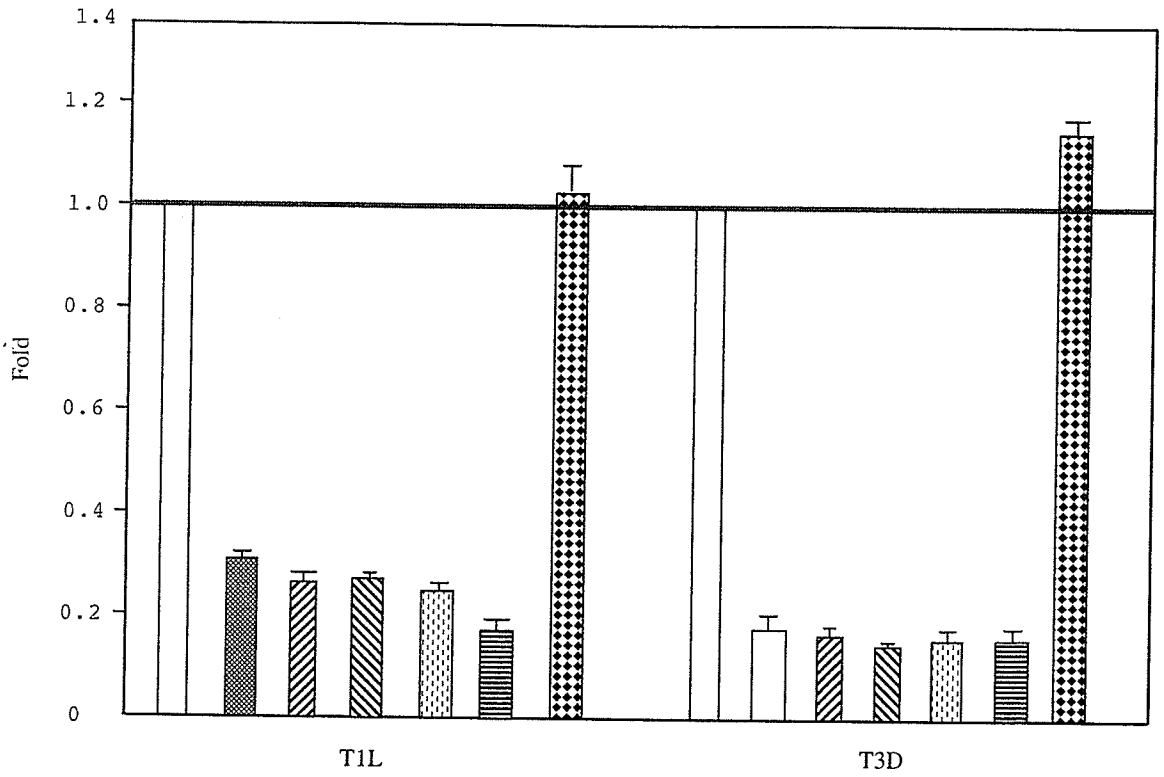


Figure 4.6. Inhibition of reovirus replication after transfection of dsRNA genomic size classes. Mouse L929 cells were transfected with different genomic parts of reovirus dsRNA. One day later they were infected with reovirus T1L or T3D at dose of MOI= 5 PFU/cell and harvested on 3 days PI. The virus titers of the transfected cells were divided by the control. The data were from three independent experiments (n=3). Error bars indicate standard deviation of the means. Infected only with no dsRNA added as control; Small reovirus genes-transfected (50ng/well); Medium reovirus genes-transfected (50ng/well); Large reovirus genes-transfected (50ng/well); Total reoviral dsRNA-transfected (1μg/well). Total reoviral dsRNA-transfected (5μg/well); Total reoviral dsRNA added directly (5μg/well).

Figure 4.7.

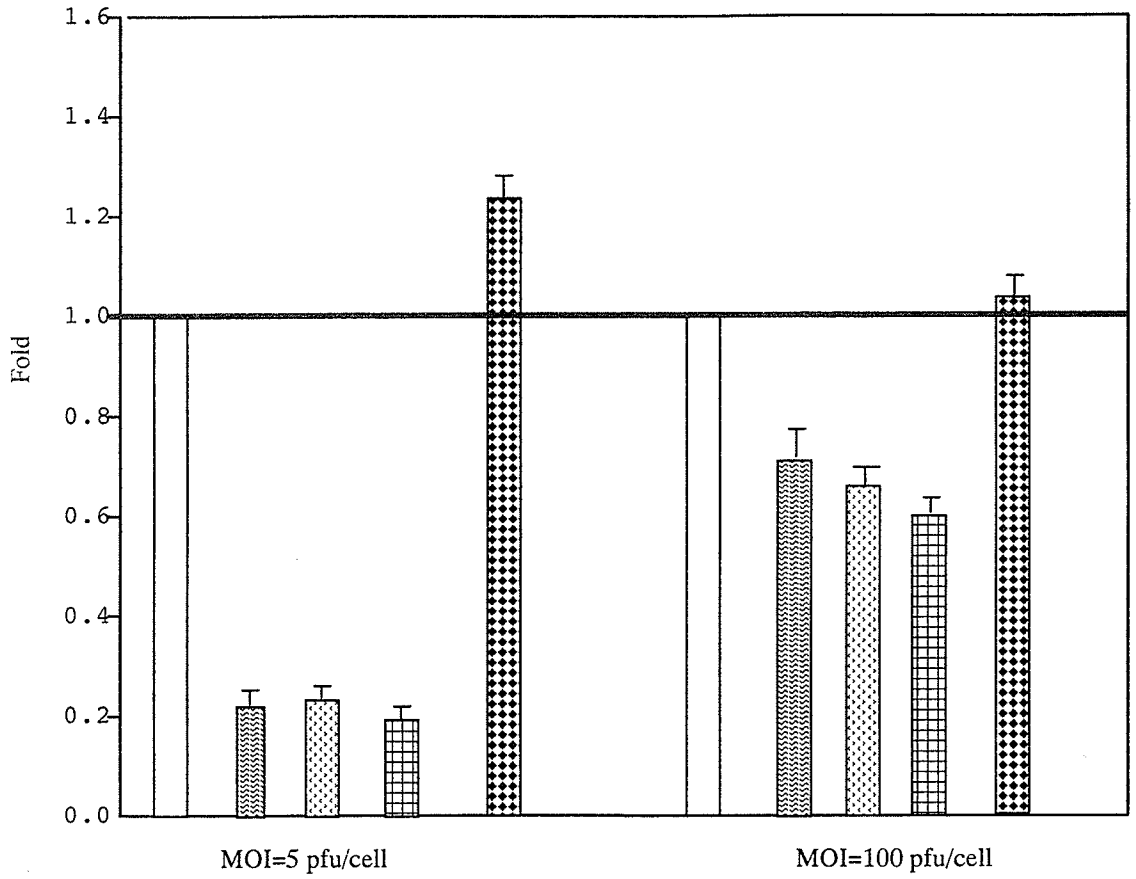


Figure 4.7. Inhibition of reovirus dsRNAs transfected on the replication of reovirus at two infectious doses. Mouse L929 cells were transfected with full length reovirus dsRNA at 1, 5 or 10μg/well. One day later they were infected with reovirus T1L at doses of MOI= 5 or 100 PFU/cell and harvested on 3 days PI. The virus titers of the transfected cells were divided by the control. The data were from three independent experiments (n=3). Error bars indicate standard deviation of the means. ▭ Infected only with no dsRNA added as control; ▨ dsRNA-transfected (1μg/well); ▩ dsRNA-transfected (5μg/well); ▦ dsRNA-transfected (10μg/well); ▩ dsRNA added directly(10μg/well).

titers in the cells transfected with dsRNA and infected at MOI=100 PFU/cell decreased to about half of the control (Figure **4.7**).

Full discussion for the experiments and results is in Chapter 7. Here I can make the following conclusions.

4.3. Conclusion.

1. Two sets of reovirus siRNAs did not seem to inhibit reovirus growth. It is possible that the selection of these parts of siRNAs was not satisfactory or the siRNAs did not function on virus replication. It indicates that siRNA function is very complex.
2. Long full-length genomic reovirus dsRNA could inhibit reovirus replication.
3. Reovirus S, M and L genomic dsRNA fragments, which used separately, could inhibit replication.

Chapter 5. *REOVIRUS CORE TRANSFECTION.*

5.1. Introduction.

The mammalian orthoreoviruses (reovirus) are naked particles of intermediate structural complexity with 8 different proteins organized in two concentric capsids (Nibert and Schiff, 2001). The capsid shells can produce two naturally occurring subviral particles, which are the Intermediate (or sometimes called "Infectious") subviral particle (ISVP) and the core. The core contains one copy of each of the genome segments, and also multiple copies of specific enzymes including an RNA-dependent RNA polymerase and mRNA-capping enzyme.

Both the ISVP and the core are produced *in vivo* by proteolysis. During infection, the intact reovirus virion attaches to specific receptors on the cell membrane surface and enters the cytoplasm by endocytosis. Proteolysis then sequentially converts the virion first into the ISVP then the core (Nibert and Schiff, 2001). The ISVP also appears capable of directly penetrating the cell membrane (Tosteson et al., 1993; Lucia-Jandris et al., 1993). Uncoated core particles serve as the metabolic machine for synthesizing viral mRNA, and eventually, new virions (Coombs, 1998; Chandran and Nibert, 2003).

ISVP and core particles also may be produced *in vitro* by proteolysis (Coombs, 1998a; Yin, et al. 1996). Intact virions are infectious, as determined by standard plaque assays (Coombs et al., 1994). Likewise, some ISVPs are also infectious. Comparisons of particle-to-PFU ratios suggest that some ISVPs may have a higher specific infectivity than their corresponding intact virions (Nibert et al., 1995), presumably because of altered σ1 conformation on these ISVPs (Furlong et al., 1988). However, the σ1 cell attachment protein of some strains of reovirus is destroyed by proteolysis, resulting in loss of infectivity of corresponding ISVP (Nibert, et al. 1995). Irrespective of virus strain, purified core particles are poorly infectious, presumably because they lack entry signals present in outer capsid proteins σ1 and μ1 (Dryden et al.. 1993; Chandran and Nibert, 2003). This suggests that methods that allow the core to gain access to the cell cytosol could bypass such a cell entry block. Indeed, micro-injection has been used as means of introducing core particles into cells and promoting virus replication (Moody and Joklik, 1989). Micro-injection can be a tedious process, not suited to manipulating large numbers of cells.

There are several methods used to transfect DNA or RNA into cells. Currently, the most common methods are lipofectamine and calcium phosphate ($CaPO_4$) (Chen and Okayama, 1987, and 1988). There are a few reports that viruses such as poliovirus (Wilson, et al. 1977) and HIV (Hodgon and Solaiman, 1996) can be transfected into cells in liposomes to reproduce new daughter virions.

siRNA, viral genome dsRNA or DNA plasmids were successfully transfected into cells by using lipofectamine reagents in the previous experiments (see previous chapters). Therefore, whether these transfection methods would allow the non-infectious reovirus core particle to bypass the cellular membrane and result in a full viral replication cycle is valuable to investigate. How the expression of GADD45α protein is induced by reovirus compositions (whether intact virion, or subviral core), or whether expression of GADD45α protein is induced by the binding process of the reovirus outer capsid protein with the cell receptors could be determined.

5.2. Results

5.2.1. Reovirus core particles lack outer capsid proteins σ1, σ3, and μ1. To confirm that proteolytic treatments completely removed outer capsid proteins and generated core particles, aliquots of virions and cores were resolved in SDS-PAGE and stained to visualize proteins (Figure **5.1**). Although major core proteins λ1 and λ2 comigrate, and minor core protein μ2 comigrates with major outer capsid protein μ1C (as has been previously described (Nibert and Schiff, 2001; Coombs, 2002) intact virions clearly contained all 8 reovirus structural proteins. In contrast, my core particles lacked detectable σ1, σ3, μ1, and μ1C. Aliquots of the particles also were examined by electron microscopy and expected particle morphologies confirmed (Figure **5.2A** and **B**).

5.2.2. Reovirus core particles are non-infectious. To confirm loss of infectivity of the newly generated core particles, the capacities of both intact virions and cores were tested to generate plaques when applied to L929 cells. The amount of each type of particle was determined by OD_{260} enumeration. Both T1L and T3D virions had typical particle- to – PFU ratios of about 400-500. These was scaled to a value of 0 $\log_{10}(=1)$ for comparative purposes (Figure **5.3**). By contrast, the infectivity of cores were generally $10^5 - 10^7$ fold less than the infectivity of virions as determined by relative particle to PFU ratios, irrespective of the virus serotype (Figure **5.3**). Similarly, reovirus virions were capable of

Figure 5.1.

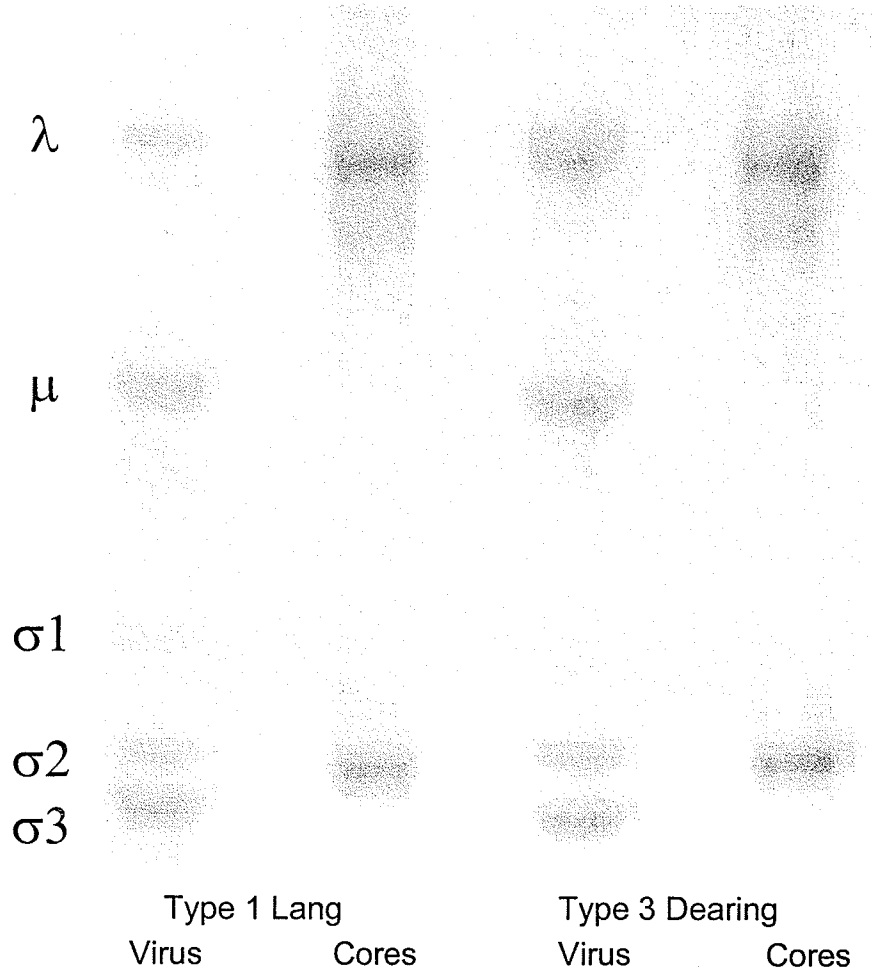


Figure 5.1. SDS-PAGE analysis of protein content of purified reovirus virion and core particles. Approximately $1x10^{11}$ purified reovirus virion and core particles were resolved in 7.5% SDS-PAGE. Identities of major viral proteins are indicated to the left. Note loss of detectable outer capsid protein μ1C and σ3 in core lanes.

Figure 5.2.

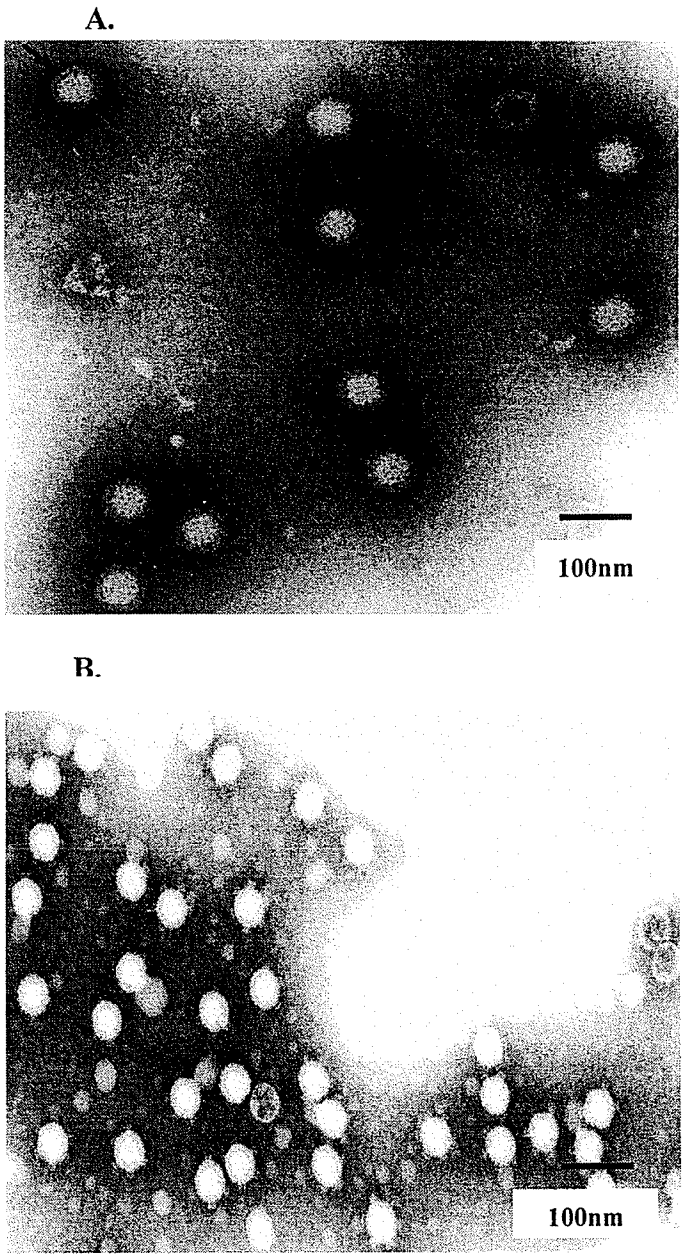


Figure 5.2. Electron micrographs of gradient purified reovirus virion and core particles. Reoviral virions or cores were purified by CsCl gradient centrifugation and mounted on 400-mesh formvar-coated copper grids by drop method, negatively stained with 1.2mM phosphotungstic acid (pH7.0) and examined in a Philips model 201 transmission electron microscope as previously described (Hazelton and Coombs, 1995). (**A**) virion particles and (**B**) core particles. Bar =100nm.

Figure 5.3.

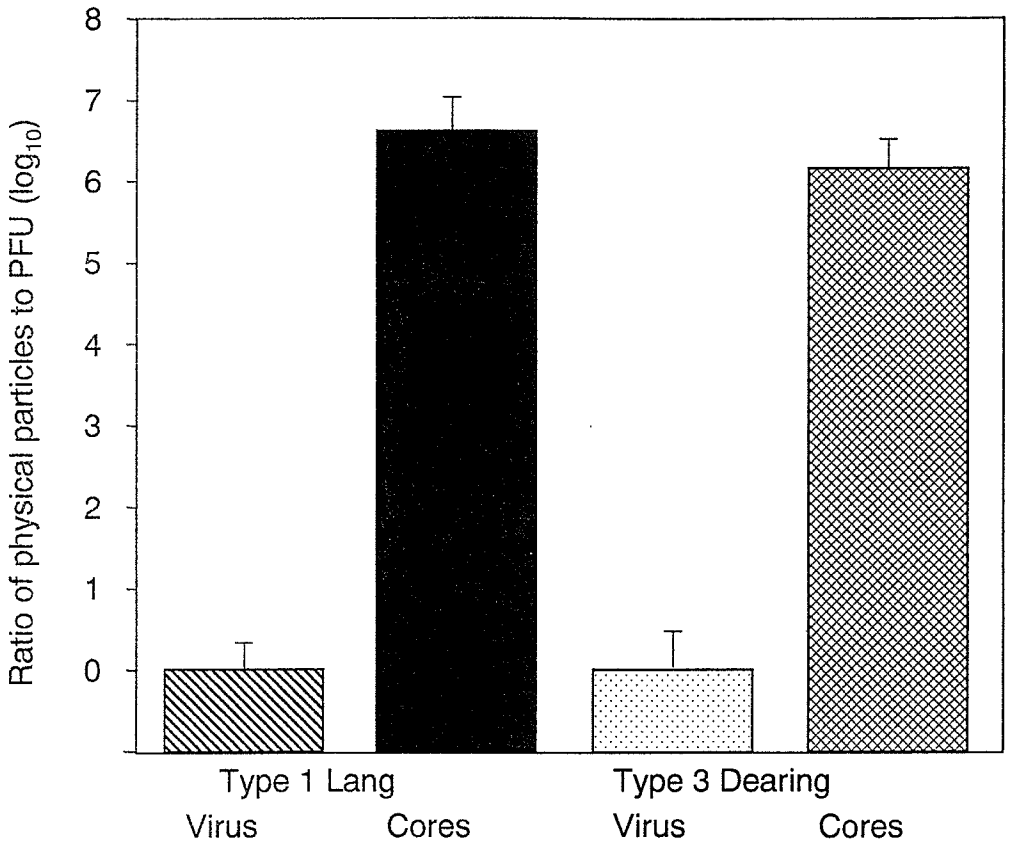


Figure 5.3. Relative particle to PFU ratios of T1L and T3D virion and core particles. Plaque assays of gradient-purified T1L and T3D virions and cores were performed on L929 cell monolayers. Plaques were counted and compared to actual number of physical particles, as determined by OD_{260}. Data is expressed as relative particle-to-PFU ratios, scaled to a value of log_{10} (=1) for virions. The data represents the average of a minimum of three experiments (n=3) and the error bars represent one standard deviation.

Figure 5.4.

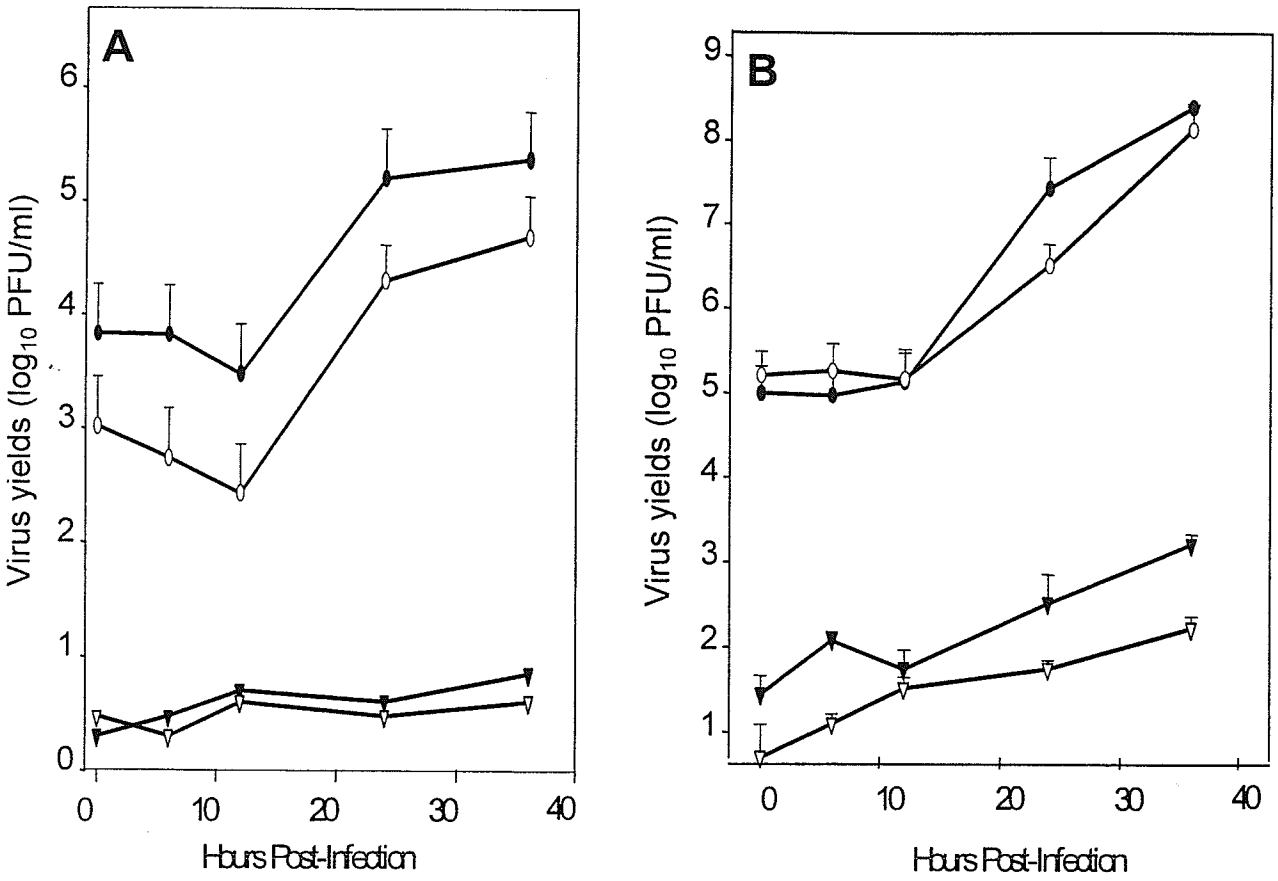


Figure 5.4. Replication capacity of reovirus T1L or T3D virion and core particles

L929 cell monolayers were infected with 1 or 100 particles of purified T1L or T3D virion or core particles per cell respectively and harvested at indicated times post-infection (PI). Virus titers were determined by plaque assay (A: 1 particle/cell; or B: 100 particles/cell). The data represents the average of a minimum of three experiments (n=3) and the error bars represent one standard deviation. ●T1L-virion; ○ T3D-virion; ▼ T1L-core and ▽ T3D-core particles.

infecting and replicating in L929 cell monolayers, and demonstrated a rise in virus titer during incubation, whereas application of core particles to cells did not result in substantial viral replication (Figure **5.4**).

5.2.3. Pretreatment of cores with lipofectamine dramatically increases capacities of cores to replicate. Sub-confluent cell monolayers were exposed to two doses (1 or 100 particles per cell) of virus virions or cores that had been treated with lipofectamine 2000™, using the manufacturers's instructions (Invitrogen), and as described above in section **2.7.1.** Cells in the groups transfected with cores showed more than 70% cytopathic effect (CPE) by 72 hours post-transfection. The cells to which core particles were added directly did not demonstrate CPE at the time point, consistent with the inability of cores to infect and replicate. Supernatants and cells from various treatments were harvested at 72 hours post-treatment, and virus titers were measured by plaque assays as described in **2.2.2.** Yields of infectious progeny from cells that had been exposed to core particles without transfection reagents was negligible, both in mouse L929 cells (Figure **5.5**) and in monkey Vero cells (Figure **5.6**), consistent with early results (Figure **5.1**) that showed cores are essentially non-infectious. Premixing core particles with lipofectamine significantly enhanced production of infectious progenies. Final virus yields were not significantly affected by the two tested doses (1 or 100 core particles per cell) (Figure **5.5** and **5.6**). These results indicated that lipofectamine transfection of reovirus core particles assisted passage of the core particles through the cell membrane and into the cytoplasm. They also confirm that reovirus core particles by themselves cannot pass the cell membrane. The very low titers of viruses detected when core particles were added directly to cells in the absence of lipofectamine (~10^2 PFU/ml), probably reflect that gradient-purified core particles were not 100% pure and still contain a very small number of reovirus ISVPs or virion particles. The effect of the lipofectamine transfection on intact virion particles was also examined. Lipofectamine transfection resulted in only minor changes in replication capacity of intact virions (Figure **5.5** and **5.6**). Although there were only minor differences in the final yields of infectious virus when L929 cells were transfected with cores or viruses, paradoxical effects were seen when Vero cells were exposed to intact virions (Figure **5.6**). There appeared to be a dose response in infectious virus yield and, at low particle treatment (1 particle/per cell),

Figure 5.5.

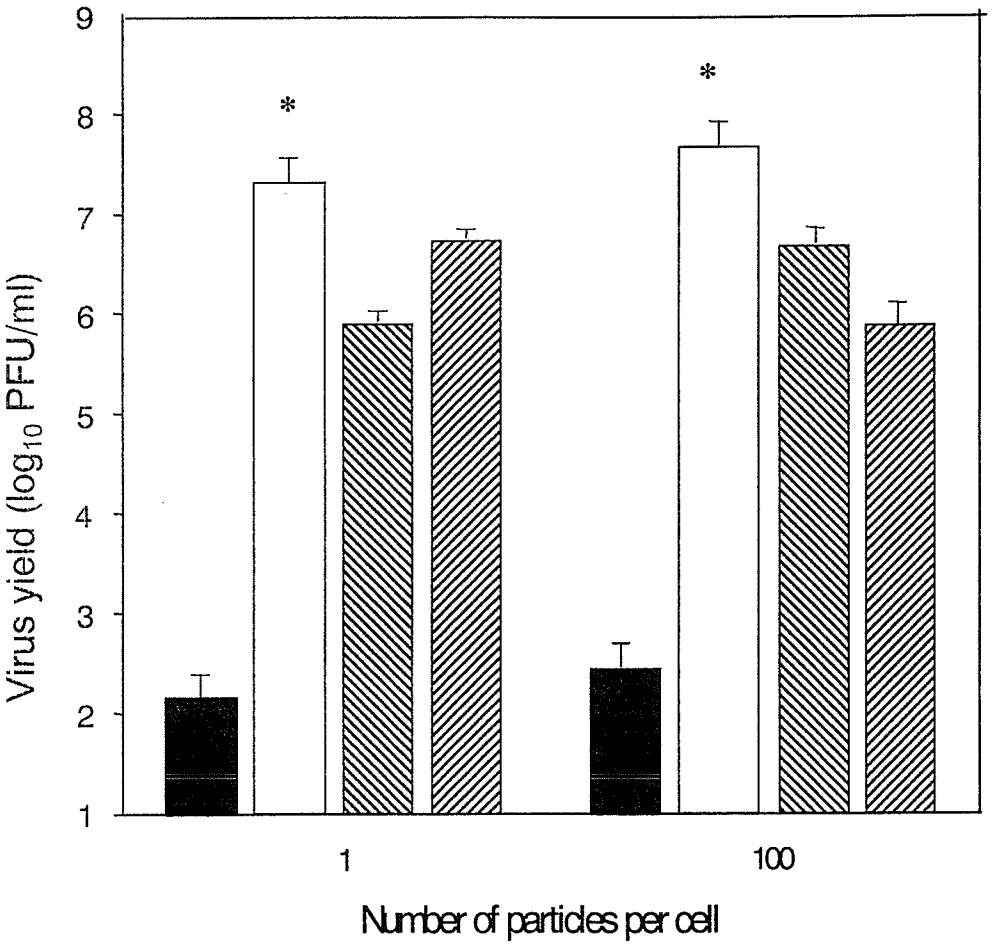


Figure 5.5. Reovirus T1L virion or core particles transfected with Lipofectamine 2000 in L929 cells. T1L virion or core particles were transfected into L929 cells with lipofectamine 2000 or were added directly to L929 cells. After 72 hours, cells were harvested and virus yields were determined by plaque assays. The data represents the average of a minimum of three experiments (n=3) and the error bars represent one standard deviation. ▬ Cores not transfected; ▭ Cores transfected with Lipofectamine 2000, ▨ Virions infected and ▨ Virions transfected with Lipofectamine 2000. * The virus titers in the cells transfected with viral cores were significantly higher than the un-transfected controls by *t-test* ($p < 0.05\%$)

Figure 5.6.

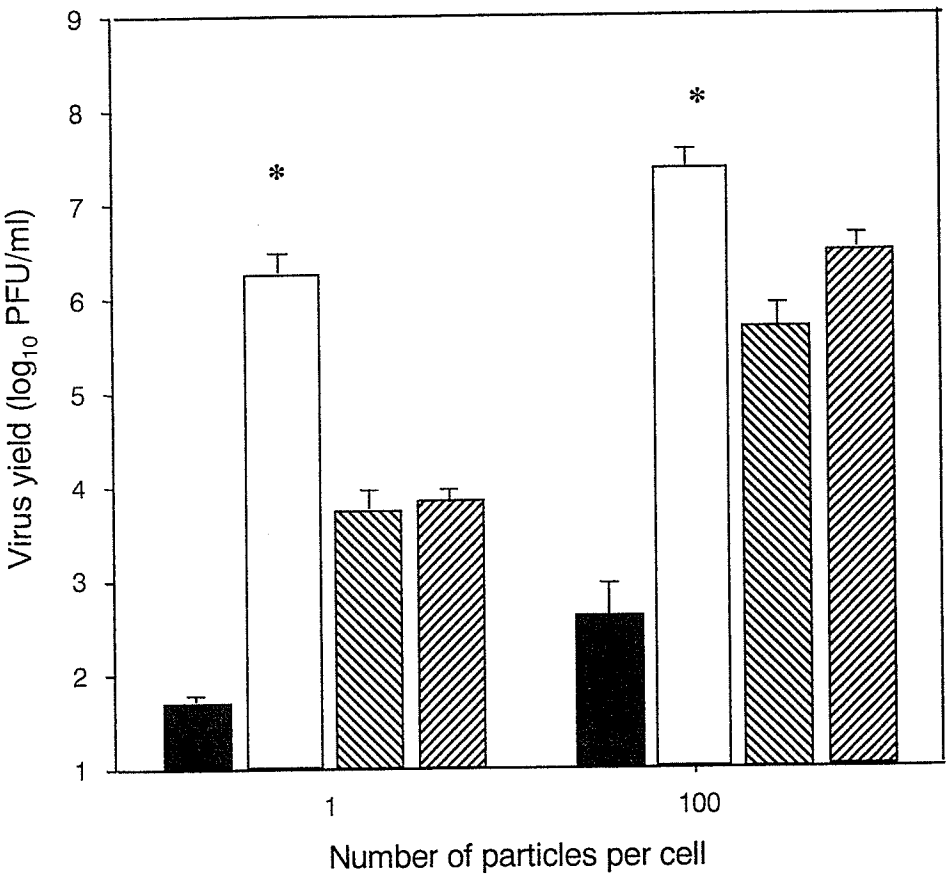


Figure 5.6. Reovirus T1L virion or core particles transfected with Lipofectamine 2000 in Vero cells. T1L virion or core particles were transfected into Vero cells with Lipofectamine 2000 or were added directly to Vero cells. After 72 hours, cells were harvested and virus yields were determined by plaque assays. The data represents the average of the minimum of three separate experiments (n=3) and the error bars represent one standard deviation. ☐ Cores not transfected; ■ Cores transfected with Lipofectamine 2000; ▨ Virions infected; and ▨ Cores transfected with Lipofectamine 2000. * The virus titers in the cells transfected with viral cores were significant higher than that the un-transfected control by *t-test* ($p < 0.05\%$)

transfected core appeared to replicate ~ 100-fold better that transfected virions.

The two standard strains of reovirus (T1L and T3D) often show difference in biological characteristics. For example, reovirus strain T1L viruses infect cells and form filamentous factories to produce new viruses, but T3D forms globular factories (Parker, et al. 2002). Reovirus T3D can induce higher levels of cell apoptosis than T1L (Tyler, et al. 1995). I then determined if there were any differences in lipofectamine 2000 transfection of T1L or T3D was determined. As with T1L core transfection, the virus titers from cells transfected with reovirus T3D cores, or transfected or infected with virion particles, reached nearly 10^8 (Figure **5.7**); thus, there were no obvious differences between the two reovirus strains T1L and T3D. Lipofectamine transfection non-specifically assists the infectious entry of reovirus core particles through the cellular membrane. These results also confirm the core particles contain all the information and necessary materials for replication to produce new viruses once they are able to gain access to the cytosol.

5.2.4. Transfection of cores with calcium phosphate ($CaPO_4$) also enhances core replication. Because $CaPO_4$ has been successfully used in nucleic acid transfection, we determined whether calcium phosphate transfection could also act to facilitate core entry. Calcium phosphate reagents (Invitrogen) were used to transfect reovirus T1L core particles into L929 cells according to the manufacturer's instructions (as described in **2.7.2**). After 3 days of incubation at 37°C, the cells were examined and harvested, and the virus titers in the culture were determined as described in **2.2.2.** Similarly to the lipofectamine 2000 reagent transfection, addition of $CaPO_4$ increased the specific infectivity of core particles, but only ~300 fold (Figure **5.8**). Thus, lipofectamine transfection was more efficient than calcium phosphate transfection in L929 cells.

Full discussion for the experiments and results is in Chapter 7. Here I can make the following conclusions.

5.3. Conclusion.

1. Purified reovirus core particles were not infectious when directly added to cell.
2. Transfection reagents enhance core particle entry into cells and the core can replicate to reach almost same level of viruses as reovirus virions.

Figure 5.7.

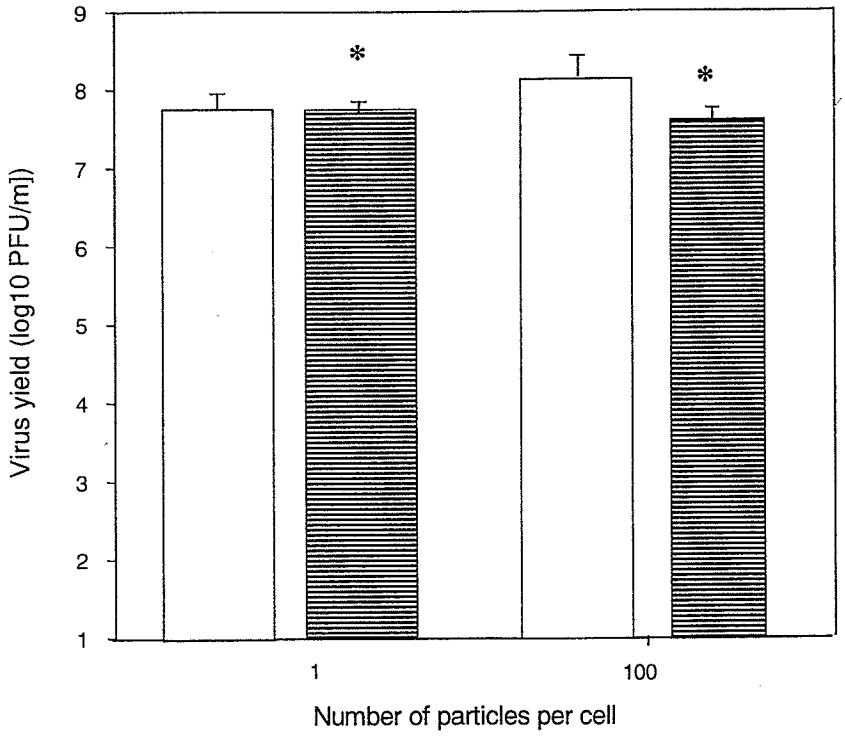


Figure 5.7. Comparison of reoviruses T3D and T1L core particles transfected with Lipofectamine 2000. Reovirus T3D or T1L core particles were transfected into L929 cells with Lipofectamine 2000. After 72 hours, cells were harvested and virus yields were determined by plaque assays. The data represents the average of a minimum of three experiments (n=3) and the error bars represent one standard deviation. ☐ T1L cores transfected with Lipofectamine 2000; and ▤ T3D cores transfected with Lipofectamine 2000. * The virus titers in the cells transfected with reovirus T1L cores were not significantly different from those that transfected with reovirus T3D cores by *t-test* (p = 0.35)

Figure 5.8.

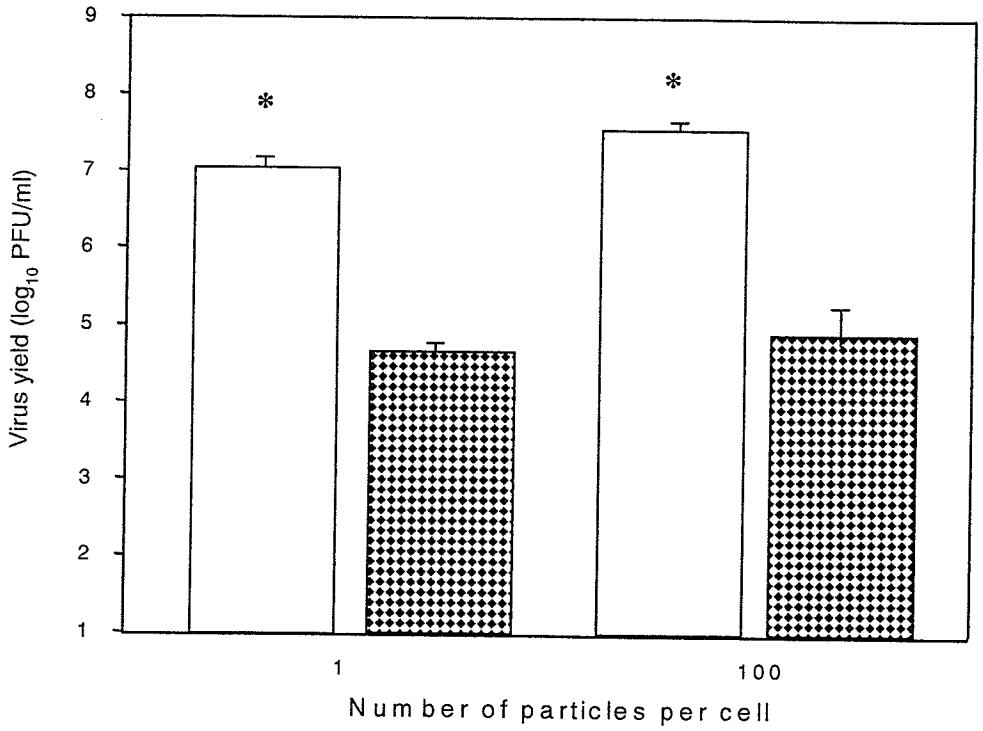


Figure 5.8. reovirus T1L core particles transfected with Lipofectamine 2000 or calcium phosphate. Two doses of reovirus T1L core particles were transfected with lipofectamine 2000 or calcium phosphate into L929 cells. 72 hours later, cells were harvested and virus yields measured. The data represents the average of minimum of three experiments (n=3) and the error bars represent one standard deviation. ▭ Cores transfected with Lipofectamine 2000; and ▩ Cores transfected with calcium phosphate. * The lipofectamine transfection is significantly efficient than the Ca^{2+} transfection by *t-test* ($p < 0.05$).

3. Lipofectamine reagent transfection of reovirus cores was significantly more efficient than Calcium phosphate ($CaPO_4$) methods.

4. These transfection methods may pave the way to improve genetic manipulation.

(The experiments described in this chapter have been published *(Jiang and Coombs, 2005. J. Virol. Meth. 128:88-92)*)

Chapter 6. *SEQUENCING OF A NEW MAMMALIAN REOVIRUS STRAIN TYPE 2 WINNIPEG (T2W).*

6.1. Introduction.

Reovirus has a wide geographic distribution and host range and can infect virtually all mammals including human beings. There are various reports of pneumonia and other respiratory diseases in both naturally and experimentally infected primates (Tyler, 2001) and there have been several reports describing reoviruses isolated from children with meningitis (Joskw and Aronson, 1958; Johansson et al., 1996; Tyler et al., 2004). In Dr. Coombs' Lab, the isolation and partial characterization of a new reovirus strain isolated from the central nervous system of an immunocompromised 8-week-old female infant was previously described (Hermann et al., 2004). This reovirus strain was identified as a reovirus serotype 2 member by virus neutralization and RNA-gel electrophoresis studies and was named serotype 2 Winnipeg (T2W) (Hermann et al., 2004).

In the previous researches of this thesis, two mammalian reoviruses Type 1 Lang (T1L) and Type 3 Dearing (T3D) were used to infect cells and observed the cellular response, performed siRNA assays as well as prepare the cores for transfection. However, reovirus type 2 strains generally do not grow as well as the other two reovirus serotypes (T1 and T3). The type 2 reoviruses have not been used to determine their functions on cell response including apoptosis either. Reovirus T2W strain replicate slower, and to lower titers than the type 2 prototype.strain T2 Jones (T2J) in various cell lines (data not shown). Because transfection improved infectivity of viral cores, which also are poorly infectious (Chapter **5**), an attempt was made to use the same method to grow more T2W. However, purified T2W virions transfected with Lipofectamine reagents did not show improved infectivity compared to direct virus infection, the virus titers in transfected cells were similar to the virus infection (data not shown). As we know most reovirus infections of humans are sunclinical and association of reovirus with human disease are also very rare. Only a few of reports showed some reovirus are observed in the samples (). Thus reovirus T2W isolated from the brain tissue of one sicked child could represent a clinically relevant emerging virus. The pathogenesis and molecular characteristics of the virus are valuable to investigate. First I tried to analyze its genotype by sequencing the S genes due to that the S1 gene and σ1 protein of reovirus determine the serotype of reovirus, and

there also currently were full geneome sequences of ≥12 MRV clones for each of the S genes, whereas there are only 3 or 4 full genome sequence available for most of the M and L genes in Genbank characteristics so I decided to sequence the T2W S1, S2, S3 and S4 gene sequences and to deduce σ1, σ2, σNS and σ3 protein sequences. Therefore, T2W S gene sequences and σ proteins were compared to all previously reported mammalian reovirus gene and protein sequences in order to better type the new strain and attempt to better understand the variation of T2W. In addition, the new reovirus strain could be typed by genotyping and be used for investigating whether the reovirus type 2 strain could induce apoptosis and activate the expression of cellular GADD45α protein.

6.2. Results.

6.2.1. Sequences of the reovirus T2W S1, S2, S3 and S4 genes: The complete sequences of the T2W S1, S2, S3 and S4 genes were determined in both directions. The genes (described in Figure **6.1)** were 1435, 1331, 1198 and 1196 nucleotides long, respectively; and submitted to Genbank (Genbank codes in Table **2.2**). All T2W S-class genes contain the conserved nucleotides 5'GATC- at the 5' terminus and 5'- TCATC 3' at the 3' terminus as shown in Figure **6.1** (identical to the terminal sequences of other mammalian reovirus genes in GenBank). The T2W S2, S3 and S4 genes are the same lengths as other mammalian reovirus S2, S3 and S4 genes (Figure **6.1**), and corresponding T2W σ2, σNS and σ3 proteins that the genes encode are the same lengths as the cognate proteins of the 3 mammalian prototype reovirus strains T1L, T2J and T3D (Figure **6.2B**, **C** and **D**). However, the T2W S1 gene and encoded σ1 protein differ in size from the S1 genes and σ1 proteins of the 3 prototype strains (Figure **6.1A** and **6.2A**).

6.2.2. Sequence analysis of the reovirus S1 gene and σ1 protein: The reovirus S1 gene encodes virus attachment protein σ1 (Lee, et al. 1981). The viral σ1 protein is unique to each prototype of mammalian reovirus and determines the serotype and is also the major genetic determinant of neurovirulence in infected mice (Tyler et al., 1986). The full-length sequences of the T2W S2, S3 and S4 genes were determined by the 3'end ligation method (Lambden et al., 1992; Yin et al., 2004) described in Materials and Methods (**2.10.2**), but T was unable to obtain full-length sequence of the T2W S1 gene by this method. The 5' end of the S1 sequence was not identified no matter what methods

Figure 6.1.

A. T2W S1 gene sequence:

```
T2W-S1          GCTATTCGTACTGATGT---CGGATCTGCGTCAGCTCATAAGGAGAGAGATTCTAGCTCT 57
T1L-S1          GCTATTCGCGCCTATGGA--TGCATCT--CTCATTACAGAGATACGGAAAATAGTACTCC 56
T2J-S1          GCTATTCGCACTCATGT---CGGATCTAGTGCAGCTCATAAGAAGGGAGATCTTACTGTT 57
T3D-S1          GCTATTGGT-CGGATGGATCCTCGCCTACGTGAAGAAGTAGTACGGCTGATAATCGCATT 59
                ****** *  *  ***          **     *      *          *

T2W-S1          TG-TA---GCGACGAGTGAT----GGCGGAG----CGAAA---GAAGTCGAGGAAATCAA 102
T1L-S1          AACTATCTGTATCAAGCAATGG--CTCCCAG----TCAAA--AGAAATCGAGGAAATCAA 108
T2J-S1          AACTG---GGAATGGAGAATCA--GCCAACT----CGAAACACGAGATCGAGGAAATTAA 108
T3D-S1          AACGAGTGATAATGGAGCATCACTGTCAAAAGGGCTTGAATCAAGGGTCTCGGCGCTCGA 119
                           **      *           **       **  **   *  *

T2W-S1          GAAACAGATACAACAATTGCGTAATGACGTCGATGGGATCAATCACAAACTGTCTGCGCA 162
T1L-S1          GAAACAAGTCCAGGTCAACGTTGATGATATCAGGGCTGCCAATATTAAACTCGACGGACT 168
T2J-S1          GAAACAAATTAAAGACATCTCTGCTGATGTCAACAGGATCAGTAACATCGTTGATTCAAT 168
T3D-S1          GAAGACGTCTCAAATACACTCTGATACTATCCTCCGGATCACCCAGGGACTCGATGATGC 179
                ***         *          *  *    **          **          *

T2W-S1          GCAAGGAGAACTCGCTAGCATTACTGCGAGAGTGTCAGCCATTGAGTCAGGATTTAGTAC 222
T1L-S1          TGGAAGACAGATTGCTGACATCAGCAATAGCATCTCAACCATTGAGTCAAGATTGGGTGA 228
T2J-S1          CCAAGGACAACTGGGTGGATTATCTGTACGCGTGTCAGCCATTGAATCGGGAGTTAGTGA 228
T3D-S1          AAACAAACGAATCATCGCTCTTGAGCAAAGTCGGGATGACTTGGTTGCATCAGTCAGTGA 239
                      *    *         *         *          * * *   *   * *  **

T2W-S1          ACTTAGCAATAGAGTTACTGACAATGAGCGGTCTATCTCGCAACTGTCAGGAGACGTGGG 282
T1L-S1          GATGGATAATCGACTTGTGGGTATCTCGAGTCAGGTCACGCAATTATCTAACTCAGGTAG 288
T2J-S1          GAACGGCAATCGAATTGATAGACTCGAGCGAGATGTCTCCGGCATATCGGCTAGCGTTAG 288
T3D-S1          TGCTCAACTTGCAATCTCCAGATTGGAAAGCTCTATCGGAGCCCTCCAAACAGTTGTCAA 299
                        * * *               *     **       *          *

T2W-S1          CGCAATCAATGGTAGTCTATCCACATTGGGTAACAGAATCGATGTTGCAGAACAAGGAAT 342
T1L-S1          CCAGAACACTCAGAGCATATCCTCATTGGGTGACAGAATCAATGCTGTCGAACCACGAGT 348
T2J-S1          CGGAATCGATTCGCGTTTATCCGAGCTGGGTGACCGAGTCAATGTTGCAGAACAGCGAAT 348
T3D-S1          TGGACTTGATTCGAGTGTTACCCAGTTGGGTGCTCGAGTGGGACAACTTGAGACAGGACT 359
                        *    *  *  **    *****    ** *          **     ** *

T2W-S1          TAGCCAGCTGGTTACTAGGACGGACGATCTCACTAGCAGAACATCAGCTTTGGAAGCAAC 402
T1L-S1          TGACAGTCTGGATACGGTCACGTCTAATCTCACTGGACGAACATCCACTTTGGAGGCAGA 408
T2J-S1          TGGCCAGTTGGATACAGTCACGGATAATCTCCTTGAGCGAGCATCAAGACTGGAAACTGA 408
T3D-S1          TGCAGACGTACGCGTTGATCACGACAATCTCGTTGCGAGAGTGGATACTGCAGAACGTAA 419
                *       *                  *****  *    **           **

T2W-S1          GAGTTCATCATTAACAACTGATCTGGCTTCTTTGAATACACGTGTCACGACGGAG----- 457
T1L-S1          TGTTGGAAGCTTACGGACAGAACTAGCAGCGCTAACAACACGGGTGACAACTGAGGTTAC 468
T2J-S1          AGTATCAGCCATTACTAATGACCTTGGATCATTGAATACGAGGGTGACGACTGAA----- 463
T3D-S1          CATTGGATCATTGACCACTGAGCTATCAACTCTGACGTTACGAGTAACATCCATA----- 474
                      *    *    *  ** **     *  * *      * ** **  *

T2W-S1          ------------CTCAATGATATACGTGCAAAC---GTGACAGCCATCTCTGGAAGAGT 501
T1L-S1          AAGGTTAGATGGTCTAATCAATAGTGGCCAGAATTCGATTGGTGAGCTATTCACAAGACT 528
T2J-S1          ------------TTGAACGATGTCCGCCAAACT---ATTGCTGCGATAGACACGCGTCT 507
T3D-S1          ----------------CAAGCGGATTTCGAAT-------CTAGGATATCCACG----- 504
                             *          *          *

T2W-S1          TGATGTATTGGAACGTGACGCTGTCACGAGTGTGGGTCAGGGATTGGAAAAGACGGGAAA 561
T1L-S1          ATCCAATGTGGAGACGTCTATGGTGACGACGGCTGGACGGGGACTGCAGAAAAACGGAAA 588
T2J-S1          CACGACACTGGAGACCGATGCCGTGACGTCGGTTGGTCAAGGGCTTCAGAAGACTGGGAA 567
T3D-S1          -------TTAGAGCGCACGGCGGTCACTAGCGCGGG---AGCTCCCCTCTCAATCCGTAA 554
                      * **          ** **    *  **    *           *   * **

T2W-S1          TTCTGTCAAGGTGATAGCTGGAAACGGTATGTGGTTTAATAGCCAGAATCAAATTCAGCT 621
T1L-S1          CACCTTGAACGTCATTGTAGGTAATGGAATGTGGTTTAATAGTTCTAATCAATTGCAGCT 648
T2J-S1          CTCGATTAAGGTTATTGTGGGTACGGGGATGTGGTTCGACCGCAATAATGTTCTGCAGTT 627
T3D-S1          TAACCGTATGACCATGGGATTAAATG--ATGGACTCACGTTGTCAGGGAATAATCTCGCC 612
                      *     ** *     *  *  ***   *      *          *   *

T2W-S1          AGATTTATCTGCACAAATGAAAGGTGTTGGATTTGAAGGGTCAGGTATGATAGCAAAGAT 681
T1L-S1          CGACCTTTCGGGGCAATCAAAAGGGGTGGGATTTGTCGGCACAGGAATGGTGGTTAAGAT 708
T2J-S1          ATTCTTATCGAACCAGCAGAAAGGGTTGGGATTCATAGACAATGGAATGGTAGTGAAAAT 687
T3D-S1          AT-CCGATTGCCAGGAAATACGGGTCTGAATATTCAAAATGGTGGACTTCAGTTTCGATT 671
                       *          *  **  *     *          **  *          *
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T2W-S1      CGACGGCACGTACTTCATGTATAACAGCAGTGGTCAAATCACATTGAAAGACAACATATC 741
T1L-S1      TGATACTAATTATTTTGCTTACAATAGTAATGGAGAGATTACATTGGTGAGTCAAATCAA 768
T2J-S1      AGATACCCAGTATTTCAGCTTCGATAGCAATGGCAACATAACTCTGAACAACAACATAAG 747
T3D-S1      TAATACTGATCAATTC-CAGATAGT--TAATAATAACTTGACTCTCAAGACGACTGTGTT 728
              *       * **           * *    *  * **  *          *

T2W-S1      AGGG-CTTCCA--TCCAGGACATCAGCACTTGAGTCACTAAAAATAGATACTGTCTTACC 798
T1L-S1      TGAA-TTGCCA--TCGCGCGTATCAACACTGGAATCAGCGAAAATCGATTCAGTTTTACC 825
T2J-S1      TGGT-CTGCCG--GCGCGAACAGGTTCCCTCGAGGCATCTCGTATCGATGTGGTAGCGCC 804
T3D-S1      TGATTCTATCAACTCAAGGATAGGCGCAACTGAGCAAAGTTACGTGGCGTCGGCAGTGAC 788
             *    *  *    *  *   *    *    **   *       * *     *      *

T2W-S1      TCCGTTGGTAGTGCAGGAATCGCAGGGATCTAGGTCACTGCGTTTGTTGTACGATGCAGT 858
T1L-S1      TCCATTAACCGTACGCGAAGCGAGCGGCGTACGTACCCTGAGCTTTGGTTATGATACGAG 885
T2J-S1      ACCGCTTGTGATACAGTCTACTGGTAGCACTCGGCTACTGCGTCTCATGTACGAGGCTGT 864
T3D-S1      TCCCTTGAGATTAAAC------AGTAGCACGAAGGTGCTGGATATGC--TAATAGACAGT 840
             **  *     *              *          ***    *    **  *  *

T2W-S1      CGATTTTAGAGTAGTAAATCAAGCATTCACATTAGTGACGAGGGCATCGATTCCGACCTA 918
T1L-S1      CGATTTTACAATCATCAACTCCGTACTGTCGTTACGGTCACGTTTGACTCTTCCGACATA 945
T2J-S1      GGACTTCGTGGTTACTAACAACGTTCTCACACTGAGAAATCGATCGGTCACGCCAACATT 924
T3D-S1      TCAACACTTGAAATTAATTCTAGTGGACA-GCTAACTGTTAGATCGACATCCCCGAATTT 899
              *             *     *         *        *          ** *  *

T2W-S1      TCGGTTTCCTTTGGATTACGATGCTTCGGCGAATATTGTCACGCTTAGCCCGAACTATCG 978
T1L-S1      CAGGTACCCTCTGGAGCTCGACACAGCAAATAATAGAGTGCAGGTGGCAGATCGTTTTGG 1005
T2J-S1      CAAGTTTCCTCTGGAGTTGAATAGTGCTGATAACTCAGTGAGCATTCATAGAAATTACCG 984
T3D-S1      GAGGTATCCGATAGC------TGATGTTAGCGGCGGTATCGGAATGAGTCCAAATTATAG 953
              **  **  * *                          *     *          *  *

T2W-S1      TATTAGAACTGGCCAATGGACTGGACAACTGCAATATCAAACTCCAACTGTCAATTGGAG 1038
T1L-S1      CATGCGCACGGGTACTTGGACGGGACAATTGCAATATCAGCACCCACAATTGAGTTGGAG 1065
T2J-S1      CATTAGACTTGGGCAATGGTCAGGTCAATTGGAATATCACACGCCGAGTTTGCGTTGGAA 1044
T3D-S1      GTTTAGGCAGAGCATGTGGATAGGAATTGTCTCCTATTCTGGTAGTGGGCTGAATTGGAG 1013
              *  *     *    ***   **     *    ***           *   *****

T2W-S1      CGTCCCAGTGACGGTGAAATTGATGCGAGTGAATGACTGGTTAATATTAAGTTTCTCCAG 1098
T1L-S1      AGCAAATGTCACTTTGAATTTGATGAAGGTGGATGATTGGTTGGTGTTGAGCTTTTCTCA 1125
T2J-S1      TGCTCCCGTCACGGTTAATTTGATGCGAGTAGACGATTGGCTCATTTTGAGTTTTACTCG 1104
T3D-S1      GGTACAGGTGAACTCCGACATTTTTATTGTAGATGATTACATACATATATGTCTTCC--A 1071
             *     ** *      *  *  *    **  * ** *   *     *  *  *  *

T2W-S1      ATTCTCAACGGGCG--CTATCCTCGCATCTGGTAAGCTCGTTTTAAACTTTGTTACTGGG 1156
T1L-S1      GATGACGACTAACT--CAATAATGGCAGATGGGAAATTTGTGATTAATTTTGTGTCTGGG 1183
T2J-S1      GTTTTCGACGAGCGG-CGATCTTAGCGTCAGGAAAGTTTGTATTGAACTTCGTAACTGGT 1163
T3D-S1      GCTTTTGACGGTTTCTCTATAGCTGACGGTGGAGATCTATCGTTGAACTTTGTTACCGGA 1131
             *    **       * **    *     **  *  *     * ** ** **  * **

T2W-S1      TTATCTTCAGGGTGGCAGACTGGTTCAACTGAGCCATCGAGTACGACTGACCCA-TTGTC 1215
T1L-S1      TTATCTTCTGGATGGCAGACGGGGGATACTGAACCATCGTCAACTATTGATCCA-TTG-C 1241
T2J-S1      TTGTCTCCAGGGTGGGCGACTGGGAGTACCGAGCCCTCGACAACTACTAACCCA-CTGTC 1222
T3D-S1      TTGTTACCACCGTTACTTACAGGAGACACTGAGCCCGCTTTTCATAATGACGTGGTCACA 1191
            ** *   *    *     ** **    ** ** **  *       * * *

T2W-S1      TACGACGT-----TCGCGGCAATTCAATTCGTGAATGGTT-CAAGTCGAGTTGATGCCTT 1269
T1L-S1      TACGACAT-----TTGCCGCGGTCCAATTTCTAAATAACGGTCAA-CGCATTGATGCGTT 1295
T2J-S1      AACGACGT-----TTGCTGCAATTCAGTTCATCAATGGGTGCATCTCGCGTAGACGCCTT 1277
T3D-S1      TATGGAGCACAGACTGTAGCTATAGGGTTGTCGTCGGGTGGTGCGCCTCAGT-ATATGAG 1250
             * *          *  **  *    **                 *      *

T2W-S1      TCGAATATTAGGAGTCACAGAATGGAATGCTGGTGAGTTGGAAGTATCTGAACTATGGTG 1329
T1L-S1      TAGGATCATGGGAGT-ATGGAATGGACGGATGGAGAATTAGAGAT-TAAGAATTATGGTG 1353
T2J-S1      TAGAATCTTGGGAGTCGCAGAGTGGAATGCCGGGGAACTAGAGAT-CACGAATCATGGCG 1336
T3D-S1      TAAGAATCTGTGGGTGGAGCAGTGGCAGGATGGAGTACTTCGGTTACGTGT-TGAGGGGG 1309
            *   *   *  * **     * ***   *  ** *   *     *    *   * ** *

T2W-S1      GGACGTACACCGCTCACACTGGGGTTGATTGGGCACCGATGACTTTGATGTATCCATGT- 1388
T1L-S1      GCACATACACCGGTCATACTCAAGTATATTGGGCTCCGTGGACGATCATGTATCCATGC- 1412
T2J-S1      GAACATATACAGCGCATACCAATGTCGACTGGGCGCCGATGACCATTATGTACCCATGT- 1395
T3D-S1      GTGGCTCAATTACGCACTCAAACAGTAAGTGGCCTGCCATGACCGTTTCGTACCCGCGTA 1369
            *    *  *     **  *        * *** *  *   ***  *   *** **  *
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T2W-S1      --CTGGGCTGACGATCCGAGTACGCCACTCGGCACA-GTGGGCATTCATC 1435
T1L-S1      --AATGTGAGGTGAATCTAGCGCG--AATCGGCACAAGGGGTCAATCATC 1458
T2J-S1      --CTGGGCTGAGGATCCGGGTGCTCCACTCGGCACA-GTGGCGACTCATC 1442
T3D-S1      GTTTCACGTGAGGATCAGACCACCC--CGCGGCACT-GGGGCATTTCATC 1416
```

B. T2W S2 gene sequence:

```
T2W-S2      GCTATTCGCTGGTCAGTTATGGCACGCGCTGCGTTCCTATTCAAGACCGTTGGATTCGGT 60
T1L-S2      GCTATTCGCTGGTCAGTTATGGCTCGCGCTGCGTTCCTATTCAAGACTGTTGGATTTGGT 60
T2J-S2      GCTATTCGCTGGTCAGTTATGGCACGCGCCGCGTTCCTATTCAAGACCGTTGGATTTGGT 60
T3D-S2      GCTATTCGCTGGTCAGTTATGGCTCGCGCTGCGTTCCTATTCAAGACTGTTGGGTTTGGT 60
            ********************** ***** ***************** ***** ** ***

T2W-S2      GGGCTGCAAAATGTTCCCATTAACGATGAGTTGGCATCTCATTTGCTTAGGGCAGGCAAT 120
T1L-S2      GGTCTGCAAAATGTGCCAATTAATGATGAGTTATCGTCACATCTACTTCGAGCCGGTAAT 120
T2J-S2      GGGCTGCAAAATGTACCTATTAACGATGAGCTTGCTTCACATCTGTTACGAGCTGGAAAT 120
T3D-S2      GGTCTGCAAAATGTGCCAATTAACGACGAACTATCTTCACATCTACTCCGAGCTGGTAAT 120
            ** *********** ** ***** ** **  *  * ** *** *  *  * ** ** ***

T2W-S2      TCTCCATGGCAATTGACTCAGTTTCTAGATTGGATCAGTCTTGGAAGAGGGCTAGCAACA 180
T1L-S2      TCGCCATGGCAGCTGACTCAGTTCTTAGATTGGATAAGTCTTGGAAGAGGATTAGCTACA 180
T2J-S2      TCTCCATGGCAGCTGACGCAATTCCTTGACTGGATTAGTCTCGGGAGAGGATTGGCTACC 180
T3D-S2      TCACCATGGCAGTTAACACAGTTTTTAGACTGGATAAGCCTTGGGAGGGGTTTAGCTACA 180
            ** ********  * ** ** **  * ** ***** ** ** ** ** **  * ** **

T2W-S2      TCCGCTCTTGTACCGGCAGCTGGCTCACGTTATTATCAGATGAGCTGTTTATTGAGTGGT 240
T1L-S2      TCAGCTCTTGTTCCAACCGCTGGTTCAAGATATTATCAGATGAGTTGTTTATTGAGTGGT 240
T2J-S2      TCTGCATTAGTGCCTACGGCGGGATCGCGTTACTATCAAATGAGTTGCCTACTGAGTGGT 240
T3D-S2      TCGGCTCTCGTTCCGACGGCTGGGTCAAGATACTATCAAATGAGTTGCCTTCTAAGTGGC 240
            ** **  * ** **  * ** ** **  * ** ***** ***** **  *  * *****

T2W-S2      ACATTGCAAATTCCATTTAGGCCGAATCATCGTTGGGGTGACGTTCGTTTCCTTCGCCTA 300
T1L-S2      ACCCTCCAGATTCCATTTCGTCCTAATCATCGATGGGGAGATATTAGGTTTCTGCGTCTA 300
T2J-S2      ACGCTGCAAATTCCTTTTCGACCAAATCATAGATGGGGAGACGTACGATTTCTACGTCTG 300
T3D-S2      ACTCTCCAGATTCCGTTCCGTCCTAACCACCGATGGGGAGACATTAGGTTCTTACGCTTA 300
            **  * ** ***** **  * ** ** **  * ***** **  *  * **  * **  *

T2W-S2      GTATGGTCTTCACCCACACTAGATGGATTAGTAGTTGCTCCTCCACAAGTGCTTGCTCAA 360
T1L-S2      GTGTGGTCAGCTCCTACGCTTGACGGGTTGGTTGTTGCCCCACCGCAGGTCTTAGCTCAG 360
T2J-S2      GTATGGTCTGCTCCTACCCTCGACGGTTTAGTTATTGCACCACCGCCCATTCTTGCGCAA 360
T3D-S2      GTGTGGTCAGCTCCTACTCTCGATGGATTAGTCGTAGCTCCACCACAAGTTTTGGCTCAG 360
            ** *****  * ** ** ** ** ** ** **  * ** ** ** *   *  * ** **

T2W-S2      CCGGCATTACAGGCACTAGCTGATCGAGCATACGACTGTGATGATTATCCCTTCCTTGCG 420
T1L-S2      CCAGCGTTACAGGCGCAGGCAGATCGAGTGTATGATTGTGATGATTACCCATTCTTAGCA 420
T2J-S2      CCTGCGATACAGGCGCAAGCGGATAGAGCTTACGACTGTGATGACTATCCTTTCCTTGCA 420
T3D-S2      CCCGCTTTGCAAGCACAGGCAGATCGAGTGTACGACTGCGATGATTATCCATTTCTAGCG 420
            ** **  * ** ** *  ** *** ***  ** ** ** ***** ** ** **  * **

T2W-S2      CGTGACCCTCGATTCAAACATCGTATATTCCAGCAACTGAGTGCGATTACTTTGTTAAAC 480
T1L-S2      CGTGACCCGAGATTTAAGCATCGAGTGTATCAACAATTGAGCGCCGTAACTCTGCTTAAC 480
T2J-S2      CGTGATCCCCGCTTCAAACATAGAGTATATCAGCAACTTAGTGCGATTACCCTGCTTAAT 480
T3D-S2      CGTGATCCAAGATTCAAACATCGGGTGTATCAGCAATTGAGTGCTGTAACTCTACTTAAC 480
            ***** **  * ** ** *** *  * *  ** *** * ** **  * **  *  * **

T2W-S2      TTAACAGGGTTTGGGCCCATATCGTTCGTTAGAGTAGATGAAGACATGTGGAGCGGTGAC 540
T1L-S2      TTGACGGGGTTTGGTCCAATTTCCTATGTTCGAGTAGACGAGGATATGTGGAGTGGGGAT 540
T2J-S2      CTGACTGGCTTCGGTCCGATATCATACGTGCGGGTTGATGAGGACATGTGGAGTGGTGAC 540
T3D-S2      TTGACAGGTTTTGGCCCGATTTCCTACGTTCGAGTGGATGAAGATATGTGGAGTGGAGAT 540
             * ** ** ** ** ** ** ** * **  * ** ** ** ** ******** ** **

T2W-S2      GTGAATCAGTTGATGATGAATTATTTCGGCCATACATTTGTGGAGATTGCTTATACTTTA 600
T1L-S2      GTGAACCAGCTTCTCATGAACTACTTCGGGCATACGTTTGCGGAAATTGCGTATACATTA 600
T2J-S2      GTGAGCCAGTTACTCATGAATTATTTTGGTCACACTTTCGCCGAAATCGCGTACACATTA 600
T3D-S2      GTGAACCAGCTTCTCATGAACTATTTCGGGCACACGTTTGCAGAGATTGCATACACATTG 600
            ****  *** *  * ***** ** ** ** ** ** ** *  ** ** ** ** ** **

T2W-S2      TGTCAGGCATCAGCCAATCGTCCCTGGGAGCATGATGGCACTTATGCTAGAATGACTCAA 660
T1L-S2      TGTCAAGCTTCAGCCAATAGACCTTGGGAGCATGATGGTACGTATGCGAGGATGACTCAA 660
T2J-S2      TGTCAAGCTTCGGCTAACCGACCATGGGAACATGATGGAACATATGCGCGTATGACTCAA 660
T3D-S2      TGTCAAGCCTCGGCTAATAGGCCTTGGGAATATGACGGTACATATGCTAGGATGACTCAG 660
            ***** ** ** ** **  * ** *****  **** ** ** *****  * ********
```

```
T2W-S2      ATTATATTGTCGTTATTTTGGTTATCTTACGTTGGGGTGATTCACCAGCAGAACACTTAC 720
T1L-S2      ATTATACTGTCCTTATTTTGGTTATCATATGTCGGTGTAATTCATCAGCAGAACACGTAC 720
T2J-S2      ATAGTGTTGTCGTTGTTCTGGTTATCGTATGTCGGAGTAATTCATCAACACAACACGTAT 720
T3D-S2      ATTGTGTTATCCTTGTTCTGGCTATCGTATGTCGGTGTAATTCATCAGCAGAATACGTAT 720
            **  *  * ** ** ** *** **** ** ** ** ** ***** ** ** ** ** **

T2W-S2      CGAACGTTTTATTTTCAGTGTAATCGTCGTGGGGACGCTGCTGAGGTTTGGATACTATCG 780
T1L-S2      CGGACGTTCTATTTCCAATGCAATCGGCGCGGTGATGCTGCTGAAGTATGGATTCTTTCC 780
T2J-S2      CGTACGTTCTATTTCCAATGTAATAGACGTGGTGATGCCGCGGAGGTGTGGATACTGTCG 780
T3D-S2      CGGACATTCTATTTTCAGTGTAATCGGCGAGGTGACGCCGCTGAGGTGTGGATTCTTTCT 780
            ** ** ** ***** ** ** *** * ** ** ** ** ** ** ** ***** ** **

T2W-S2      TGCTCATTAAATCACTCTGCCCAGATTAGACCTGGTAATCGTAGTCTATTTGTGGTCCCT 840
T1L-S2      TGCTCATTAAACCACTCCGCCCAGATTAGACCGGGTAATCGCAGTCTATTCGTCATGCCA 840
T2J-S2      TGCTCATTGACTCACTCGGCACAGATAAGGGCTGGAAATCGTAGCCTTTTCGTCATGCCC 840
T3D-S2      TGTTCGTTGAACCATTCCGCACAAATTAGACCGGGTAATCGTAGCTTATTCGTTATGCCA 840
            ** ** ** *  ** ** ** ** ** **  * ** ***** **  * ** **  * **

T2W-S2      ACTAGTCCGGATTGGAACATGGATGTTAATCTTATTTTGAGTTCCACCCTTACCGGCTGT 900
T1L-S2      ACAAGTCCAGATTGGAATATGGACGTCAATTTGATTTTAAGTTCGACGTTGACAGGGTGC 900
T2J-S2      ACTAGCCCTGATTGGAACATGGATGTGAATTTGATTCTCAGTTCAACACTTACTGGATGC 900
T3D-S2      ACTAGCCCAGATTGGAACATGGACGTCAATTTGATCCTGAGTTCAACGTTGACGGGGTGT 900
            ** ** ** ******** ***** ** *** * **  * ***** **  * ** ** **

T2W-S2      CTTTGCTCAGGGACACAACTCCCTTTAATAGATAACAACTCTGTTCCGAACGCGTCACGC 960
T1L-S2      TTGTGTTCGGGCTCTCAGTTACCGCTTATTGACAATAACTCAGTGCCTGCGGTTTCGCGT 960
T2J-S2      ACTAGCCCTGATTGGAACATGGATGTGAATTTGATTCTCAGTTCAACACTTACTGGATGC 900
T3D-S2      TTGTGTTCGGGTTCACAGCTGCCACTGATTGACAATAATTCAGTACCTGCAGTGTCGCGT 960
                 * ** ** **  * **  * **  * ** ** ** ** ** ** **    *  **  *

T2W-S2      AACATCCATGGATGGACTGGACGTGGGGGTAATCAATTACATGGTTTCCAGGTGCGTAGA 1020
T1L-S2      AACATCCATGGTTGGACTGGCAGAGCTGGCAACCAGCTGCATGGTTTCCAAGTGCGACGA 1020
T2J-S2      AACATTCATGGATGGACGGGCCGAGGTGGTAATCAATTGCATGGGTTCCAAGTTCGACGC 1020
T3D-S2      AACATCCATGGCTGGACTGGTAGAGCTGGTAACCAATTGCATGGGTTCCAGGTGAGACGA 1020
            ***** ***** ***** **  * *  ** ** **  * ***** ***** **  *  *

T2W-S2      ATGGTAACTGAGTTCTGTGATCGTCTTAGACGGGATGGGGTGATGACTCAAGCTCAGCAG 1080
T1L-S2      ATGGTGACTGAATTCTGTGACAGATTAAGACGCGATGGAGTTATGACTCAAGCTCAGCAA 1080
T2J-S2      ATGATTACTGAATATTGCGATAGGCTGAGGCGTGATGGCGTGATGACCCCCGCGCAGCAG 1080
T3D-S2      ATGGTGACTGAATTTTGTGACAGGTTGAGACGCGATGGTGTCATGACCCAAGCTCAGCAG 1080
            *** * ***** *  ** **  *  * ** ** ***** ** ***** *  ** *****

T2W-S2      AACCAGGTCGAAGTACTGGCTGATCAGACTCAGCAGTTTAAGAGAGACAAACTTGAGACA 1140
T1L-S2      AATCAAATTGAAGCGTTGGCAGATCAAACTCAACAGTTTAAGAGGGATAAACTTGAGGCG 1140
T2J-S2      ATGCAAATAGAAGCGTTAGGGGATCAGACGCAGCAGTTCAAGCGTGACAAGCTTGAGGCC 1140
T3D-S2      AATCAAGTTGAAGCGTTGGCAGATCAGACTCAACAGTTTAAGAGGGACAAGCTCGAAACG 1140
            *  **  * ****   * *  ***** ** ** ***** *** * ** ** ** **  *

T2W-S2      TGGGCTCGTGAAGATGATCAGTATAATCAAGCTCACCCTAATTCGACGATGTTCAGAACT 1200
T1L-S2      TGGGCTAGGGAAGATGATCAGTATAATCAGGCTAATCCGAATTCTACGATGTTCCGTACG 1200
T2J-S2      TGGGCTTTGGAGGACGATCAATATAATCGCGCGCATCCAAACTCGACAATGTTTAGGACT 1200
T3D-S2      TGGGCGAGAGAAGACGATCAATATAATCAGGCTCATCCCAACTCCACAATGTTCCGTACG 1200
            *****    ** ** ***** *******  **  * ** ** ** ** *****  * **

T2W-S2      AAGCCATTCACTAATGCTCAATGGGGGAGAGGTAATACAGCGGCTACTAGTGCGGCAATT 1260
T1L-S2      AAGCCATTTACGAATGCGCAATGGGGACGAGGAAATACCGGAGCGACTAGTGCCGCAATT 1260
T2J-S2      AAGCCATTTACCAATGCTCAATGGGGCCGGGGAAATACAGCAGCGACTAGCGCAGCGATT 1260
T3D-S2      AAACCATTTACGAATGCGCAATGGGGACGAGGTAATACGGGGGCGACTAGTGCCGCGATT 1260
            ** ***** ** ***** ********  * ** ***** *  ** ***** ** ** ***

T2W-S2      GCAGCTCTCATCTAATCGTCTTGAAGCGTGAGGGTCCCCCCACACCCTCCGCGACTGACC 1320
T1L-S2      GCAGCCCTTATCTAATCGTCTTGGAGTGAGGGGATCCCCCCACACCCCTCACGACTGACC 1320
T2J-S2      GCGGCTCTCATCTAACCGTCTGGAAGCGTGGGGGTCCCCCCACACCCTCCTCGGCTGACC 1320
T3D-S2      GCAGCCCTTATCTGATCGTCTTGGAGTGAGGGGGTCCCCCCACACACCTCACGACTGACC 1320
            ** ** ** **** * ***** * ** * * ** ********** *  * ** ******

T2W-S2      ACTTATTCATC 1331
T1L-S2      ACACATTCATC 1331
T2J-S2      ACCTATTCATC 1331
T3D-S2      ACACATTCATC 1331
            **  *******
```

C. T2W S3 gene sequence:

```
T2W-S3      GCTAAAGTCACGCCTGTTATCGTCACTATGGCTTCTTCACTCAGAGCAGCGATCTCCAAG 60
T1L-S3      GCTAAAGTCACGCCTGTTGTCGTCACTATGGCTTCCTCACTCAGGGCTGCGATCTCTAAG 60
T2J-S3      GCTAAAGTCACACCTGTTATCGTCGCCATGGCTTCCTCACTCAGAGCTGCGATCTCAAAG 60
T3D-S3      GCTAAAGTCACGCCTGTCGTCGTCACTATGGCTTCCTCACTCAGAGCTGCGATCTCCAAG 60
            ********** *****  ***** * ******** ******** ** ******** ***

T2W-S3      ATTAAACGTGATGATCTTGGTCAGCAAGTGGGTCCCGGCTATGTCATGCTGAGATCATCT 120
T1L-S3      ATCAAGAGAGATGATGTTGGTCAGCAAGTTTGTCCAAATTATGTCATGCTTAGATCATCG 120
T2J-S3      ATCAAGCGCGATGATGTCGGTCAGCAAGTATGCCCTAACTATGTCATGTTGAGGTCTTCT 120
T3D-S3      ATCAAGAGGGATGACGTCGGTCAGCAAGTTTGTCCTAATTATGTCATGCTGCGGTCCTCT 120
            ** **  * *****  * **********  * **    ********* *  * ** **

T2W-S3      GTGACAACCAAAGTTGTTCGTAACGTGGTTGAGTACCAAATTAGGACTGGTGGTTTCTTT 180
T1L-S3      GTCACAACGAAGGTGGTACGAAACGTTGTTGAGTATCAAATCCGTACAGGTGGATTCTTT 180
T2J-S3      GTGAATACCAAAGTGGTGAGAAATGTGGTTGACTACCAGATTAAAACTGGCGGCTTCTTC 180
T3D-S3      GTCACAACAAAGGTGGTACGAAATGTGGTTGAGTATCAAATTCGTACGGGCGGATTCTTT 180
            ** *  ** ** ** **  * ** ** ***** ** ** **    ** ** ** *****

T2W-S3      TCTTGTCTGTCAATGTTGCGTCCGCTCCAGTATGCCAAGCGTGAGCGGTTGTTAGGCCAG 240
T1L-S3      TCGTGCCTAGCTATGTTGAGACCGCTCCAGTATGCTAAACGTGAGCGTCTGCTTGGACAA 240
T2J-S3      TCCTGTATCGCAATGCTGCGTCCTTTGCAATATGCCAAGCGTGAAAGATTGCTGGGTCAA 240
T3D-S3      TCGTGCTTAGCTATGCTAAGGCCACTCCAGTACGCTAAGCGTGAGCGTTTGCTTGGTCAG 240
            ** **  *  * *** *  * **  * ** ** ** ** ***** *  ** * ** **

T2W-S3      CGCAATTTGGAACGCATATCCTCTAGAGATATTCTTCAGACTCGTGATCTGCATTCCTTG 300
T1L-S3      AGGAATCTGGAACGCATATCGACTAGGGACATTCTTCAGACACGCGATTTGCACTCATTG 300
T2J-S3      CGCAACCTAGAACGTATCGCTGCCCGAGACGTGCTGCAAACCAGGGATCTACACTCACTA 300
T3D-S3      AGGAATCTGGAACGTATATCGACTAGGGATATCCTTCAGACTCGTGATTTACACTCACTA 300
             * **  * ***** **  *  *  * **  * ** ** **  * *** * ** **  *

T2W-S3      TGTATGCCTACTCCTGATGCTCCTATGTCAAACTATCAGGCATCGACTATGAGAGAGCTC 360
T1L-S3      TGCATGCCAACTCCTGACGCGCCAATGTCCAATCATCAGGCAGCCACCATGAGAGAGCTG 360
T2J-S3      TGCATGCCCACGCCTGATGCTCCGATGACTAATTACCAGGCTTCCACCATGAGAGAATTA 360
T3D-S3      TGTATGCCAACTCCTGATGCGCCAATGTCTAATCATCAAGCATCCACCATGAGAGAGCTG 360
            ** ***** ** ***** ** ** *** * **  * ** **  * ** ******** *

T2W-S3      ATATGTAGTTATTTTAAGACAGATCATGCTGATGGTCTGAGATATGTACCAATGGATGAT 420
T1L-S3      ATCTGCAGCTACTTCAAGGTCGACCACACTGATGGGTTGAAATATATACCCATGGATGAG 420
T2J-S3      GTTTGTGACCACTTCAAGGTTGATCACGTGGATGGACTACGCTATGTCCCCATGGATGAC 420
T3D-S3      ATTTGCAGTTACTTCAAGGTCGATCATGCGGATGGGTTGAAATATATACCCATGGATGAG 420
             * **     * ** ***   ** **    *****  *    *** * ** ********

T2W-S3      CGATATTCGCCCGTCTCGCTGGCTAGGCTTTTTACTATGGGAATGGCTGGACTTCACATT 480
T1L-S3      AGATATTCTCCATCATCGCTTGCCAGATTGTTCACTATGGGTATGGCTGGACTACACATT 480
T2J-S3      AGGTACTCCCCCTCTTCCTTAGCTCGACTGTTTACAATGGGAATGGCTGGTCTCCACATC 480
T3D-S3      AGATACTCTCCGTCATCACTTGCCAGATTGTTTACCATGGGCATGGCTGGGCTGCACATT 480
             * ** ** **    **  * **  *  * ** ** ***** ******** ** *****

T2W-S3      ACTACAGACCCTTCATATAAGAGAGTGCCGATAATGCATTTGGCGGCAGACCTTGATTGC 540
T1L-S3      ACCACTGAGCCTTCCTACAAACGTGTGCCCATCATGCACTTGGCAGCAGATTTGGACTGC 540
T2J-S3      ACGACTGAGCCTGCGTATAAGCGAGTCCCAATTATGCACCTTGCTGCTGATCTTGACTGC 540
T3D-S3      ACCACTGAGCCATCTTATAAGCGTGTTCCGATTATGCACTTAGCTGCGGACTTGGACTGT 540
            ** ** ** **  * ** **  * ** ** ** *****  * ** ** **  * ** **

T2W-S3      ATGACATTGGCCTTGCCTTACATGATCACTCTCGATGGTGACACAGTTGTTCCTGTCGCT 600
T1L-S3      ATGACGTTAGCTTTACCCTACATGATTACACTTGATGGTGACACAGTGGTACCTGTCGCC 600
T2J-S3      ATGACATTTGCACTTCCATACATGATCACTGTTGATGGTGATACTGTAGTACCTGTGGCT 600
T3D-S3      ATGACGCTGGCTCTACCTTACATGATTACGCTTGATGGTGATACTGTGGTTCCTGTCGCT 600
            *****  * **  * ** ******** **  * ******** ** ** ** ***** **

T2W-S3      CCTGTGGCTTCTGCTGAGCAGTTGTTGGATGATGGCTTCAAGGGCTTGGCTTGTATGGAC 660
T1L-S3      CCAACGCTTTCTGCAGAACAGCTTTTGGATGATGGACTTAAGGGATTAGCATGCATGGAT 660
T2J-S3      CCAACTTTACCAGCTGAACGCCTTTTGGATGACGGTTTTAAAGGATATGGTTGTTTAGAC 660
T3D-S3      CCAACACTGTCAGCGGAACAGCTTCTGGACGACGGACTCAAAGGATTAGCATGCATGGAT 660
            **        * ** ** *   *  **** ** **  * ** ** *  *  **  * **

T2W-S3      ATTTCATACGGTTGTGAGGTCGACGCCAATAATCGGTCAGCTGGAGATCAGAGTATGGAC 720
T1L-S3      ATCTCATACGGATGTGAGGTGGACGCTAGCAACCGATCAGCTGGTGATCAGAGCATGGAT 720
T2J-S3      ATCTCATATGGATGTGAGGTTGATGCCAACAACCGGTCAGCGGGAGATCAAAGTATGGAC 720
T3D-S3      ATCTCCTATGGATGTGAGGTGGACGCGAATAGCCGGCCGGCTGGTGATCAGAGTATGGAC 720
            ** ** ** ** ******** ** ** *  *  **  * ** ** ***** ** *****
```

```
T2W-S3      TCTTCGCGTTGTATCAACGAGCTGCACTGTGATGATACGGCTGAGGCGATATGCATACTA 780
T1L-S3      TCTTCACGATGCATCAATGAGTTATATTGCGAGGAAACGGCAGAAGCTATCTGTATACTC 780
T2J-S3      TCATCCAGATGCATTAATGAGCTGTATACCGCTGAAACAGCTGAGGCCATTTGTATACTT 780
T3D-S3      TCTTCACGCTGCATCAACGAGTTGTATTGCGAGGAGACAGCAGAAGCCATCTGTGTGCTT 780
            ** **  * ** ** ** *** *  *    *  ** ** ** ** ** ** **  * **

T2W-S3      AAGACATGTCTGATACTTAACTGCATGCAGTTTAAGTTAGAGATGGACGACCTGGCACAT 840
T1L-S3      AAAACATGTCTTGTGCTGAACTGTATGCAATTCAAACTTGAGATGGATGATTTAGCACAC 840
T2J-S3      AAAACTTGTTTGATCCTGAATTGTATGCAGTTTAAGTTGGAGATGGACGACTTGGCACAC 840
T3D-S3      AAGACATGCCTTGTGTTAAATTGCATGCAGTTTAAACTTGAGATGGATGACCTAGCACAT 840
            ** ** **  *  *  * ** ** ***** ** **  * ******** **  * *****

T2W-S3      AACAGCGTAGAGCTGGATAAAGTGCAGATGATGATTCCGTTCAGTGAGCGTATCTTTAGG 900
T1L-S3      AACGCTACTGAGCTGGACAAGATACAGATGATGATACCTTTTAGTGAACGTGTTTTCAGA 900
T2J-S3      AATGGATTCGAATTAGATAAGGTACAGATGATGATACCATTCAGCGAGAGAGTGTTTCGG 900
T3D-S3      AACGCTGCTGAGCTGGACAAGATACAGATGATGATACCCTTCAGTGAGCGTGTTTTTAGG 900
            **       **  * ** **  * ********** ** ** ** **  *  * **  *

T2W-S3      ATGGCCTCCGCGTTCGCCACAATCGATGCGCAGTGCTTCCGTTTCTGTGTCATGATGAAA 960
T1L-S3      ATGGCTTCTTCATTTGCTACCATTGATGCCCAGTGTTTCAGGTTTTGTGTGATGATGAAG 960
T2J-S3      ATGGCTTCCGCATTTGCTACCATAGATGTTCAGTGTTTCAGATTTTGTCTTTTGATGAAG 960
T3D-S3      ATGGCCTCGTCCTTTGCGACTATTGATGCCCAGTGTTTTAGGTTTTGCGTGATGATGAAG 960
            ***** **  * ** ** ** ** **** ***** **  * ** **  *  *******

T2W-S3      GACAAGAATTTGAAAGTTGATATGCGAGAGACTATGAGGACATGGAGGGGACCCAGCTCC 1020
T1L-S3      GATAAGAATTTGAAGATAGACATGCGTGAAACGATGAGACTTTGGACTCGATCGGCGTTG 1020
T2J-S3      GATAAGAACCTCAAAATTGATATGAGGGAGACCATGCGCTTGTGGACGCGCGCTGGCTCG 1020
T3D-S3      GATAAAAATCTGAAAATAGATATGCGTGAAACGACGAGACTGTGGACTCGTTCAGCATCA 1020
            ** ** **  * **  * ** *** * ** ** * * *    ****   *  *    *

T2W-S3      GATGACGCTATTGTTATCTCATCATTGACTGTCACCCTGGATAGGGGTAGAAGGGTTGCT 1080
T1L-S3      GACGATTCAGTGGTTACGTCATCTTTGAGTATTTCGCTGGATCGAGGTCGATGGGTAGCA 1080
T2J-S3      GACGACGCCATCTCTACATCTTCCCTGACTATTTCACTGGACCGTGGACGGTGGGTGGCG 1080
T3D-S3      GATGATTCTGTGGCCACGTCATCTTTAAGTATTTCCTTGGACCGGGGTCGATGGGTGGCG 1080
            ** **  *  *    *  ** **  * * * *  *  **** * **  *  **** **

T2W-S3      TCTGATGCAAATGACACTCGACTGCTCGTATTCCCTATTCGTGTGTAATGGGTGAGTGAG 1140
T1L-S3      GCTGATGCTACTGATGCTAGATTGCTGGTGTTTCCAATTCGCGTGTAATGGGTGAGTGAG 1140
T2J-S3      ATGGATATGAACGAGGTGCGTCTCTTAGTGTTCCCTGCTCGTGTGTAATGGGTGAGTGAT 1140
T3D-S3      GCTGACGCCAGTGATGCTAGACTGCTGGTTTTTCCGATTCGCGTGTAATGGGTGAGTGAG 1140
               **    *  **     *  *  * ** ** **   *** ****************

T2W-S3      CTGATGTGATCACTGAGGCATGTGCCGATGCTTCGGTGGTGGGTGACGTACATTCATC 1198
T1L-S3      CCGATGTGGTCGCCAAGACATGTGCCGGTTTCTTGGTGGTGGGTGGCGCCTAATCATC 1198
T2J-S3      GCTAGGCGATCGTCAGGGCATGTGCCGGTGCCCTGGTGGAGGGTGGCGCTCAATCATC 1198
T3D-S3      CTGATGTGGTCGCCAAGACATGTGCCGGTGTCTTGGTGGTGGGTGACGCCTAATCATC 1198
               * * * **     * ********* *     ***** ***** **   * *****
```

D. T2W S4 gene sequence:

```
T2W-S4      GCTATTTTTGCCTCTTCCTAGACGTCGTCGCAATGGAGGTGTGCTTGCCAAACGGTCACC 60
T1L-S4      GCTATTTTTGCCTCTTCCCAAACGTTGTCGCAATGGAGGTGTGCTTGCCCAACGGTCATC 60
T2J-S4      GCTATTTTTGCCTCTTCCTAGACGTTGTCGCAATGGAGGTGTGCCTGCCTAACGGTCATC 60
T3D-S4      GCTATTTTTGCCTCTTCCCAGACGTTGTCGCAATGGAGGTGTGCTTGCCCAACGGTCATC 60
            ****************** * **** ******************** **** ******** *

T2W-S4      AAGGATGGGACAAGACAATCTCAGCACAACTTTATATGATGGTGTGAAGTGGCGCCATCG 180
T1L-S4      AGATCGTGGACTTGATTAACAACGCTTTTGAAGGTCGTGTATCAATCTACAGCGCGCAAG 120
T2J-S4      AGATCGTCGACTGGATCAATAACGCATTTGAAGGACGAGTGTCAATTTACAGTGCTCAGC 120
T3D-S4      AGGTCGTGGACTTGATTAACAACGCTTTTGAAGGTCGTGTATCAATCTACAGCGCGCAAG 120
            ** **** **    ** ** ***** ***** **  * ** ** ** ***    * **

T2W-S4      AAGGATGGGACAAGACAATCTCAGCACAACTTTATATGATGGTGTGAAGTGGCGCCATCG 180
T1L-S4      AGGGATGGGACAAAACAATCTCAGCACAGCCAGATATGATGGTATGTGGTGGTGCCGTCG 180
T2J-S4      AAGGATGGGACAAAACAATCTCAGCACAGCCGGACATGATGGTGTGTGGTGGCGCCGTCG 180
T3D-S4      AGGGATGGGACAAAACAATCTCAGCACAGCCAGATATGATGGTATGTGGTGGCGCCGTCG 180
            * ********** ************** *   * ******** **  **** *** ***

T2W-S4      TAAGCATGCATTGTCTAGGCGTCGTTGGATCTCTCCAGCGCAAAATAAAGCATTTGCCTC 240
T1L-S4      TTTGCATGCATTGTCTAGGTGTTGTTGGATCTCTGCAACGCAAGCTGAAGCATTTGCCTC 240
T2J-S4      TATGCATGCATTGTCTAGGTGTGGTTGGGTCACTGCAGCGAAAGCTGAAGCACTTGCCCC 240
T3D-S4      TTTGCATGCATTGTCTAGGTGTTGTTGGATCTCTACAACGCAAGCTGAAGCATTTGCCTC 240
            *  ***************** ** ***** ** ** ** ** **  * ***** ***** *
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T2W-S4      ATCATAGATGCAATCAACAGTTGAGACAACAAGATTACGTTGACCTGCAGTTTGCTGACC 300
T1L-S4      ACCATAGATGTAATCAACAGATTCGTCATCAGGATTACGTCGATGTACAGTTCGCAGATC 300
T2J-S4      ATCATAAGTGCAACCAACAGCTACGCCAACAGGACTACGTTGATGTGCAGTTCGCTGATC 300
T3D-S4      ACCATAGATGTAATCAACAGATCCGTCATCAGGATTACGTCGATGTACAGTTCGCAGACC 300
            * ****  ** ** ****** *  * ** ** ** ***** **  * ***** ** ** *

T2W-S4      GTGTTACGGCTCACTGGAAACGTGGGATGCTCTCATTTGTTTCTCAAATGCATGCTCTGA 360
T1L-S4      GTGTTACTGCTCACTGGAAGCGGGGTATGCTGTCCTTTGTTGCGCAGATGCACGCGATGA 360
T2J-S4      GGGTTACTGCTCACTGGAAACGTGGTATGCTATCATTTGTCTCTCAGATGCATGCTATCA 360
T3D-S4      GTGTTACTGCTCACTGGAAGCGGGGTATGCTGTCCTTCGTTGCGCAGATGCACGAGATGA 360
            * ***** ********** ** ** ***** ** ** **  * ** ***** *   * *

T2W-S4      TGAATGACGTCCAACCAGAACAATTGGATCATGTAAGAGTCCAAGGTGGTAAATTGGTTG 420
T1L-S4      TGAATGACGTATCACCAGAGGATCTAGACCGTGTGCGTACTGAGGGAGGTTCACTAGTGG 420
T2J-S4      TGAACGATGTGACACCCGAAGAGCTTGAAAGAGTGAGAACTGACGGAGGTTCTTTGGCTG 420
T3D-S4      TGAATGACGTGTCGCCAGATGACCTGGATCGTGTGCGTACTGAGGGAGGTTCACTAGTGG 420
            **** ** **    ** **  *  * **    **  *     * ** ***    * *  *

T2W-S4      AATTGGACTGGCTACAGGTCGATCCGGGGTCAATGTTCAGATCGATACATGCCAATTGGA 480
T1L-S4      AGTTAAACTGGCTTCAGGTTGATCCAAATTCAATGTTTAGATCAATACACTCAAGTTGGA 480
T2J-S4      AGCTTAACTGGCTACAAGTGGATCCTGGCTCAATGTTTCGATCAATTCATTCCAGCTGGA 480
T3D-S4      AGCTGAACCGGCTTCAGGTTGACCCAAATTCAATGTTTAGATCAATACACTCAAGTTGGA 480
            *  *  ** **** ** ** ** **    ********  **** ** **  * *  ****

T2W-S4      CTGACCCCTTGCAGGTTGTGGAAGACTTGGATACCCAACTTGACAGATATTGGACCGCCC 540
T1L-S4      CAGATCCTCTGCAGGTAGTGGATGATCTTGACACTAAGCTGGATCAATACTGGACGGCCC 540
T2J-S4      CTGATCCCCTTCAAGTGGTTGAGGATCTCGATACTCAGCTAGACCGCTATTGGACAGCGT 540
T3D-S4      CAGATCCTTTGCAGGTGGTGGACGACCTTGACACTAAGCTGGATCAGTACTGGACAGCCT 540
            * ** **  * ** ** ** ** **  * ** **  * ** **    ** ***** **

T2W-S4      TCAATCTAATGATTGATTCATCCGACCTGGTACCCAACTTTCTTATGCGAGATCCTTCTC 600
T1L-S4      TGAATCTGATGATTGATTCATCCGACTTGGTGCCCAACTTCATGATGAGAGACCCATCAC 600
T2J-S4      TAAATCTAATGATTGACTCATCAGACTTGGTGCCGAACTTTATGATGCGTGATCCATCGC 600
T3D-S4      TAAACCTGATGATCGACTCATCCGACTTGATACCCAACTTTATGATGAGAGACCCATCAC 600
            * ** ** ***** ** ***** *** ** * ** *****  * *** * ** ** ** *

T2W-S4      ATGCTTTTAATGGTGTTAAGTTGGAGGGTGACGCCAGACAGACCCATTTCTCGCGGACTT 660
T1L-S4      ATGCATTCAATGGTGTGAGACTGGAGGGAGACGCCCGCCAAACTCAATTCTCTAGGACTT 660
T2J-S4      ATGCTTTTAATGGGGTGAAGTTGGAAGGTGAGGCACGACAGACTCAGTTTTCTCGTACAT 660
T3D-S4      ACGCGTTCAATGGTGTGAAACTGGAGGGAGATGCTCGTCAAACCCAATTCTCCAGGACTT 660
            * ** ** ***** ** *   **** ** ** ** *  * ** ** ** ** ** **  * ** *

T2W-S4      TCGAGCCTCGATCCAGTCTAGAATGGGGGGTAATGGTGTATGACTATTCGGAGCTCGAAA 720
T1L-S4      TCGATTCGAGATCGAGTTTAGAATGGGGTGTGATGGTTTACGATTACTCTGAGTTAGAGC 720
T2J-S4      TCGATTCAAGATCGAACTTGGAGTGGGGCGTCATGATCTATGACTATTCTGAATTAGAAA 720
T3D-S4      TTGATTCGAGATCGAGTTTGGAATGGGGTGTGATGGTTTATGATTACTCTGAGCTGGAGC 720
            * **  *  **** *   * ** ***** ** *** * ** ** ** ** ** **  * **

T2W-S4      GCGATCCACTAAAAGGCCGCGTGTATCGTCGTGAGCTTGTCACTCCAGCTCGTGATTTCG 780
T1L-S4      ATGATCCATCGAAGGGCCGTGCTTACAGGAAGGAATTGGTGACGCCAGCACGAGACTTCG 780
T2J-S4      GAGATCCCCTAAAAGGGCGAGCCTACAGGAAGGAGGTTGTTACACCAGCGAGAGATTTTG 780
T3D-S4      ATGATCCATCGAAGGGCCGTGCTTACAGAAAGGAATTGGTGACGCCAGCTCGAGATTTCG 780
              *****    ** ** ** *  **  *    **  * ** ** *****  * ** ** *

T2W-S4      GACACTTTGGATTATCGCATTATTCCCGGGCAACTACACCATTGCTGGGCAAGATGCCTG 840
T1L-S4      GTCATTTTGGATTATCCCACTACTCTAGGGCGACTACCCCAATCCTTGGAAAGATGCCAG 840
T2J-S4      GTCACTTTGGACTGTCGCATTACTCTCGCGCTACAACGCCGATTCTTGGCAAGATGCCAG 840
T3D-S4      GTCACTTTGGATTATCCCATTATTCTAGGGCGACTACCCCAATCCTTGGAAAGATGCCGG 840
            * ** ****** * ** ** ** **  * ** ** ** **  * ** ** ******** *

T2W-S4      CTGTGTTCTCGGGCATGCTTACCGGGAACTGTAAAATGTACCCTTGCATTGAAGGAACCG 900
T1L-S4      CTGTATTCTCGGGAATGTTGACTGGGAATTGTAAAATGTATCCATTCATCAAAGGAACGG 900
T2J-S4      CCGTCTTTTCCGGGATGCTCACTGGAAACTGCAAAATGTACCCCTTCATAAAAGGGACTG 900
T3D-S4      CCGTATTCTCAGGAATGTTGACTGGGAACTGTAAAATGTATCCATTCATTAAAGGAACGG 900
            * ** ** ** ** *** * ** ** ** ** ******** ** * ***  **** ** *

T2W-S4      CGAAGGTGAAAACGGGGAAGAAGCTGGGTGATGCTGTTAATAACATTTGGGGCGTTGAAA 960
T1L-S4      CTAAGTTAAAGACAGTGCGCAAGCTAGTGGATTCAGTCAATCATGCGTGGGGCGTCGAGA 960
T2J-S4      CCAAATTAAGAACGGTCAAAAAGCTAGTAGATGCTGTGAATCATACGTGGGGCTCTGAGA 960
T3D-S4      CTAAGCTGAAGACAGTGCGCAAGCTAGTGGAGGCAGTCAATCATGCTTGGGGTGTCGAGA 960
            * **  * *  ** *     ***** *  **  * ** *** *    *****    ** *
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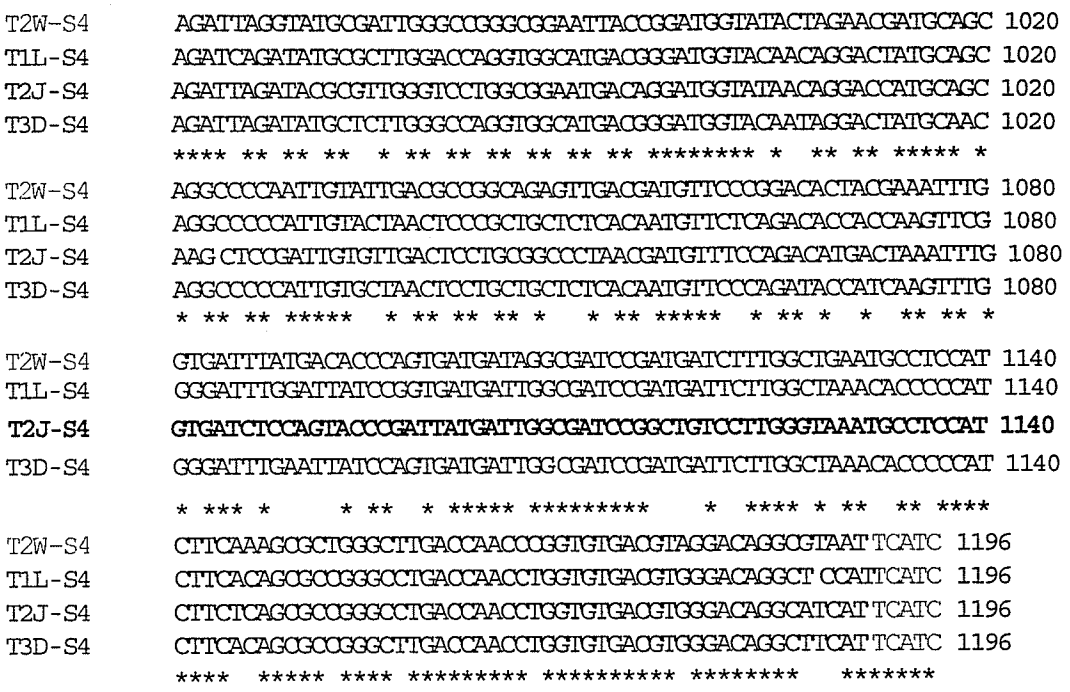


Figure 6.1. Alignments of the reovirus T2W S gene sequences. The reovirus T2W S gene sequences were aligned with 3 prototype strains of mammalian reoviruses (T1L, T2J and T3D). S1 (A), S2 (B), S3 (C), and S4 (D). Alignments were performed by Clustal-W (1.82) program. Asterisks indicated the nucleotides in 4 virus sequences are identical. The 4 nucleotides in the 5' terminal and 5 nucleotides in the 3' terminal indicate nucleotides that are conserved in the gene sequences of all mammalian reovirus strains.

were taken. Therefore, the TA-cloning method was used as described (in **2.10.3**) and 5 independent clones were picked and sequenced. The sequences of the 5 clones were completely identical, and the full sequence of T2W S1 gene was thereby achieved. When compared to other sequences at the nucleotide level by Laligne program analyses (Table **6.1A**), the T2W S1 gene showed most identity (94.3% and 93.4%) to the sequences of reovirus strains T2/Netherlands/1984 (T2N84) and T2/Netherlands/1973 (T2N73), 55.5% identity to reovirus type 2 prototype strain T2J; about 48% identity with reovirus type 1 prototype T1L and 3 other type 1 strains; but only about 24-28% identity with all the type 3 viruses. The predicted T2W S1 gene product (σ1 protein) was 447 amino acids in length, and showed most identity (91%) to the type 2 viruses T2N84 and T2N73, 51% identity with prototype T2J reovirus; 47-49% identity with 4 type 1 reoviruses, and only about 21% identity with type 3 reovirus σ1 proteins. To define the evolutionary relationship of the T2W S1 gene with the S1 genes of other reovirus strains whose sequences are deposited in GenBank, we constructed phylogenetic trees by use of variation in the S1 gene nucleotide sequences and the neighbor-joining algorithm (Figure **6.3A**). The most noteworthy characteristics of the S1 phylogenetic tree is that the T2W gene S1 sequence is more closely related to the S1 gene of other type 2 reovirus strains, and most divergent from the type 3 reovirus strains. Alignment of the T2W σ1 protein with the T1L, T2J and T3D σ1 proteins with T-coffee (Notredame et al., 2000) showed that there was great diversity of T2W σ1 with all 3 reovirus prototype strains. The T2W σ1 protein was more related to the σ1 protein of T2J and T1L than with that of T3D (Figure **6.3A**).

6.2.3. Sequence analysis of T2W S2, S3, and S4 genes: Compared to the great diversity of the T2W S1 gene sequence of this clone with the S1 genes of other reovirus strains, the sequences of the T2W S2, S3 and S4 genes showed much higher identity with those of other reovirus strains in GenBank.

The T2W S2 gene sequence showed 75.4%, 74.5% and 73.5% identity with the 3 reovirus prototypic strains T1L, T2J and T3D respectively, and about 73-75% identity with the other 9 reovirus strains (Table **6.1B**). Comparison of the amino acid sequences of the σ2 proteins predicted for all 13 reoviruses in GenBank indicated the T2W σ2 sequence was closer to strain T3D (95%) and T1L (94.7%), and least related to T2J

Table 6.1. Comparisons of mammalian reovirus S gene and σs proteinidentities

A (S1 gene-σ1 protein)

	T2W	T1L	T2J	T3D	T3C18	T1C23	T3C31	T3C43	T3C44	T3C45	T1C50	T3C8	T3C84	T3C87	T3C9	T3C93	T3Co86	T1N85	T2N73	T2N84
T2W	****	47.7	54.9	27.1	27.7	46.5	27.8	25.7	26	25.9	47.1	24.9	26	26.8	26.4	26.8	25.4	48.3	93.4	94.3
T1L	48.8	****	45.4	24.1	23.9	67.4	24.5	25.9	25.2	26.1	95.3	24.8	25	25.4	23.4	24.9	24.2	90.5	48.1	47
T2J	51.4	41.1	****	26.5	27	46	25.1	25	24.5	25.2	47.1	25.1	24.3	24.4	25.4	24.9	25.7	47	52.2	53.1
T3D	20.8	73.8	24	****	76.6	24.2	98.2	98.2	98.2	97.6	23.6	82.5	97.7	98.2	78.3	97.8	65.1	22.6	24.5	24.9
T3C18	21.5	20.9	19.3	87	****	23.3	93.6	77	77.2	76.3	22.9	76.8	76.6	76.7	89.8	76.2	63.6	24.4	26.5	26.5
T1C23	47.2	76.6	42.4	20.2	20	****	23.1	24.7	24.6	24.6	67.1	26.6	24.7	24.5	23.2	24.9	25.2	69.7	47.4	47
T3C31	21.3	21.5	20.6	87.9	94.7	20.7	****	78.2	78.3	78.1	22.2	77.8	77.8	77.8	91	77.5	64.6	24.7	27.2	27.2
T3C43	20.8	22	20.1	98.7	87	20	87.5	****	99.7	99.6	23.7	82.5	99	99.1	78.2	99	65.6	24	24.7	25
T3C44	21.3	22	20.8	98.5	87	20	87.5	99.3	****	99.6	24	82.3	99	99.1	78.1	99	65.5	23.9	24.8	25
T3C45	20.8	22	20.3	98	86.4	20.2	87.3	98.9	98.7	****	23.8	82.1	98.9	99	78.2	98.9	65.3	23.9	24.9	25.1
T1C50	47.9	96	40.6	21.5	21.1	74.7	22.2	21.8	21.8	21.8	****	23.6	23.1	24.3	25.4	23.1	23.1	89	48.4	48.3
T3C8	20.4	20.9	19.5	91.2	87.7	19.3	88.4	91.2	91	90.5	20.4	****	81.9	82.3	77.4	81.7	63.9	23.5	24.2	24.3
T3C84	20.8	22	20.1	98.2	86.6	20	87	99.1	98.9	98.5	21.8	90.8	****	98.8	77.7	99.4	65.1	23.8	24.8	25.1
T3C87	20.8	21.8	20.1	98.5	86.8	20	87.3	99.3	99.1	98.7	21.5	91	98.9	****	77.7	98.8	65.4	24	24.8	25
T3C9	21	21.5	19.8	86.4	93	20.9	94.1	86.4	86.4	86.2	22	87.5	85.9	86.2	****	77.5	64.5	26	24.8	25
T3C93	20.6	21.8	20.1	97.8	86.2	20.4	86.6	98.7	98.5	98	21.5	90.3	99.1	98.5	98.5	****	65	23.8	24.9	25.2
T3Co	22.4	21.8	21.6	74.3	73.8	20	74.9	74.5	74.5	74.3	21.8	74.5	74.3	74.7	74.5	73.8	****	26.2	23.6	26
T1N85	49	97.2	42.1	21.8	20.9	77.2	21.5	22	22	22	93.6	20.9	22	21.8	21.5	22	22	****	48.8	48.6
T2N73	91.5	50.7	50.4	21.8	22	49.6	22.2	21.8	22	21.5	49.8	21.3	21.8	21.8	21.3	21.5	21.5	50.9	****	98
T2N84	91.5	50.7	50.9	22.4	22.4	49.1	22.6	22.4	22.6	22.2	49.8	21.8	22.4	22.4	21.8	22.2	21.5	50.7	97.8	****

B (S2 gene -σ 2 protein).

	T2W	T1L	T2J	T3D	T3C9	T3C87	T3C84	T3C8	T1C62	T1C49	T3C31	T3C18	T1C11
T2W	****	75.4	74.5	73.5	73.7	74.9	74.5	74.4	74.5	75.1	73.1	74.9	75.3
T1L	94.7	****	74.8	85.2	84.5	97.1	97.2	90.9	97.9	99.5	83.8	98.7	97.7
T2J	92.8	94.3	****	74.9	71.4	75	74.7	73.9	73.8	74.8	74.5	74	73.9
T3D	95	98.8	94	****	84.6	84.3	84.2	83.1	83.8	84.6	96.3	83.8	82.4
T3C9	94.5	98.1	93.3	97.8	****	83.9	84.2	84.2	84.2	84.2	84.3	83.9	84.2
T3C87	94.5	98.8	94	98.6	97.8	****	98.7	90.5	95.4	96.6	83.9	95.9	94.9
T3C84	94	98.3	93.8	98.1	97.4	99	****	90.5	95.5	96.7	83.8	96.1	94.7
T3C8	94.3	98.8	93.5	98.3	97.6	98.1	97.6	****	90.1	90.8	82.7	90.5	91.1
T1C62	94	98.8	93.5	98.1	97.8	98.1	97.6	98.1	****	97.5	83.8	98.3	97.9
T1C49	94.5	99.8	94	98.6	97.8	98.6	98.1	98.6	98.6	****	83.7	98	97.3
T3C31	95	98.3	93.5	99.5	97.4	98.2	97.6	97.8	97.6	98.1	****	83.4	83.1
T3C18	94.5	99	93.8	98.6	98.1	98.3	97.8	98.3	98.8	98.8	98.1	****	98.6
T1C11	94.7	99.5	94.3	98.8	98.6	98.8	98.3	98.8	99.3	99.3	98.3	99.5	****

C (S3 gene-σNS protein)

	T2W	T1L	T2J	T3D	T3A	T3F	T3C11	T3C18	T3C31	T1C62	T3C8	T3C 84	T3C9	T1N67	T1N84	T1N85	T2N73	T2N84	T3N83	T2SV59	T2To90
T2W	****	72.5	67.8	71.9	71.7	71.6	72.5	72.5	71.7	72.6	72.3	71.6	73.2	72.9	72.7	71.5	73.2	72.3	73.6	71.7	73
T1L	88	****	67.4	83.9	85.6	96.9	96.9	96.9	96.5	97.5	84.5	84.1	85.8	85.6	90.7	84.8	90.5	90.3	90.6	94.7	89.5
T2J	85	86.3	****	68.5	67.8	66.9	66.8	66.8	66.5	66.6	68.1	67.8	67.3	67.6	68.4	66.9	68.6	67.4	68.2	66.8	67.9
T3D	87.7	97	86.1	****	98.6	85.2	84.1	84.1	85.1	84.4	87.7	98.9	91.9	91.9	85	90.7	84.8	84	84.3	85.5	84
T3A	87.2	95.9	85.5	98.9	****	83.9	82.7	82.7	83.5	83.1	86.6	98.9	90.9	91	84.4	90.5	84.1	83.3	83.8	84.3	83.6
T3F	87.7	97.8	86.1	97.5	96.4	****	96.5	96.5	97.4	97	85.2	84.2	85.6	85.3	91	85.5	90.7	90.4	90.8	94.9	90.1
T3C11	87.4	97.3	85.8	96.7	95.6	98.4	****	100	96.4	98.6	84.2	82.8	84.2	84.2	90.4	84.4	90.1	89.8	90.3	94.2	89.7
T3C18	88	97.8	86.3	97.3	96.2	98.9	98.9	****	96.4	98.6	84.2	82.8	84.2	84.2	90.4	84.4	90.1	89.8	90.3	94.2	89.7
T3C31	87.4	97.8	85.8	97.3	96.2	98.9	98.4	98.9	****	96.9	85.8	84	85	85.1	90.8	85.1	90.2	90.3	90.4	94.4	89.8
T1C62	88	97.8	86.3	97.3	96.2	98.9	98.9	99.5	98.9	****	84.7	83.4	84.7	84.6	91	84.6	90.7	90.3	90.8	94.9	90.1
T3C8	87.7	95.9	85.2	97	95.9	97.3	96.4	97	97	97	****	87.1	87.7	87.6	84.7	87.9	84.2	84.2	84.2	85	84
T3C84	88	96.7	86.3	99.7	99.2	97.3	96.4	97	97	97	96.7	****	91.5	91.3	84.6	90.8	84.2	83.5	84	84.5	84.2
T3C9	89.1	97.8	86.3	98.1	97	97.3	96.4	97	97	97	96.7	97.8	****	91.3	85.8	90.5	90.8	90.3	85.9	85.8	83.8
T1N67	88.8	97.3	86.1	98.1	97	97.3	96.4	97	97	97	96.7	97.8	99.5	****	98	84.1	83.5	83.4	84.1	85.9	83.4
T1N84	88.5	97.3	85.8	97.5	96.4	98.4	97.8	98.4	98.4	98.4	97.3	97.3	98.1	98.1	****	98.5	98	97.6	84.1	91.3	97.1
T1N85	88.3	97.5	86.3	97.5	97	98.6	98.1	98.6	98.6	98.6	97.8	97.8	97.3	97.3	97.3	****	83.5	84	84.2	85.8	83.2
T2N73	87.7	97.5	85.8	97.5	96.4	98.6	98.1	98.6	98.6	98.6	97.3	97.3	97.3	98.6	98.6	98.9	****	97.2	97.5	90.5	96.9
T2N84	87.7	96.7	85.2	96.7	95.6	97.8	97.3	97.8	97.8	97.8	96.4	97	97	98.4	98.4	98.1	98.1	****	85.8	90.8	96.4
T3N83	88.8	97.3	86.1	98.1	97	97.3	96.4	97	97	97	96.7	99.5	99.5	98.1	98.1	97.3	97.3	97	****	90.3	98.9
T2SV59	88	96.7	86.1	96.7	95.6	97.8	97.3	97.8	97.8	97.8	96.4	96.4	96.4	97.8	97.8	98.1	98.1	97.3	96.4	****	89.4
T2To90	88	96.7	85.5	96.7	96.2	97.8	97.3	98.7	98.7	98.7	97.3	97	96.7	96.7	98.1	99.2	98.1	97.3	96.7	97.3	****

D (S4 gene-σ3 protein).

	T2/V	T1L	T2J	T3D	T3C10D	T1C15	T3C18	T1C23	T3C31	T3C44	T3C45	T1C50	T3C8	T3C84	T3C87	T3C9	T3C88
T2/V	****	72.1	73.4	72.7	71.6	70.8	70.2	69.9	71.9	69.9	70.1	69.9	69.9	70.2	71.9	72.1	70.2
T1L	85.5	****	76.2	98.1	92.7	99.7	85.7	85.4	92.8	87.2	87.2	86.2	89.1	87	98.3	88.9	87.2
T2J	84.9	90.7	****	74.8	73.6	74.5	75.7	75.6	74.4	75	75.2	75.9	75.1	75	74	75.4	75.1
T3D	84.4	97	89.6	****	98.5	92.7	86.1	85.7	94.4	87	87.1	86.4	89.3	87	98.1	88.7	87.1
T3C10D	85.2	97.3	90.4	98.1	****	92.5	85.8	85.4	94.2	86.8	86.9	86.2	89	86.8	98.1	88.5	86.9
T1C15	84.9	99.5	90.1	96.4	96.7	****	86.1	85.6	94	86.8	86.9	86.3	89.1	86.8	97.5	88.2	86.8
T3C18	85.5	96.4	91.5	96.2	96.7	95.9	****	99.5	85.8	96.4	96.5	93.8	85.7	96.9	86.2	88.9	96.8
T1C23	84.9	95.9	91.5	95.6	95.9	95.3	98.9	****	85.6	96.1	96.2	93.5	85.5	96.5	85.9	88.7	96.2
T3C31	84.9	97	90.7	96.4	96.7	97	96.4	95.9	****	86.7	86.8	86.1	89.3	86.7	94.4	88.6	86.8
T3C44	86	96.4	92.1	96.2	96.4	95.9	98.9	98.4	96.4	****	99.7	96.4	85.7	99.4	87.5	82.6	99.4
T3C45	86	96.4	92.1	96.2	96.4	95.9	98.9	98.4	96.4	99.5	****	96.5	85.8	99.5	87.4	82.4	99.5
T1C50	84.9	95.9	91	95.6	95.9	95.3	98.9	98.4	95.9	98.4	98.4	****	86.2	96.9	86.6	82.1	96.8
T3C8	85.8	96.7	91.2	96.4	96.7	96.2	97.5	97	96.7	97.5	97.5	97	****	86.1	91	88.2	86.2
T3C84	85.8	96.7	91.8	96.4	96.7	96.2	99.2	98.6	96.7	99.2	99.2	98.6	97.8	****	87.2	82.7	99.8
T3C87	86	97	90.7	97.8	98.1	96.4	96.7	96.2	97	96.7	96.7	96.2	97.5	97	****	84	87.3
T3C9	85.5	95.9	90.1	95.6	96.9	95.3	95.9	95.3	95.9	95.9	95.9	95.3	95.9	96.2	96.2	****	82.6
T3C88	85.8	97.3	91.8	96.4	96.7	96.7	99.2	98.6	96.7	99.2	99.2	98.6	97.8	99.5	97	96.2	****

• Values for S gene sequences shown in bold above diagonal: values for σ proteins shown below diagonal.
Values determined using Lalign ( ).

(92.8%) (Table **6.1B**), as confirmed by analysis of the phylogenetic tree of the S2 genes (Figure **6.3B**). For a more detailed amino acid comparison, I aligned the T2W σ2 protein with the T1L, T2J and T3D σ2 proteins with T-Coffee (Notredame, et al. 2000). The sequences of the σ2 proteins were highly conserved, and all were 418 amino acids in length. The T2W σ2 protein had 22 amino acid differences, compared to the prototype T1L, T2J, and T3D σ2 proteins, and the differences appeared randomly distributed (Figure **6.2B**).

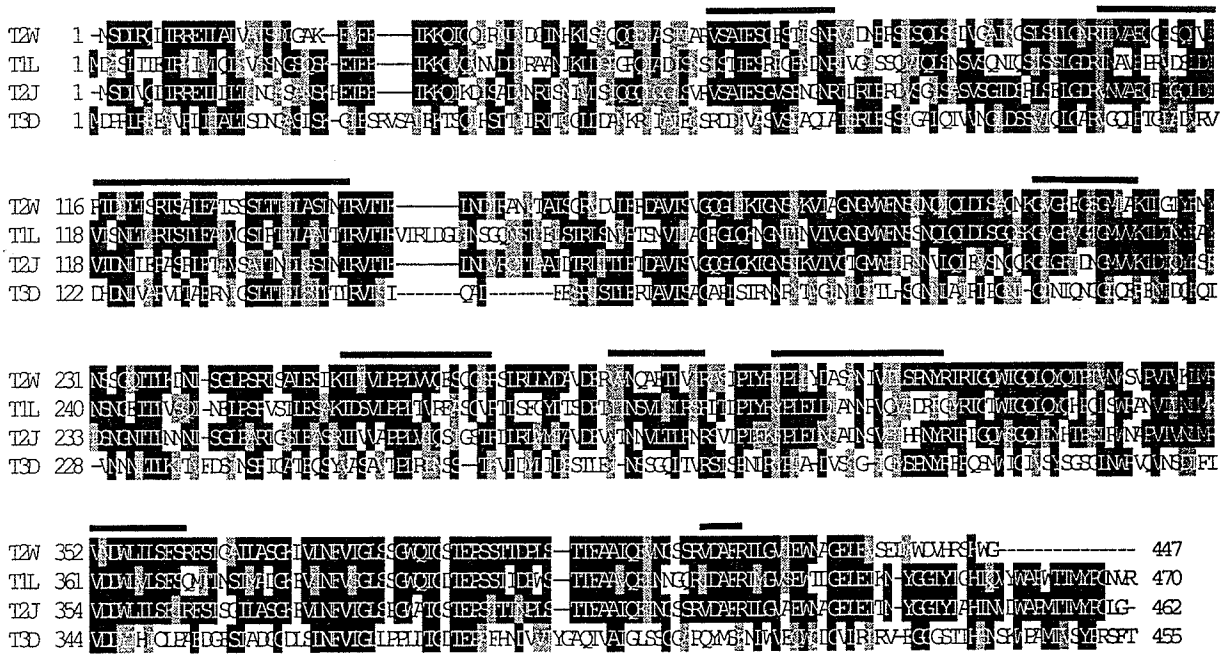
The T2W S3 gene sequence was about 71-74% identical to each of 19 other reovirus strains including two serotype prototypic strains T1L and T3D, but least similar to the sequence of type 2 reovirus prototype reovirus T2J (67.8% identity) (Table **6.1C**). The amino acids of the σNS protein encoded by the T2W S3 gene showed 87-89% identity to other reoviruses, except for reovirus T2J with 85% identity (Table **6.1C**). Phylogenetic tree evolution of the S3 gene showed an overall similar pattern as that generated by the S2 gene. T2W was closer to other reovirus strains than to T2J (Figure 6.3C). The sequences of the T2W σNS protein (Figure 6.2C) showed more variation to other reoviruses than did T2W σ2 protein (Figure 6.2B). There were 27 amino acids in the T2W σNS protein that differed from these of other reoviruses. The highest variations of the amino acid sequences were in 3 areas (amino acid 105-135; 195-245 and 325-355).

The T2W S4 gene sequence had the highest identity with prototype reovirus T2J (73.4%) and showed about 69.9-72.7% identity with the S4 gene sequences of the 15 other reoviruses in Genbank (Table **6.1D**). Analysis of the amino acid sequence of the σ3 protein encoded by the S4 gene showed T2W σ3 had about 84-86% identity to the σ3 proteins of the other 16 reoviruses in GenBank (Table **6.1D**) and 85.5%, 84.9% and 84.4% identity to three prototype reoviruses T1L, T2J and T3D (Table **6.1D**). The T2W σ3 protein sequences had 38 amino acids that differed from these in the prototype reoviruses T1L, T2J and T3D (Figure **6.2D**).

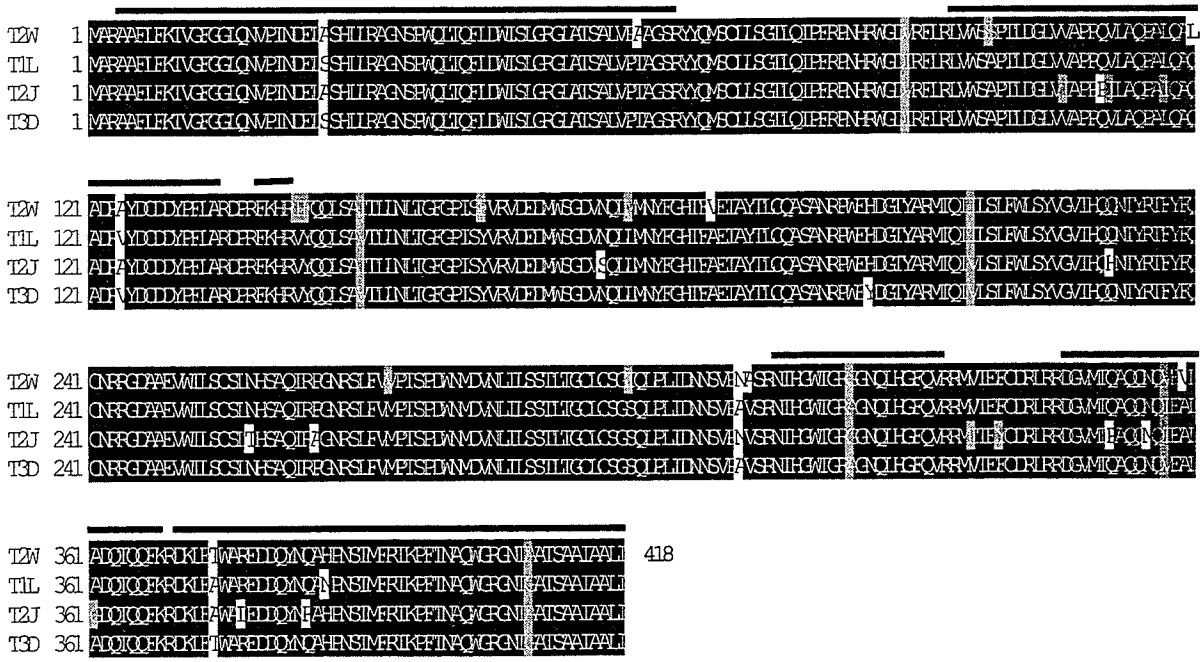
6.2.4. Further analysis of the predicted secondary structures of the σ1, σ2, σNS and σ3 proteins of T2W: The MLRC secondary structure prediction program (Combet, et al. 2000) was used to further analyze the predicted secondary structures of the T2W σ1, σ2, σNS and σ3 proteins and to compare them to the T1L, T2J and T3D σ1, σ2, σNS and σ3

Figure2

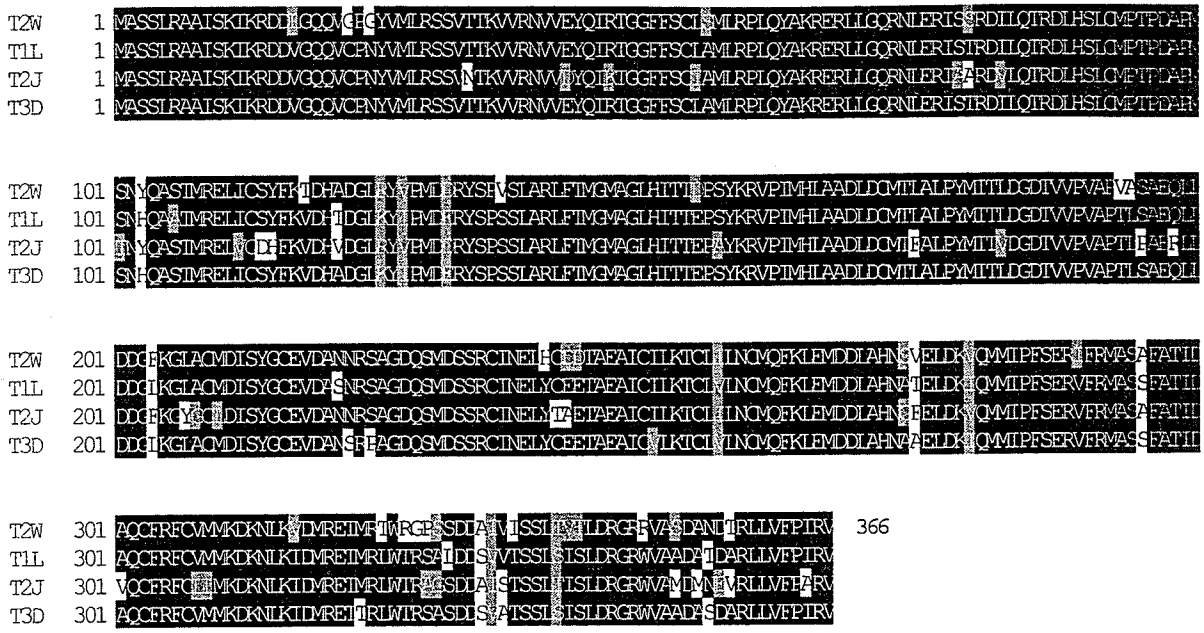
A α1



B α2



C. σNS


T2W 1 MASSLRAAISKIKRDDVGQQVCEGYVMLRSSVITKVVRNVVEYQIRTGGFFSCLAMLRPLQYAKRERLLGQRNLERISTRDILQIRDLHSLCMPTPDAR
T1L 1 MASSLRAAISKIKRDDVGQQVCENYVMLRSSVITKVVRNVVEYQIRTGGFFSCLAMLRPLQYAKRERLLGQRNLERISTRDILQIRDLHSLCMPTPDAR
T2J 1 MASSLRAAISKIKRDDVGQQVCENYVMLRSSVNTKVVRNVVEYQIRTGGFFSCLAMLRPLQYAKRERLLGQRNLERISARDVLQIRDLHSLCMPTPDAR
T3D 1 MASSLRAAISKIKRDDVGQQVCENYVMLRSSVITKVVRNVVEYQIRTGGFFSCLAMLRPLQYAKRERLLGQRNLERISTRDILQIRDLHSLCMPTPDAR
T2W 101 SNYQASTMRELICSYFKVDHADGLRYYEMDERYSPSSLARLFTMGMAGLHITTEPSYKRVPIMHLAADLDCMTLALPYMITLDGDIVVPVAPVVASAEQLL
T1L 101 SNHQASTMRELICSYFKVDHTDGLKYYEMDERYSPSSLARLFTMGMAGLHITTEPSYKRVPIMHLAADLDCMTLALPYMITLDGDIVVPVAPTLSAEQLL
T2J 101 TNYQASTMRELICDFFKVDHVDGLRYYEMDERYSPSSLARLFTMGMAGLHITTEPAYKRVPIMHLAADLDCMTLALPYMITMDGDIVVPVAPTLEAERLL
T3D 101 SNHQASTMRELICSYFKVDHADGLKYYEMDERYSPSSLARLFTMGMAGLHITTEPSYKRVPIMHLAADLDCMTLALPYMITLDGDIVVPVAPTLSAEQLL
T2W 201 DDCFKGLACMDISYGCEVDANNRSAGDQSMDSSRCINELHCEETAEAICILKTCLTLNCMQFKLEMDDLAHNCVELDKYCQMMIPFSERTFRMASAFATII
T1L 201 DDCLKGLACMDISYGCEVDASNRSAGDQSMDSSRCINELYCEETAEAICILKTCLYLNCMQFKLEMDDLAHNATELDKYCQMMIPFSERVFRMASSFATII
T2J 201 DDCFKGYGCIDISYGCEVDANNRSAGDQSMDSSRCINELYTEETAEAICILKTCLTLNCMQFKLEMDDLAHNCFELDKYCQMMIPFSERVFRMASAFATII
T3D 201 DDCLKGLACMDISYGCEVDANSRFAGDQSMDSSRCINELYCEETAEAICTLKTCLYLNCMQFKLEMDDLAHNCAELDKYCQMMIPFSERVFRMASSFATII
T2W 301 AQCFRFCVMMKDKNLKYIDMREIMFTWRGESSDDATVLSSLVKTLDRGFRVASDANDTRLLVFPIRV 366
T1L 301 AQCFRFCVMMKDKNLKTIDMREIMRLWIRSATDDSYVTSSLSTSLDRGRWVAADATDARLLVFPIRV
T2J 301 VQCFRFCLIMKDKNLKTIDMREIMRLWIRFGSDDATSTSSLTTSLDRGRWVAMDMNEVRLLVFFARV
T3D 301 AQCFRFCVMMKDKNLKTIDMRETTRLWIRSASDDSYATSSLSTSLDRGRWVAADASDARLLVFPIRV

D. σ3


T2W 1 MEVCLPNGHQIVDLINNAFEGRVSIYSAQEGWDKTISAQLVMVVCGAVVCMHCLGVVGSLQRKLKHLPHHRCNQCLRCQDYVDLQFADRVTAHWKRGMI
T1L 1 MEVCLPNGHQIVDLINNAFEGRVSIYSAQEGWDKTISAQPDMVVCGGAVVCMHCLGVVGSLQRKLKHLPHHRCNQCLRHQDYVDVQFADRVTAHWKRGMI
T2J 1 MEVCLPNGHQIVDLINNAFEGRVSIYSAQEGWDKTISAQPDMVVCGGAVVCMHCLGVVGSLQRKLKHLPHHRCNQCLRCQDYVDVQFADRVTAHWKRGMI
T3D 1 MEVCLPNGHQIVDLINNAFEGRVSIYSAQEGWDKTISAQPDMVVCGGAVVCMHCLGVVGSLQRKLKHLPHHRCNQCLRHQDYVDVQFADRVTAHWKRGMI
T2W 101 SFVSQMHAMNDVQPEQLLEVFVSGGKLVELNWLQVDEQSMERSIHSNWTDPLQVVMDLDICLLRYWTALNLMIDSSDLVPNFMMRDPSHAFNGVKLEGL
T1L 101 SFVAQMHAMNDVSPEDLLRVRIEGGSLVELNWLQVDENSMERSIHSSWTDPLQVVMDLDIKLLCYWTALNLMIDSSDLVPNFMMRDPSHAFNGVRLEGL
T2J 101 SFVSQMHAMNDVTPEHLLRVRIEGGSLAELNWLQVDEQSMERSIHSSWTDPLQVVMDLDICLLRYWTALNLMIDSSDLVPNFMMRDPSHAFNGVKLEGE
T3D 101 SFVAQMHEMNDVSPEDLLRVRIEGGSLVELNRLQVDENSMERSIHSSWTDPLQVVMDLDIKLLCYWTALNLMIDSSDLIPNFMMRDPSHAFNGVKLEGL
T2W 201 ARQIHFSRITFLERSSLEWGVMVYDYSELESDFIKGRVYRFELVIPARDFGHFGLSHYSRAITPLLGKMPAVFSGMLTGNCKMYFCLFGTAKVKTCRKICL
T1L 201 ARQIQFSRITFDSRSSLEWGVMVYDYSELEHDFSKGRAYRKELVIPARDFGHFGLSHYSRAITPLLGKMPAVFSGMLTGNCKMYPFIKGTAKLKTVRKLVI
T2J 201 ARQIQFSRITFDSRSNLEWGVMIYDYSELEHDFIKGRAYRKELVIPARDFGHFGLSHYSRAITPLLGKMPAVFSGMLTGNCKMYPFIKGTAKLRTVRKLVI
T3D 201 ARQIQFSRITFDSRSSLEWGVMVYDYSELEHDFSKGRAYRKELVIPARDFGHFGLSHYSRAITPLLGKMPAVFSGMLTGNCKMYPFIKGTAKLKTVRKLVI
T2W 301 AVNNIWGVEKIRYALGPGGMTGWYRIMQQAPIVLTPAELIMFDITKFGDFCHPVMIGDFMIFG 365
T1L 301 SVNHAWGVEKIRYALGPGGMTGWYRIMQQAPIVLTPAALIMFSDITKFGDILYPVMIGDFMILG
T2J 301 AVNHTWGSEKIRYALGPGGMTGWYRIMQQAPIVLTPAALIMFPDVTKFGDLQYPFMIGDFAVLG
T3D 301 AVNHAWGVEKIRYALGPGGMTGWYRIMQQAPIVLTPAALIMFPDITKFGDLIYPVMIGDFMILG

Figure 6.2. Alignments of the deduced reovirus T2W σ protein amino acid sequences. The deduced reovirus T2W σ protein amino acid sequences were aligned with 3 prototype strains of mammalian reoviruses (T1L, T2J and T3D): σ1 (A), σ2 (B), σNS (C), and σ3 (D). Alignments were performed by T-Coffee programs and visualized with BoxShade. Amino acid residues that are identical in at least 2 of the sequences are indicated with black background shading, grey background shading indicates conserved amino acid substitutions in at least 2 of the sequences. The amino acid sequences of T2W σ1, σ2, and σ3 proteins identified by mass spectrometry are indicated by horizontal lines above the sequences individually.

Figure 6.3.

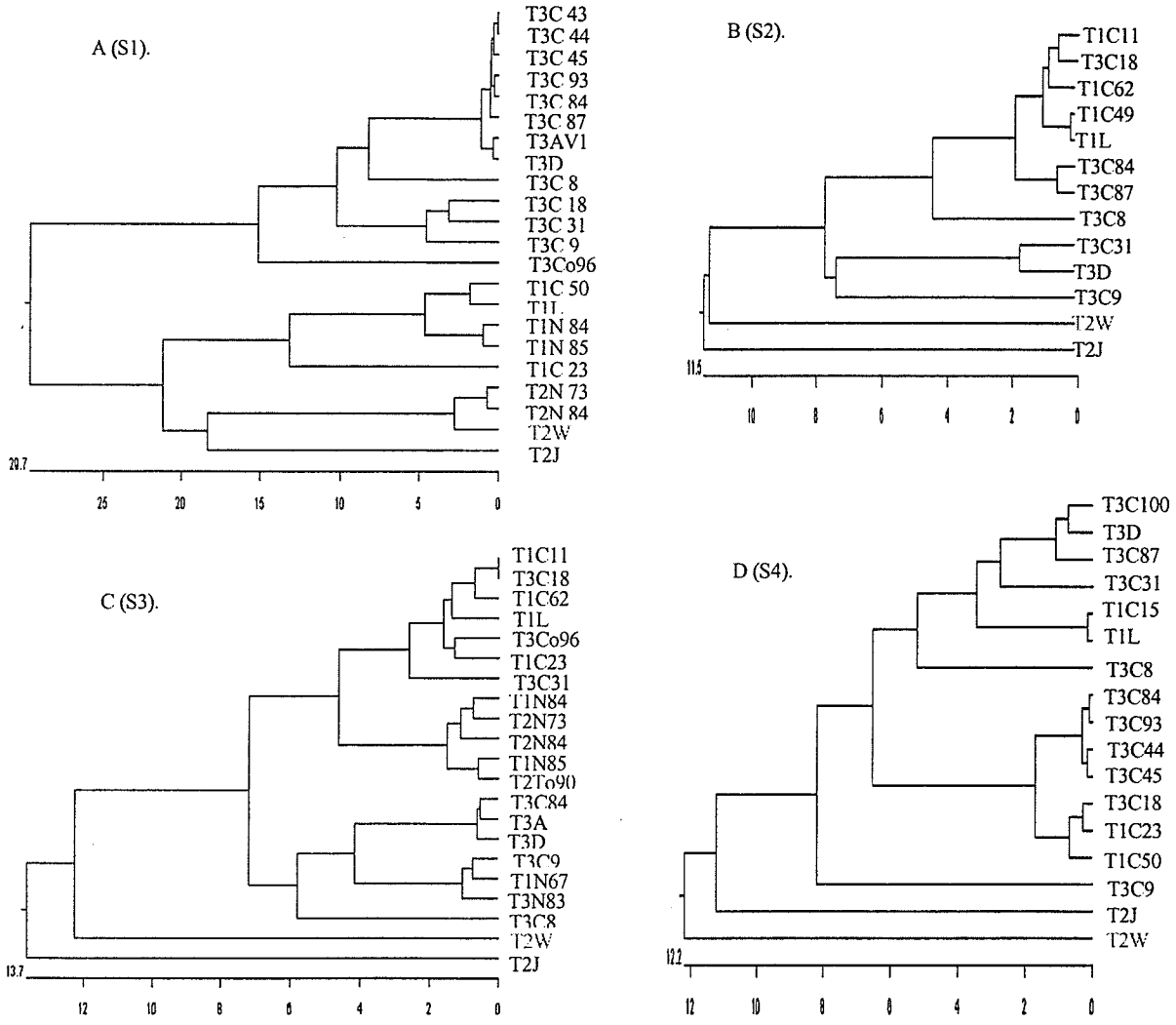


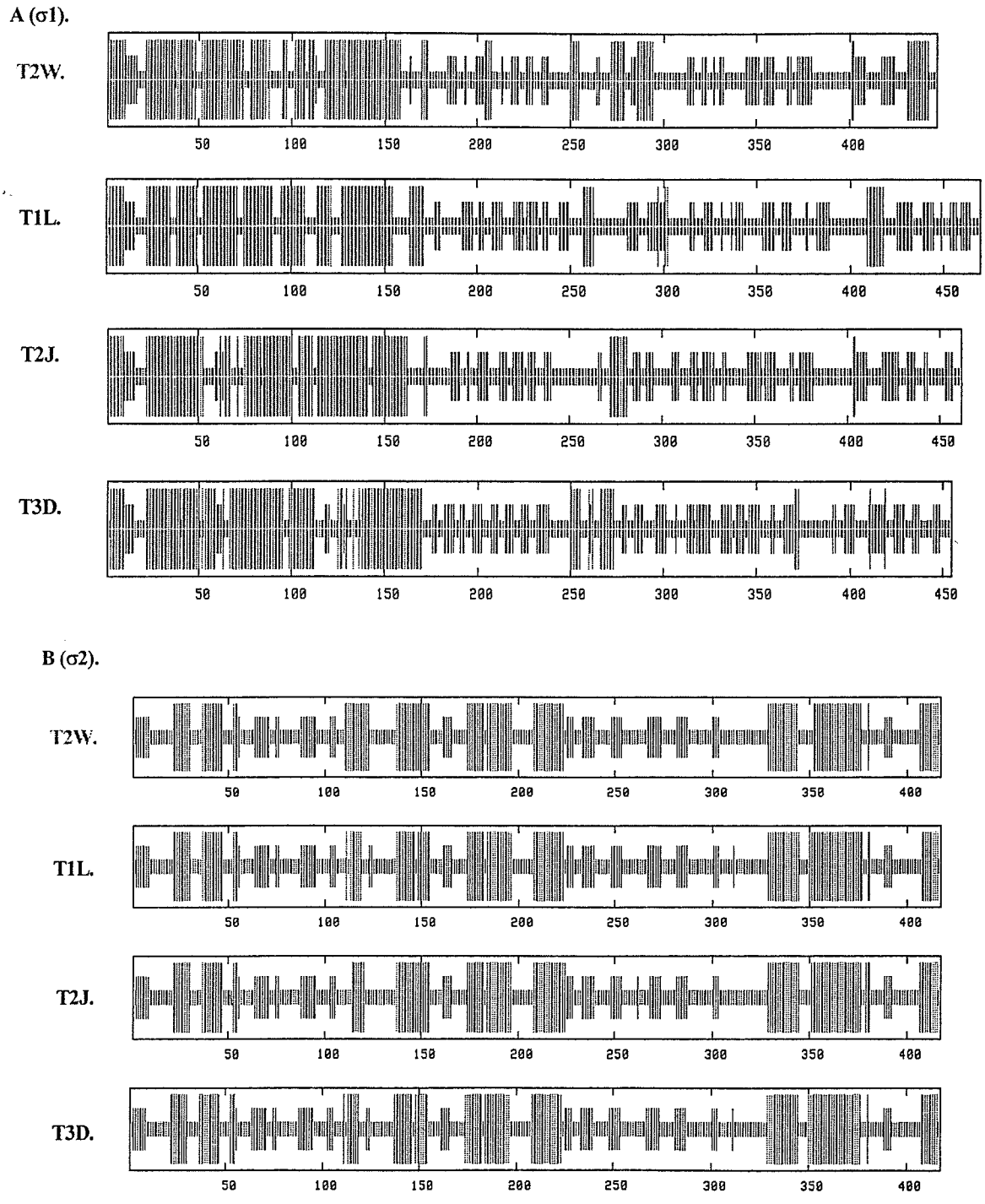
Figure 6.3. Phylogenetic tree analyses of reovirus T2W genes. Phylogenetic tree analyses of reovirus T2W genes with all mammalian reoviruses in Genbank: S1 (A), S2 (B), S3 (C), and S4 (D), and homologous genes in other mammalian reoviruses (based on available GenBank sequence data). Lines are proportional in length to nucleotide substitutions. Alignments were performed by Clustal-W (Thompson, et al. 1994) and visualized by Treeview (Felsenstein J. 1993). GenBank accession numbers and abbreviation of the analyzed reoviruses are defined in the Table **2.2** legend.

proteins. The σ1 proteins were the most divergent structurally, in keeping with having the most divergent sequences. The T2W σ1 N-terminal 150 amino acids, corresponding to the "stalk", consisted primarily of α-helix and was very similar to this region in the σ1 proteins of the other reoviruses (Figure **6.4A**). The region from amino acids 200 to 300 in T2W σ1 contained more predicted α-helix structure than this region in the other viruses, and the structure of the region from amino acids 300 - 400 was predicted to be similar in all viruses. The C-terminal end of T2W σ1 protein showed a stronger α-helix prediction than this region in the three prototype reovirus σ1 proteins.

The predicted secondary structures of the T2W σ2, σNS and σ3 proteins were very similar to the cognate T1L, T2J, and T3D proteins (Figure **6.4B, C, and D**). The T2W σ2 differed from other reoviruses only in 2 areas: one was the region of amino acids 250-350 that contained 4 β-sheets instead of 5 β-sheets found in the prototype reoviruses, and the other was the region of amino acids 100-150, which had no β-sheet similar to T2J in contrast to the one β-sheet found in T1L and T3D (Figure **6.4B**). The T2W σNS protein showed 3 minor variations in the predicted secondary structure compared to other reoviruses: less α-helix in the region from amino acids 230-300, one longer α-helix between 2 β-sheets of amino acids 310-320, and more β-sheets between amino acids 325-340 (Figure **6.4C**). The T2W σ3 protein predicted secondary structure had 4 deviations compared to the prototype reoviruses, one longer α-helix region between amino acids 1-50, an α-helix and β-sheet part between amino acids 110-120, 4 β-sheets and 2 α-helixes between amino acids 170-250, and one β-sheet and α-helix part between amino acids 300-350 (Figure **6.4D**).

6.2.5. Confirmation of predicted reovirus T2W σ protein sequences by mass spectrometry: Mass spectrometry was used to analyze the amino acids of reovirus T2W proteins σ1, σ2 and σ3 and compare them with the same proteins of reoviruses T1L and T3D. The σ1 proteins of the three reoviruses were distinctly different (Figure **6.5A**). Profound, Moverz and ExPASy (Wilkins et al., 1997) database searches were used to identity each peak in each separation and identities of many peaks were confirmed by tandem mass spectrometry. Peptides identified by MS and MS/MS were located within the T2W σ1 sequence to confirm the nucleotide-deduced sequences (Figure **6.3A**). These

Figure 6.4.


A (σ1).
T2W.
50 100 150 200 250 300 350 400
T1L.
50 100 150 200 250 300 350 400 450
T2J.
50 100 150 200 250 300 350 400 450
T3D.
50 100 150 200 250 300 350 400 450
B (σ2).
T2W.
50 100 150 200 250 300 350 400
T1L.
50 100 150 200 250 300 350 400
T2J.
50 100 150 200 250 300 350 400
T3D.
50 100 150 200 250 300 350 400

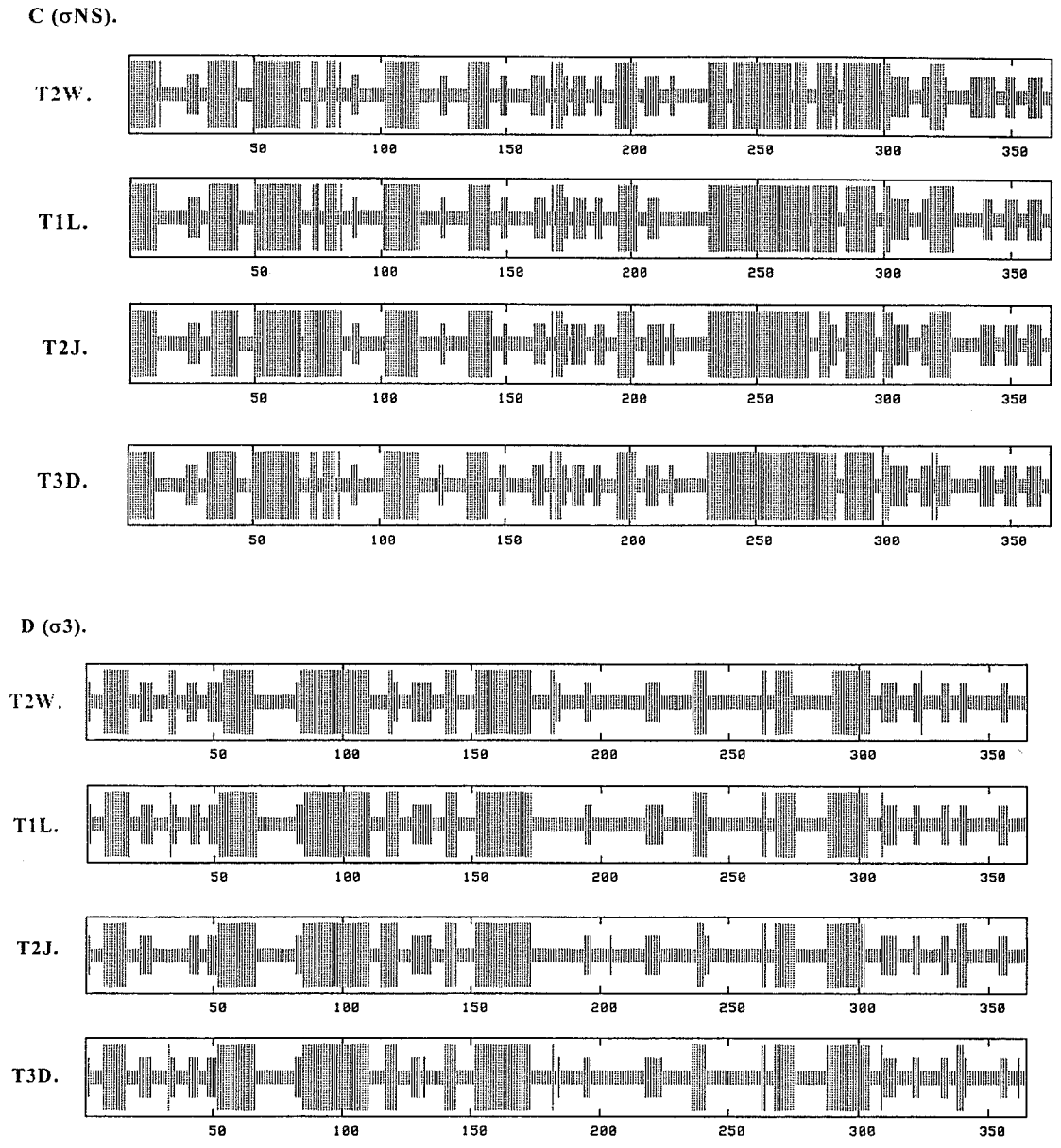


Figure 6.4. Secondary structure predictions of σ proteins of reovirus T2W.

Comparisons of secondary structure predictions of σ1 protein of reovirus T2W and the 3 prototype strains T1L, T2J and T3D. Secondary structure predictions made with Network Protein Sequence Analysis (Combet et al., 2000); A (σ1 protein), B (σ2 protein), C (σNS protein), and D (σ3 protein); tall vertical lines (α-helix), medium lines (β-sheet), and short lines (random coil).

confirmatory peptides were located throughout the T2W σ1 sequences, from amino acids 61 to 420, and accounted for 138 of the 447 amino acids (30.9% protein coverage). Previously determined T1L and T3D σ1 protein sequences were also confirmed by this approach (Table **6.2A**).

Tryptic peptide spectra of the T1L, T2W, and T3D σ2 and σ3 proteins (Figure **6.5B** and **C**) also showed some differences, but far less than the σ1 proteins, in keeping with the greater predicted σ1 variability (Figure **6.5A**). The T2W σ2 and σ3 tryptic mass spectra were also used to locate peptides throughout the T2W σ2 and σ3 sequences (Figure **6.2B** and **D**). Because it is a non-structural protein, I was unable to use this approach to confirm the T2W S3 gene and σNS protein.

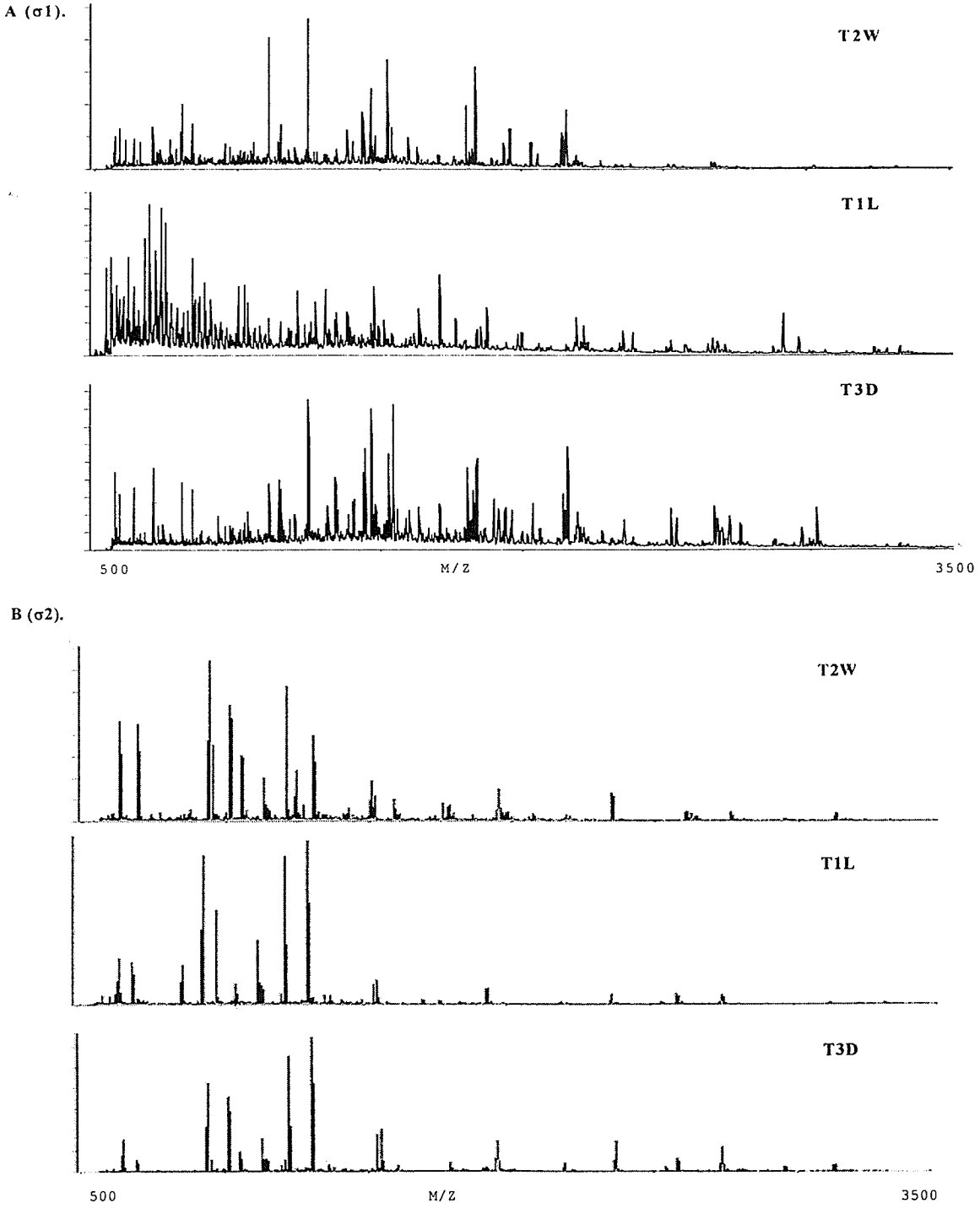
Full discussion for the experiments and results is in Chapter 7. Here I can make the following conclusions

6.3. Conclusion.

1. Reovirus Type 2 Winnipeg (T2W) is a new type 2 virus, as confirmed by the higher identity of the S1 gene to other serotype 2 reoviruse S1 genes.
2. The T2W S1 and S4 genes are most similar to T2J, but T2W S2 and S3 genes are most divergent from T2J.
3. T2W is a novel reovirus because all S genes differ from previous reported reovirus strains.
4. The virus σ protein sequences were also analyzed by mass spectrometry and further confirmed this reovirus is a new type 2 virus strain.

(One article was written from some of this research and accepted by *Virus Genes* for publication; to appear in October 2006 issue).

Figure 6.5.



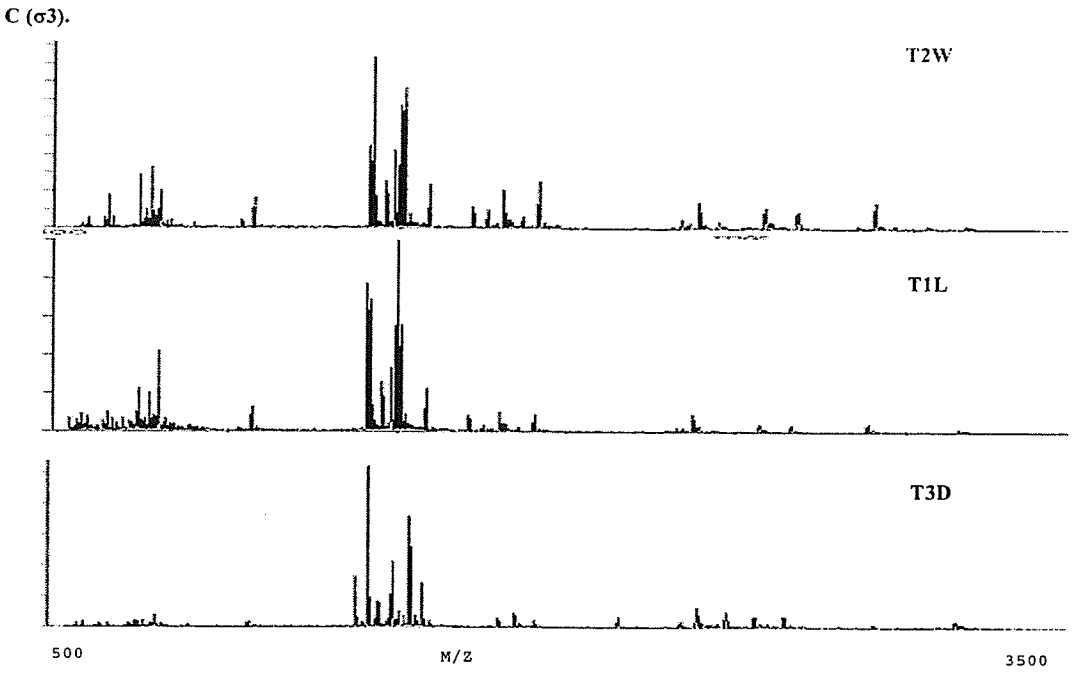


Figure 6.5. Tryptic mass spectrometry of T2W σ proteins. Comparisons of tryptic mass spectrometry of T2W, T1L, and T3D σ1 proteins (A), σ2 proteins (B), and σ3 proteins (C). Each virus was extracted from $6x10^8$ infected cells purified in CsCl gradients, viral proteins resolved by SDS-PAGE, and the σ1, σ2, and σ3 protein bands excised and subjected to in-gel digestion with trypsin. Spectra were acquired on a prototype MALDI-Qq-TOFMS.

Chapter 7. *DISCUSSION.*

There are two major purposes of the research in this thesis: the cellular response to reovirus infection, especially tumor supressor p53 and p53-related proteins, and further understanding of reovirus replication by molecular biological methods. The experiments showed that reovirus induced apoptosis or cell arrest in different cell types, and cellular protein GADD45α was induced to express by reovirus infection. GADD45α siRNA could inhibit expression of GADD45 protein and decreased the level of reovirus replication. Then I used reoviral siRNA to test whether the technical method could interfere with reovirus replication; and used the transfection methods to transfect reoviral core particles into cells in order to examine the hypothesis that transfection could enhance reovirus core particles entering cells and increase virus replication. Furthermore, a new type 2 reovirus strain T2W was sequenced to analyze the difference of the S genes.

7.1. Reovirus induced apoptosis or cell arrest and cellular responses.

7.1.1. Reovirus can induce cell apoptotic change or cell arrest in different cell lines.

Reovirus induces programmed cell death (apoptosis) in a wide variety of cultured cells *in vitro* and in target tissues *in vivo,* including the heart and central nervous system (CNS) (Tyler et al., 1995; Clarke and Tyler, 2005). Reovirus induced cell apoptotic changes differ in genetics of strain–specific characterization. T3 prototype strains Abney (T3A) and Dearing (T3D) can induce apoptosis more efficiently than the serotype 1 strain Lang (T1L) in cell culture (Tyler et al., 1995). Analysis of reassortant viruses that contain mixtures of gene segments from T1L and T3D indicate that the viral S1 gene is the primary determinant of differences in the efficiency of apoptosis induction exhibited by these strains in L929 (Tyler et al., 1995), MDCK (Rodgers et al., 1997) and HeLa cells (Connolly et al., 2000). The M2 gene encoding μ1/μ1C (a virus major outer capsid protein) also functions on the efficiency of apoptosis induction (Rodgers et al., 1997; Tyler et al., 1995). The mammalian reovirus strains Type 1 Lang (T1L) and type 3 Dearing (T3D) were used to infect three cell lines (Mouse L929 cells, Monkey Vero cells and Human lung carcinoma cell lines (H1299). My results confirm these reoviruses can induce apoptosis in L929 cells (Figures **3.1** and **3.2**) and also indicate apoptosis occurs in Vero cells (Figure **3.2**). The apoptotic cells showed shrinkage, rounding, no normal cell

structure and phenomena, and detachment from the plates as in other reports (Tyler et al., 1995). However, an H1299 cell line that is p53-null was also tested to determine the role of p53 in cell response. After reovirus infection, H1299 cells became larger in size (Figure **3.2**), and the cell numbers decreased compared to the mock control (Figure **3.3**). These results indicate H1299 cells arrest after reovirus infection. Reovirus induced cell arrest was also seen in other cell lines C127, MDCK, HEK293 and Hela cells (Poggioli, et al. 2000, and 2002). These data indicate that reovirus infection of cultured cells results in different response of cells depending upon the cell type. However, the virus replication in L929 cells or H1299 cells was similar and reached very high level by 72 hours post-infection. This means that reovirus infection and replication in the different host cells does not depend strictly upon whether the cell undergoes apoptosis or arrest, and that p53 has little (if any) effect upon reovirus replication, but that p53 is associated with apoptosis (Ko and Prives, 1996; Vousden and Prives, 2003).

7.1.2. Role of p53 on reovirus infection. As a step in investigating the molecular events implicated in reovirus-induced cell death or cell arrest, I focused first on the role of p53 pathway in H1299 cells. The tumor suppressor protein p53 is an essential component of an emergency stress response that prevents the growth and survival of damaged or abnormal cells (Fridman and Lowe, 2003). p53 is a transcription factor acting as a major mediator of either growth arrest or apoptosis (Ko and Prives, 1996). p53 activates the expression of several downstream target genes including the proapoptotic bax (Myashita and Read, 1995), p21 (an inhibitor of cyclin-dependent kinases (CDKs) (El-Deiry et al., 1993), mdm2 (Momand et al., 1992), and GADD45 (growth arrest and DNA damage) (Kastan et al., 1992). There are several reports that some RNA virus infections such as HIV (Gaeden and Morrison, 2005), Influenza virus (Turpin et al., 2005), rubella virus (Megyeri et al., 1999) and HCV (Siavoshian et al., 2005) can induce p53 participation in multiple cellular processes of apoptosis. It is not known whether p53 has some function on reovirus induced apoptosis. So a stable H1299 cell line was induced to transcriptionally express human wild-type p53 (Chen et al., 1996) to check functions of p53 on reovirus infection. During experiments, the cells were induced to express wt-p53 by withdrawing tetrocycline in the media and then infected with reoviruses T1L or T3D. The results showed that p53 expression did not affect virus replication, and the

virus titers were not obviously different in the cell culture no matter p53 presence (Figure **3.3**). Reovirus infection also did not induce the expression of p53 protein (Figure **3.6**, and **3.8**). These results indicate that reovirus infection did not induce p53-participated apoptosis or cell arrest. There may be other cellular pathways play roles in reovirus infection.

7.1.3. The number of reovirus particles during infection affects reovirus induced apoptosis. Generally a very large amount of reovirus (MOI=100 PFU/cell) has been used to trigger apoptosis (Tyler et al., 1995) and experimental results presented in the current thesis also confirmed the reaction. However, reovirus naturally infects host cells at much lower MOI. Therefore, it was important to determine how cells behave with smaller (more physiologically relevant) doses of reovirus. I used three doses of reoviruses (MOI=100, 10 or 0.5 PFU/cell) to infect L929 cells, Vero cells or H1299 cells. The results showed that reoviruses could also induce apoptosis in L929 and Vero cells when the cells were infected with reoviruses at dose of MOI= 10 PFU/cell (Table **3.2**) (Figure **3.3**). This was very similar to the cell reaction when infected with dose of MOI=100 PFU/cell (Table **3.2**) (Figures **3.2** and **3.5**). But reovirus infection at dose of MOI=0.5 PFU/cell did not induce apoptosis. Therefore, reovirus-induced apoptosis depends upon doses of virus infection. A larger amount of reovirus (MOI $\geq$ 10 PFU/cell) induces stronger stress to the cells and results in cell apoptotic response in the early period of infection (36 hours PI). Smaller amounts of reovirus caused CPE in the infected cells at later period (after 48 hours PI); and the CPE was not apoptosis. For H1299 cells, different doses of reovirus infection (MOI= 100, 10, or 0.5 PFU/cell) did not cause cell apoptosis. However, a larger amount of reovirus infection (MOI $\geq$ 10 PFU/cell) caused the cells to arrest much stronger than the dose of MOI= 0.5 PFU/cell. The cell numbers in the groups infected with doses of MOI $\geq$ 10 PFU/cell were much less than at MOI= 0.5 PFU/cell. These results indicate that larger amounts of virus contacting and entering cells give the cells strong signals and suddenly change the cellular conditions, and the cells must make all necessary changes and responses to virus infection immediately; furthermore these result in the cells change the expression of some specific genes and result in arrest or apoptotic responses to reovirus infection.

7.1.4. Expression of cellular proteins in reovirus infection. During reovirus-induced

apoptosis, a variety of cellular pathways stimulated to change cell reaction and cellular gene expression have been confirmed. Until now, reovirus infection is known to be involved in activation of cellular proteins such as cellular transcription factor NF-κB (Connolly et al., 2000; Clarke et al., 2003), TRAIL-apoptotic pathway (Clarke et al., 2000; 2001), JNK and c-Jun, and mitochondrial signaling system (Rodgers, et al 1997; Kominsky et al., 2003a; 2003b). Using DNA microarrays, over cellular 20 genes were found to change their expression after or during reovirus induced apoptosis (Poggioli et al., 2002: DeBiasi et al., 2003). It is also important to know whether more cellular genes could be activated in the process of reovirus-induced cell responses. In the experiments, two sets of human multi-probe template kits: human stress –1(hstress-1) and human apoptosis-3 (hAPO-3) were used to detect the transcription level of cellular gene mRNA by RNA protection assay (RPA). When infected with reoviruses T1L or T3D at dose of MOI= 100 PFU/cell, GADD45α, p21, Bax, and mcl1gene RNAs were induced higher in T1L and T3D than the control from 8 to 24 hours PI, and p53 mRNA was not detected in L929 cells (Figure **3.6A**). In H1299 cells there was one more gene (c-fox mRNA) induced to transcribe in addition to GADD45α, p21, Bax, and mcl1 genes (Figure **3.6B**). However, bcl-x RNA of the cells infected with T3D was induced much higher than the control, but bcl-x RNA was inhibited in the cells infected with T1L from 8 to 36 hours PI (Figure **3.6B**). Other genes such as Caspase-8, FASL, DR3, TRAIL, TRADD and RIP were not induced to increase in L929 or H1299 cells infected with reovirus T1L or T3D at 8 or 16 hours PI (Figure **3.9A**). Some other genes such as FADD, FAP, FAF, RIP and bcl-2 gene mRNA (also in hstrees-1 and hAPO-3 multi-probe template sets) were not detected after reovirus infection by RPA (Figure **3.9B**). The results indicated that reovirus induced apoptosis or cell arrest would trigger some cellular pathways to respond. These indicate that different cells show different reactions, induced by different cellular proteins during reovirus infection. Some cellular genes that may be expressed by induction of reovirus also depend upon the cell types. Interestingly, reovirus infection can induce the expression of some p53 target genes such as p21, bax, and GADD45α (Figure **3.6**) and these gene expressions could result in the cell arrest or apoptosis. Therefore, the expression of GADD45α, p21 or bax in reovirus infection is not regulated by a p53 dependent pathway; and these phenomena of p53-independent expression of GADD45α

(Jin et al., 2000), p21 (Alpan and Pardee, 1996; Zeng and EJ-Deiry, 1996) are also observed in cell arrest or apoptosis induced by specific stress. There must be other pathways regulating the reaction of these genes in the infected cells. Furthermore, the difference of Bcl-x expression in H1299 cells infected with T1L or T3D and no expression in L929 cells, indicates that the different cells show different reactions during reovirus infection.

7.1.5. GADD45α response in reovirus infection. Growth Arrest and DNA Damage-inducible gene 45α (GADD45α) is a cell cycle regulator, which encodes a protein that is induced by genotoxic and certain other cellular stress (Smith et al., 1994). GADD45α protein is able to associate with multiple important cellular proteins, including proliferating cell nuclear antigen p21 (Kearsey et al., 1995), Cdc2 (Zhan et al., 1999), core histone (Carrier et al., 1999), and MTK1/MEKK4 (Takekawa and Saito, 1998). More findings suggested that GADD45 is involved in the control of cell cycle checkpoint (Wang et al., 1999) and apoptosis (Harkin et al., 1999). Therefore, GADD45 appears to be an important player in maintenance of genomic stability. The experiments here showed that reovirus induced apoptosis in L929 cells and Vero cells or cell arrest in H1299 cells definitively stimulated the transcription of GADD45α mRNA (Figure **3.6**) and expression of GADD45α protein (Figure **3.11**). The response of reovirus induced GADD45α was correlated to reovirus induced apoptosis or arrest, and depends upon the dose of reovirus infection. Doses of reovirus of MOI $\geq$ 10 PFU/cell induced apoptosis or arrest, and induced the expression of GADD45α. GADD45α was not detected in cells infected with reovirus at MOI= 0.5 PFU/cell and apoptosis or arrest was also not detected. The GADD45α protein was not expressed in the L929 cells or H1299 cells treated with UV-inactivated reovirus even at a dose of reovirus particles equal to MOI=100 PFU/cell (Figure **3.12**). This means a larger amount of living reoviruses enter the cytosol and trigger the expression of GADD45α, and the induction of GADD45α is not stimulated by the reaction of reovirus with cell receptors. The expression of GADD45α is induced by reovirus infection, and the expression showed time and dose dependent patterns. Therefore GADD45α plays some important roles during reovirus infection by controlling cellular gene activities and stopping the replication of cells; that may set up some suitable

environment and conditions for reovirus replication and for cell protection to reovirus invasion. The expression of GADD45α is one of the important cellular factors responding to reovirus infection.

7.1.6. Regulation of GADD45α response in reovirus infection. It has been shown that induction of GADD45 after DNA damage is mainly p53-dependent in human and murine cells, and the increased expression of GADD45α occurred in the wild-type p53 cells; and no GADD45α and GADD45α-mediated G2/M was observed in p53-null Li-Fraumeni fibroblasts or in normal fibroblasts coexpressed with p53 mutants (Kasfan et al., 1992; Wang et al., 1999). However, there are also several reports that indicate that some inducers induce GADD45α in the absence of activated p53 (Jin et al., 2000; O,Reily et al., 2000); and p53-dependent and -independent mechanisms have been reported to play a role in the regulation of GADD45 expression (Lakin and Jackson, 1999). Recently some research showed that NF-κB pathway functioned on the expression of GADD45α (Chen et al., 2001; Zeibini et al., 2004; Thyss et al., 2005). In our experiments, we detected that during reovirus infection the expression of GADd45α was induced and the expression of NF-κB was also induced; the patterns of GADD45α and NF-κB expression were similar and NF-κB was expressed earlier than GADD45α. However, these results showed that contradictory reactions about the NF-κB regulate expression of GADD45α. Some reports showed that NF-κB could positively regulate the expression of GADD45α under UVB irradiation (Thyss et al., 2005). Arsenite treatment inhibited expression of NF-κB and can enhance the expression of GADD45α (Chen et al., 2001), and Zernini et al. (2004) also reported that NF-κB mediated repression of GADD45α and γ is needed for cancer cell survival. My results showed that p53 protein was not detected (Figure **3.6**), but GADD45α was highly expressed in reovirus infection. This means the induction of GADD45α is not regulated by p53 (p53 independent pathway) for reovirus induced cell reaction. Reovirus infection can induce the expression of NF-κB (Connolly et al., 2000). I detected NF-κB and GADD45 proteins in the cells infected with reovirus at dose of MOI=10 PFU/cell by Western blotting. The expression patterns of NF-κB subunit p65 and GADD45α were induced similarly, and the NF-κB subunit p65 protein was expressed earlier than the GADD45α in general (Figure **3.15**). It

is possible that the expression of GADD45α induced by reovirus infection at larger doses is related with NF-κB signaling pathway induced by reoviruses. The expression of NF-κB could result in the activation of sets of cellular proteins including GADD45α protein that NF-κB regulates. However, there may be other pathways to regulate the expression of GADD45α in reovirus infection because the UV treated reovirus did not induce the expression of GADD45α protein in these experiments, and the expression of NF-κB is activated by the binding of reovirus to the cell receptors (Connolly et al., 2000).

7.1.7. GADD45α expression on reovirus replication. siRNA can be used to silence gene expression in a sequence-specific manner and the application of siRNA in mammals has the potential to allow the systematic analysis of gene function (Dykxhoorn et al., 2003). In order to further understand the function of GADD45α on reovirus replication, because GADD45α can induce cell G2/M arrest, which makes suitable conditions for virus growth, GADD45α siRNA was transfected into L929 cells or H1299 cells. The transfected cells affected reovirus replication. The virus titers in the transfected cells were lower than the controls and the siRNA from different species at 36 and 48 hours PI (Figure **3.13**). By GADD45α protein detection, GADD45α siRNA can inhibit the expression of GADD45α (Figure **3.14**). The inhibition of GADD45α by siRNA partly decreases the replication of reoviruses. This seems transcient; the viruses can adapt to the condition of lower GADD45α level and possibly by activating other pathways for replication, so that reovirus replication increases after 48 hours PI and reaches normal levels at 72 hours PI. The results also indicate that the expression of GADD45α protein can improve reovirus replication by stopping the cell cycle replication.

7.2. Function of siRNA on reovirus replication:

In many eukaryotes, double-stranded (ds) RNA inhibits gene expression in a sequence-specific manner by triggering degradation of mRNA. This effect is referred to as RNA interference (RNAi). Genetic and biochemical studies have revealed that RNAi is a very complex reaction, involving many different proteins of mostly undefined functions (Sharp, 2001). Over the past 5 years, RNA interference has emerged as a natural mechanism of silencing gene expression. Artificial introduction of siRNA duplexes into

cells can thus silence the expression of selected genes and a number of methods have been developed for getting siRNA of a desired sequence into target mammalian cells. The two most commonly used are to transfect in synthetic short double-stranded RNA molecules of the desired sequence, which then act to engage RISC directly (Elbashir et al., 2001). Alternatively, expression vectors can be introduced into cells, either by transfection or the use of virus, which direct the production of short hairpin RNA sequences that are then processed into siRNA within the cell by endogenous enzymes (Brummelkamp et al., 2002; Paddison et al., 2002). The functions of siRNAs have been used to inhibit virus growth or replication successfully as summarized in the introduction (Chapter **4.1**). In the experiments of siRNA to inhibit reovirus replication, two sets of siRNAs that are a part of the gene sequence of reovirus RdRp or the M2 gene of reovirus major outer capsid protein were selected. The design of the siRNAs was based upon "rules"; that the GC content of the siRNAs should be kept between 30-70% and have a 2 nucleotide 3' overhang of uridine residues, and 5'-phosphate and 3'-hydroxyl groups for efficiency (Agrawal et al., 2003). Neither set of siRNAs could inhibit reovirus replication. The titers of two reovirus strains T1L and T3D in the siRNA-transfected cells were not decreased as we would expect. The efficacy of siRNA-mediated suppression of gene expression depends on a number of factors, including not only the chosen siRNA sequence but also the structure of the siRNA, and the receptiveness of the cell type to siRNA uptake (Kim et al., 2005; Siolas et al., 2005). The results of my experiments indicate that reovirus replication is a complicated process, and to date, there are no successful reports about reovirus siRNAs. However, the complex of siRNA on rotavirus replication can give some ideas. Especially for rotavirus, that also belongs to f the *Reoviridae* family, there have been several reports that siRNA of rotaviral proteins VP4, VP7, NSP1, NSP4 and NSP2 could inhibit the expression of their target protein and decrease virus replication (Dector et al., 2002; Lopez et al., 2005; Silvestri et al., 2004; Silvestri et al., 2005). More interesting, different rotavirus siRNA showed different function. VP4 siRNA can inhibit the production of infectious viral progeny that are triple-layered virus particles (Dector et al., 2002). Rotaviral gene 5 encoding NSP1 (an nonessential protein) siRNA did not reduce infectious virus particles (Silvestri et al,. 2004). Rotaviral gene 8 (encoding NSP2 being a component of viral replication factories)

siRNA can inhibit NSP2 expression and resulted in inhibiting viroplasm formation, gene replication, virion assembly and synthesis of the outer viral proteins (Silvestri et al., 2004). Rotaviral gene 9 encoding VP7 (outer capsid protein) siRNA can also inhibit the production of infectious virus progeny (Silvestri et al., 2004; Lopez et al., 2005). Rotaviral gene 10 encoding NSP4 (a rotavirus glycoprotein) siRNA knocked out NSP4 expression and had no marked effect on the expression of other viral proteins or on the replication of the dsRNA genome segments, but suppressed viroplasm maturation and caused a maldistribution of nonstructural and structural proteins resulting in inhibition of packaged virus particle formation (Silvestri et al., 2005).

In 2000, Svoboda, et al. used synthetic 650bp long dsRNAs of *Mos* or *Plat* genes to inhibit targets in mouse oocytes and effectively suppress gene expression (Svoboda et al., 2000); and Wianny, et al. also reported that they injected over 550bp long dsRNA of *C-mos* or *E-cadherin* genes containing GFP into the preimplantation embryo and it caused specific inhibition (Wianny et al., 2000). There have been reports of the specific inhibition of long dsRNAs on target genes in undifferentiated mouse embryonal stem cells (Yang et al., 2001), undifferentiated embryonal carcinoma cells (Billy et al., 2001), differentiated mouse neuroblastoma cells (Gan et al., 2002), human peripheral blood mononuclear cells (Bhargava et al., 2004), rat hypothalamas (Yi et al., 2003) and muscle cells (Kong et al., 2004). Haase et al. (2005) found that TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacted with Dicer and functioned in RNA silencing. Full length dsRNA triggering sequence-specific mRNA degradation is very interesting and may have a wonderful future. I here transfected full length reoviral dsRNAs into mouse L929 cells or human H1299 cells directly, and the transfected cells were infected with reovirus T1L or T3D. The reoviral long dsRNAs could inhibit reovirus replication and virus titers were decreased by 3-10 fold (Figures **4.3**, and **4.4**). The inhibition of reovirus replication also appeared in cells transfected with different dsRNA parts of reovirus genomes (Figure **4.6**). The inhibition was not influenced by different amounts of dsRNAs (Figure **4.5**); however, the dsRNA suppression depended upon subsequent infectious doses of reovirus; and reovirus could replicate a little when the transfected cells were infected at a larger amount (MOI=100PFU/cell) (Figure **4.7**).

There are some different ideas about the inhibition of long dsRNAs on target genes that are specific RNAi inhibition or unspecific interferon function. Long double-stranded RNA (dsRNA more than 30 bp) triggers the response of non-specific interferon and by activating protein kinase PKR, which phosphorylates the translation initiation factor elF2α and leads to a global inhibition of mRNA translation (Stark et al., 1998). Furthermore, some investigations found that double-stranded (dsRNA) (Alexopoulou et al., 2001) and single-stranded RNA (ssRNA) (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004) can stimulate innate cytokine response in mammals; few studies have questioned whether siRNA may have a similar effect on immune system (Kariki et al., 2004; Sioud et al., 2003). However, Heidel et al. (2004) found that naked synthetic siRNA down-regulated an endogenous or exogenous target without inducing an interferon response. Judge et al. (2005) reported that synthetic siRNA stimulated the sequence-dependent response of mammalian innate immune system; and they also found the putative immunostimulatory motifs that allowed the design of siRNAs that can mediate RNAi but induce minimal immune activation. Stein et al. (2005) found that long double-stranded RNA triggered absence of non-specific effects of RNA interference in mouse oocytes by analyzing the global pattern of gene expression with microarray analysis. Wen et al. (2005) reported that down-regulation of specific gene expression by long dsRNA induced neural stem cell differentiation in vitro. Robalino et al. (2005) found that double-stranded RNA induced sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp. Reovirus infection could trigger the expression of non-specific interferon; and this stimulated response of interferon depends upon the difference of reovirus strains that reovirus strain T3D can induce more interferon expression than the strain T1L (Farone et al., 1996). In cardiac myocyte cultures, reovirus induction of IFN-β is determined by viral core proteins and inversely correlates with the capacity of viruses to induce CPE *in vitro* and myocarditis *in vivo* (Sherry et al., 1998); also reovirus strains T1L or T3D differed in the extent to which their replication is inhibited by interferon (Jocobs and Ferguson, 1991). There were several studies demonstrating that reovirus replicated efficiently in interferon-treated cells despite activation of antiviral effector enzymes (Danis et al. 1997; Feduchi, et al., 1988). In my experiments, reovirus long full length dsRNA can positively inhibit reovirus

replication and the function did not show difference between reovirus strains T1L or T3D. Furthermore the purified reoviral L, M and S gene dsRNAs also performed the inhibition on reovirus replication. All these differed with the reovirus-induced interferon function so that full-length reovirus genomic dsRNA may be a siRNA function in my experiments.

7.3. Infectious entry of reovirus cores into mammalian cells enhanced by transfection:

Transfection has been reported to enhance replication of some viruses in different ways. For example, complexing virus particles with lamellar liposome have increased the efficiency of productive poliovirus (Wilson et al., 1977) and HIV (Konopka et al., 1990) infections in non-permissive or semi-permissive cells. In addition, Lipofectin enhanced the infectivity of cypovirus particles in cultural insect cells by more than 7 fold (Hill et al., 1999). This thesis reports reported here that two transfection methods (Lipofectamine 2000 method and Calcium phosphate method) can convert the non-infectious, transcriptionally active reovirus core into particles that are as infectious as fully functional virions in at least two different cell lines.

These findings indicate that reovirus core particles contain all the necessary information for progeny transcription and replication. This has been inferred from observations that microinjected core particles can sustain a full replication cycle (Moody and Joklik, 1989). The results of transfection with lipofectamine reagents further confirm and extend the previous reports observation of reovirus core replication by micro-injection. In addition, microinjection is a tedious process not suited to working with large numbers of cells. The use of transfection reagents, such as lipofectamine reagents or calcium phosphate assists the reovirus care particles to pass the obstacle of cell membrane and enter the cytoplasm. The two reagents allowed transfection of reovirus core particles into the cells for replication, but with different efficiencies; Lipofectamine 2000 enhanced the production of reovirus core particles more than 10,000 fold greater than the non-transfected control group (Figure **5.3**), addition of $CaPO_4$ increased the specific infectivity of core particles, but only ~300 fold (Figure **5.4**). This may be due to sensitivity of L929 cells to Lipofectimane transfection; I noticed that more cells died in the $CaPO_4$ treatments than in lipofectamine 2000 treatments.

Finally, reovirus oncolysis has been set up and developed for gene therapy to kill some cancer cells (Hirasawa et al., 2002; Norman et al., 2002). The added advantage of being able to significantly increase the specific infectivity of reovirus cores within a larger population of target cells may greatly increase reovirus oncolysis treatments and further assist molecular and genetic manipulations.

7.4. Reovirus T2W S gene sequences:

Mammalian reoviruses share a number of common structural characteristics in the genome comprising 10 segments of dsRNA and in the number and arrangement of structural proteins within particles (Nibert and Schiff, 2001). The reovirus strain analyzed here was isolated from the central nervous system of an 8-week-old female infant with a history of active varicella, oral thrush, hypoalbuminemia, intermittent fevers, diarrhea and feeding intolerance, and this reovirus strain was identified as reovirus serotype 2 by virus neutralization and RNA-gel electrophoresis studies (Hermann et al., 2004). Sequence analyses of the T2W S1, S2, S3 and S4 genes and the encoded σ1, σ2, σNS and σ3 proteins prove that this newly isolated virus is a novel reovirus and not a reassortant virus that arose from previously identified strains.

The S1 gene segment of mammalian reovirus is bicistronic, encoding both the viral attachment protein sigma-1 (σ1) and the nonstructural protein (σ1s) from overlapping but out-of-sequence open reading frames (Ernst and Shatkin, 1985; Nibert and Schiff; 2001). σ1 is a structural protein located at the icosahedral vertices of the viral outer capsid and functions as the viral cell attachment protein (Nibert and Schiff., 2001) and the major serotype-specific antigen recognized in HAI and NT assays using type-specific polyclonal antisera (Tyler, 2001). The viral σ1 protein is unique to each prototype of mammalian reovirus and determines the serotype and also is the major genetic determinant of neurovirulence in infected mice (Tyler et al.,. 1986). Sequence analysis of the T2W S1 genome segment further supports previous observations of the mammalian reovirus protein σ1, including remarkable degree of variation in size, gene arrangement, and coding potential of polycistronic S-Class genome segments (Duncan et al., 1990; Nibert et al., 1990). The T2W S1 gene showed far greater similarity to other type 2 S1

genes (92% identity to 2N84 and T2N73, and 51% identity to serotype T2J) than it did to serotype 1 and 3 reovirus S1 genes (Table **6.1A** and Figure **6.1A**). These data confirm that T2W did not result from reassortment between any currently known Type1 viruses, Type 3 viruses or Type 2 viruses; it is, therefore, a new serotype 2 reovirus strain.

The S2 gene of mammalian reovirus encodes structural protein σ2 and the σ2 protein interacts with λ1 to form the viral core shell (Mustoe et al., 1978; McCrae and Joklik, 1987). Sequence analysis of the mammalian reovirus S2 gene showed that the gene and the encoded σ2 protein had very high similarity with other reovirus S2 genes. Notably, approximately 60 nucleotides at the 5' terminus and 30 nucleotides at the 3' terminus were markedly conserved in the 4 compared reovirus strains (Figure **6.2B**) that confirmed previous reports about the high degree of S2 gene conservation of mammalian reoviruses (Chapell et al., 1994). Analysis of the T2W S2 gene also confirmed another property of the S2 gene in nature, both the S2 gene and the σ2 protein showed the highest rate of identity with the three prototype viruses T1L, T2J and T3D among the comparisons of all 4 S genes, with 73-75.5% identity in nucleotide sequence of the S2 gene and 92.5-95% identity in amino acid sequences of the σ2 proteins (Table **6.1B**, figure **6.1B**). My data of T2W S2 gene sequence and σ2 protein further confirmed that mammalian reovirus σ2 protein, present within the internal capsid is less interfered by cellular factors and immune system pressure and remains relatively stable.

The complete T2W S3 gene is 1198 nucleotides in length encoding the 367 amino acid long σNS protein and with short noncoding regions of 23 nucleotides at the 5' terminus and 64 nucleotides at the 3' terminus, identical to reovirus prototype strains T1L, T2J and T3D (Richardson and Furuishi, 1983; Wiener and Joklik, 1987). The sequence of the T2W S3 gene and the encoded σNS protein were different from the S3 genes of each of the other 20 known reovirus isolates (Goral et al., 1996), (Table **6.1C**, and Figure **6.1C**), further confirming T2W represent a novel reovirus clone.

The S4 gene of mammalian reovirus, the smallest genome segment, encodes σ3 that is a 365 amino acid protein serving as a major constituent of the reovirus outer capsid (Giantini et al., 1984; McCrae and Joklik, 1987). The 1196 nucleotide length of the S4 gene and the 365 amino acid length of the σ3 protein of T2W were identical in length to

the 3 prototype reoviruses T1L, T2J and T3D. However, the T2W S4 gene and σ3 protein showed more variability than the S2 gene and the σ2 protein with the 3 prototype reoviruses (Table **6.1D**, Figure **6.2D**). For example, the amino acids in the zinc-binding sites (Schiff, et al. 1988) of all σ3 proteins were very conserved; however, T2W had one amino acid difference (S_{54} to A_{54}) (Figure **6.3**). Furthermore, the amino acids in the dsRNA-binding site of T2W σ3 protein showed more variability, with 3 amino acid changes (H_{240} to Y_{240}, D_{242} to E or A, and D_{243} to E_{243}) compared to T1L, T2J and T3D (Kedl et al., 1995). Whether these differences of T2W S4 gene and σ3 protein change this virus property of infection and replication needs further investigation, but these data confirm the virus T2W is not a reassortant virus strain.

7.5. CONCLUSION.

The experiments include 4 areas (1) reovirus infection induces the expression of GADD45α protein; (2) siRNA inhibition on reovirus or GADD45α protein as well as the affection of these siRNAs on GADD45α expression or reovirus replication; (3) the function of transfection of reovirus cores on reovirus replication and further to understand affection of virus outer capsid proteins and the process of the binding to cellular receptors on GADD45α response; and (4) sequencing the S genes of the new reovirus type 2 strain T2W to minoter the difference of three serotype reoviruses inducing cell response and GADD45α expression with molecular virologic methods. The following conclusions can be derived from these studies:

1. Reovirus infection can induce different cellular responses that depend on the cell types. In L929 cells and Vero cells reovirus induces apoptosis; however, cell growth arrest occurred in H1299 cells. Reovirus induced cell apoptosis or arrest also depends upon the infection doses of reovirus; the infectious doses must be larger than MOI=10 PFU/cell. The cell response is not induced by reovirus infection at dose of MOI=0.5 PFU/cell.This **is the first report that reovirus infection at the dose (MOI=10 PFU/cell) can induce apoptosis.**

2. The experiments confirmed that **reovirus replication is unrelated to the p53 pathway**. Reovirus infection does not induce the transcription of p53; and the expression of p53 in cells also does not affect virus replication.

3. **The expression of cellular genes GADD45α, p21, mcl1 and bax are induced by the infection of reovirus strains T1L and T3D**. The other genes such as p53, TRAIL, DR3, caspase-8, RIP, FAS, FASL do not respond to reovirus infection. The expression of gene Bcl-x in H1299 cells depends upon the reovirus strain: reovirus strain T3D can positively induce the expression of Bcl-x and reovirus strain T1L may inhibit the expression of Bcl-x.

4. **The expression of cellular gene GADD45α is stably induced by reovirus infection. This expression corresponds with the cell responses to reovirus infection, and also is decided by the infectious doses of reovirus (MOI ≥10 PFU/cell).** UV-inactivated reovirus particles do not induce the expression of GADD45α.

5. **The expression of GADD45α is regulated by NF-κB pathway in reovirus infection.** However, there are still other pathways to regulate GADD45α expression because dead reovirus does not induce the reaction. Inhibition of GADD45α expression by siRNA can affect reovirus replication and decrease the virus titers at 36 and 48 hours PI.

6. Two sets of tested reovirus siRNAs did not inhibit reovirus growth. It is possible that the selection of these parts of siRNAs was not satisfactory or the siRNAs did not function on virus replication. This indicates that siRNA function is very complex. However, long full-length genomic reovirus dsRNA could inhibit reovirus replication; and all of reovirus S, M and L genomic dsRNA fragments could have inhibiting ability, indicating that dsRNA could preform special inhibition as one of the siRNA functions.

7. Purified reovirus core particles were not infectious when directly added to cells. **Transfection reagents enhance core particle entry into cells and the core can replicate to reach almost same level of viruses as reovirus virions.**

8. **Reovirus Type 2 Winnipeg (T2W) is a new type 2 virus,** as confirmed by the higher identity of the S1 gene to serotype 2 reoviruse S1 genes. However, The T2W S2, S3, and S4 gene are divergent from other reovirus. T2W is a novel reovirus because all S genes differ from previous reported reovirus strains.

7.6. FUTURE DIRECTIONS.

7.6.1. Function of GADD45α on reovirus infection: In this research, reovirus infection at the dose of MOI ≥10 PFU/cell can induce apoptosis or cell arrest. These cell responses are not related to p53 protein, so these reactions involve p53-independent pathways. The expression of GADD45α that is induced by reovirus does not differ between the T1L and T3D reovirus strains. GADD45α siRNA can inhibit reovirus replication. This thesis answers some questions, but also opens some important research areas for further work. First of all, some stable cell lines in which GADD45α gene would be deleted completely, or mutated partly (the protein binding sites) could be set up, then infected with reovirus strains to monitor the virus replication situation. Through these experiments, the function of GADD45α on reovirus infection might be better understood. Secondly, it is necessary to understand which parts of reovirus structures or the process of virus replication is responsible for inducing the expression of GADD45α by using drugs and inhibitors (such as drugs that inhibit virus entering into cells, uncoating or RNA synthesis) to regulate different aspects of virus replication. Some reovirus *ts* mutants or reassortant strains may also be used to test their ability to induce apoptosis or cell arrest, and detect the the expression of GADD45σ protein. These experiments can further provide some important information to understand the mechanisms of reovirus induced GADD45α and cellular responses.

7.6.2. Cellular response on reovirus infection at small dose (MOI=0.5 PFU/cell): In the achieved experiments, smaller doses of reovirus infection (MOI=0.5 PFU/cell) did not induce the expression of GADD45α and no apoptosis could be detected. However, CPE occurred, and the virus could replicate to reach very high level in cells at the late period of infection similar to larger doses. Therefore, it is necessary to know whether there are still some other cellular proteins participating in the process, and what the proteins do and how they function, because reovirus is a potential oncolysis reagent that can kill cancer cells in vitro or in vivo. There have been wonderful results of reovirus killing cancers and oncolytic reovirus has bright future for cancer treatment. An important question raised by this research is: How many reovirus particles are needed to treat cancer patients without serious side-effects?

7.6.3. Relation of the expression of GADD45α and/or NF-κB induced by reovirus infection: In my experiments, reovirus infection could induce GADD45α expression as well as NF-κB expression. There are some different results about the NF-κB regulated expression of GADD45α. Some reports showed that NF-κB could positively regulate the expression of GADD45α under UV- irradiation (Thyss, et al. 2005). However, arsenite treatment, which inhibit NF-κB expression resulted in increasing GADD45α expression (Chen et al., 2001). Zernini et al. (2004) reported that knockout of NF-κB gene would enhance GADD45α and γ expression and they suggested a mechanism of NF-κB mediated repression of GADD45α and γ for cancer cell survival. In previous reports, NF-κB was induced to express and resulted in apoptosis after reovirus infection (Connolly, et al. 2000; Clarke and Tyler, 2005). In my experiments, I found that reovirus infection induced GADD45α expression and also confirmed NF-κB expression in cells infected with reovirus. The expression of two proteins GADD45α and NF-κB were similar, not significantly different, during reovirus infection. Therefore, it is necessary to determine whether NF-κB regulation effects GADD45α expression during reovirus infection by setting up some cell lines deleted of NF-κB gene for reovirus infection.

7.6.4. Function of full length dsRNA of reovirus on virus replication: siRNA can specifically inhibit target gene translation and there have been some reports that long dsRNA is also involved in the siRNA interference in mammalian cells or embryo (see Section **7.2**). In my experiments, reoviral full length genomic dsRNA could suppress virus replication. This may have been caused by specific or non-specific affects. Therefore, this inhibition should be tested for either the specific interference, or universal interferon function, by detectinginterferon reaction or interference to other virus' replication. If the inhibition is confirmed to be specific that might be important for siRNA research on long dsRNA molecular function.

7.6.5. Inhibition of siRNA on reovirus replication: In my experiments, two sets of siRNA did not inhibit reovirus replication. However, rotavirus siRNA can inhibit rotavirus replication at different steps of virus life cycle by using different siRNAs (see section **7.2**). If some cell lines inhibiting the specific reovirus protein by siRNA technique can be established, they could be used to test reovirus replication and

determine the GADD45α or other cellular protein responses. These experiments would provide more information to understand the mechanisms of reovirus infection and cell protection.

7.6.6. Mapping strain specific difference in Bcl-x expression in H1299 cells: In the experiments, Bcl-x was induced to express by reovirus T3D, and inhibited by T1L in H1299 cells. There are 10 genomic fragments of reovirus and each of them generally determines one protein. Different type virus strains exist with difference in genomic and protein composition. We can use reovirus reassantant strains to infect H1299 cells and analyze the experession of Blc-x protein in H1299 cells so that the genomic difference can be mapped out.

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