USE OF REOVIRUS MONOREASSORTANTS TO DEFINE LYSIS OF TRANSFOMED CELLS

by

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ABSTRACT

USE OF REOVIRUS MONOREASSORTANTS TO DEFINE

REOVIRUS LYSIS OF TRANSFORMED CELLS

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Reovirus is known to selectively destroy transformed cells while establishing a persistent infection in normal cells. For this study the transformed cell lines used were L929, WI-38 VA13 2RA, T1, N1, and MYC-3. Additionally, the normal cells WI-38 and MMEC were used to examine differential sensitivity to reovirus serotypes 1, 2, and 3. Reovirus is able to replicate efficiently in L929 cells because it induces a transition from capped to uncapped translation of viral mRNA. Reovirus maintains a persistent infection in WI-38 cells, but lyses WI-38 VA13 2RA cells. Reovirus has also been demonstrated to lyse and produce more virus per cell in N1 and MYC-3 cells than MMEC cells. However, the virus does not lyse T1 cells efficiently. Monoreassortant viruses containing nine genes from serotype one and one gene from serotype three mapped the genes responsible for this differential sensitivity to the S3 and S4 genes.

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CHAPTER 1

INTRODUCTION

1.1 Background on Reovirus

The name reovirus is an abbreviation for "respiratory enteric orphan virus", and was given the name due to the fact that even though the virus is found in the respiratory and gastrointestinal tracts, it has not been associated with a particular disease (1, 2). Reovirus is a non-enveloped, icosahedral virus with double capsids and is approximately 70 nm in diameter. Reoviridae, the family that mammalian reovirus belongs to, are unique viruses because they have a segmented double-stranded RNA (dsRNA) genome. The genome consists of 10 dsRNA segments that can be separated by polyacrylamide gel electrophoresis into 3 size classes: large (L), medium (M), and small (S). L consists of 3 genome segments (L1-L3) of about 3800 bp each, M consists of 3 genome segments (M1-M3) of about 2200 bp, and S consists of 4 genome segments (S1-S4) of about 1100-1400 bp each (3). Each of the segments in the 3 class sizes have been shown to be discrete segments and not random fragments of larger molecules due to the fact that they do not hybridize with each other and each segment is transcribed into a unique mRNA molecule and a primary polypeptide (4). Table 1

shows the correlation between genome segments and corresponding proteins of mammalian reovirus while Figure 1.1 shows the structure of the reovirus virion (5, 13).

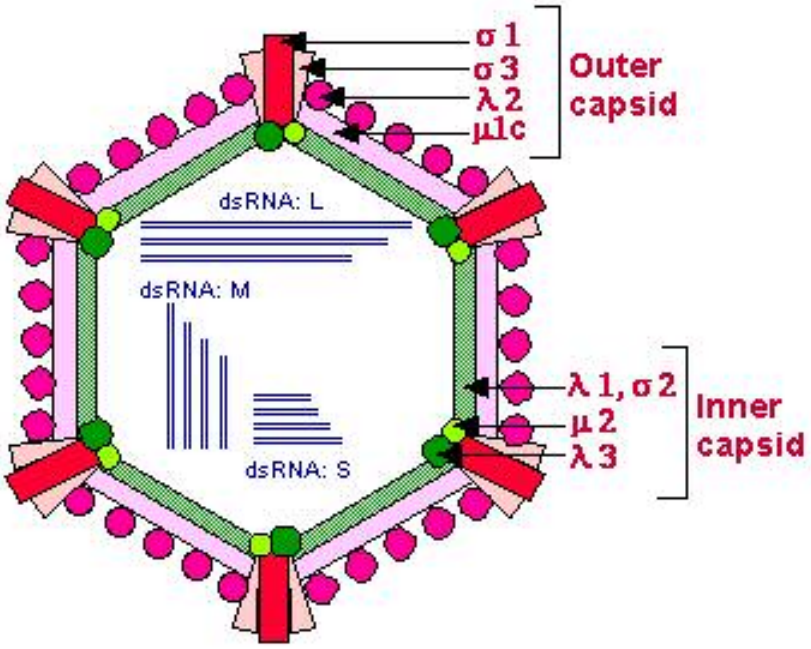

Figure 1.1 Structure of orthoreovirus

Table 1.1 Correlation between gene segments and protein

Gene segment	Polypeptide	Mass (kd)	Protein copy number per particle	Location in virus	Function
L1	λ3	142	12	Core	Fully conservative RNA dependent RNA polymerase
L2	λ2	145	60	Core spike	Guanyltransferase, methyltransferase
L3	λ1	143	120	Core	Binds dsRNA and zinc, putative NTPase, helicase and 5'-triphosphate phospohydrolase
M1	μ2	83	12	Core	NTPase, influences the morphology of inclusion bodies, interacts with cytoskeleton
M2	μ1 μ1C (T13) δ φ μ1N	76 72 59 13 4	30 600 600	Outer capsid	Multimerizes with σ3. Myristoylated N-terminus, membrane penetration.
M3	μNS	80	0	Non-structural	Binds ssRNA and virus cores, primary determinant of inclusion body formation, interacts with μ2 and σNS
S1	σ1 σ1s	49 16	36 0	Outer capsid	Cell attachment protein, hemagluttinin, primary serotype determinant, induces apoptosis
S2	σ2	47	150	Core	Binds dsRNA
S3	σNS	41	0	Non-structural	Binds ssRNA, associates with μNS
S4	σ3	41	600	Outer capsid	Sensitive to protease degradation, binds dsRNA, translation control

Reovirus replication occurs in the cytoplasm. The first step in reovirus replication is the attachment of intermediate subviral particles (ISVP) or the viral particle to cell surface receptors via the cell attachment protein σ1 (6). Sialic acid, a ubiquitous cell surface receptor, can be recognized by the viral receptor σ1. As a result, mammalian reovirus has a broad cell tropism and the only mammal that has not been shown to possess antibodies to reovirus are whales. Following attachment, the process of endocytosis internalizes the virial particles. The endosomes fuse with the lysosomes to form endolysosomes and within this vesicle that the reovirus outer capsid shell is degraded proteolytically. The virus particles are now known as ISVPs. There is evidence that ISVPs can be generated when viral particles undergo extracellular proteolysis and therefore endocytosis is not always required to infect the host cell (6). The generation of an ISVP activates the RNA dependent RNA polymerase that proceeds to transcribe the 10 capped plus strands that are not polyadenylated. Secondary transcription occurs later in infection and results in capped and/or uncapped and non-polyadenylated transcripts. ISVP products of early transcripts associate with newly made protein to form RNase-sensitive assortment complexes (6). It is within the RNA assortment complex that plus strands serve as templates for minus strands resulting in RNase-resistant replicase particles. The mechanism by which capsid assembly occurs is not well known. Release of mature virions occurs after lysis of cells. Figure 1.2 illustrates the replication cycle of reovirus (6).

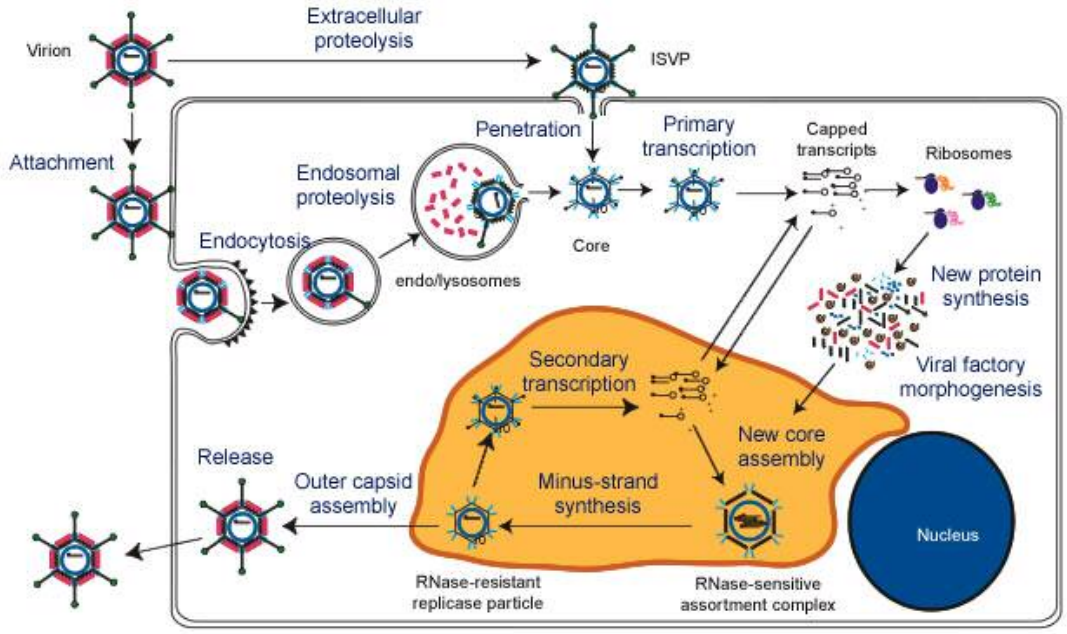

Figure 1.2 Reovirus replication cycle

1.2 Reovirus Differential Lysis

Mammalian reovirus consists of 3 serotypes: serotype 1 (ST1) strain Lang serotype 2 (ST2) strain Jones, and serotype 3 (ST3) strain Dearing. Reovirus is a benign virus in the developed world. However, in third world countries, it is a major cause of diarrhea in children that sometimes leads to death (WHO estimates ~ 300,000 per year). An intriguing fact that sets reovirus apart from many other viruses is that it is oncolytic. Reovirus oncolysis can be studied by infecting normal cells and their transformed counterparts with one, two, or all three serotypes. The use of monoreassortants in the study of reovirus lysis will be discussed later. Seven cell lines were used to study reovirus lysis. Table 1.2 shows the cell lines used.

Table 1.2 Cell lines examined

Normal Cell line	**Transformed counterpart**
	L929 (mouse-fibroblast-chemically transformed)
WI-38 (human – embryonic lung)	WI-38 VA132RA (human- embryonic lung- SV40 transformed
MMEC (mouse-mammary epithelium)	T1 (MMEC – transformed with mutant c-Ha-RAS)
	N1 (MMEC – transformed with normal c_Ha-RAS)
	MYC-3 (MMEC- transformed with normal c-myc)

Mouse L929 fibroblasts cells are chemically transformed cells and are the cell line of choice used to propagate reovirus. Reovirus mRNA possess a methylated cap structure (m^7GpppGm) at the 5′ termini when synthesized in the presence of S-adenosylmethionine (SAM) (14,15). The methylated 5′ cap structure is important in facilitating the binding of the 40S ribosomal subunit to mRNA 16). The mRNAs of L929 cells also have a capped 5′ termini and when they are infected with reovirus there is continued translation of the capped cellular mRNA. As the infection progresses (hours), viral mRNAs are no longer capped, and there is inhibition of host cell protein synthesis because the virus induces a transition from translation of capped to the

translation of uncapped viral mRNAs (17, 18). The virus therefore replicates efficiently in these cells and they provide the cell line that will be used as the control to study reovirus oncolysis. In other studies, Duncan *et al.* have shown WI-38 cells become susceptible to reovirus infection after transformation with Simian virus 40 (SV-40). They also demonstrated that reovirus mediates a lytic infection in the transformed cells WI-38 VA132RA (which will henceforth be abbreviated as 2RA) but a prolonged infection in its normal counterpart, WI-38 cells Figure 1.3 shows a comparison of the amount of virus produced in L, WI-38, and 2RA cells while figure 1.4 shows the viability of WI-38 and 2RA cells hours after infection (9).

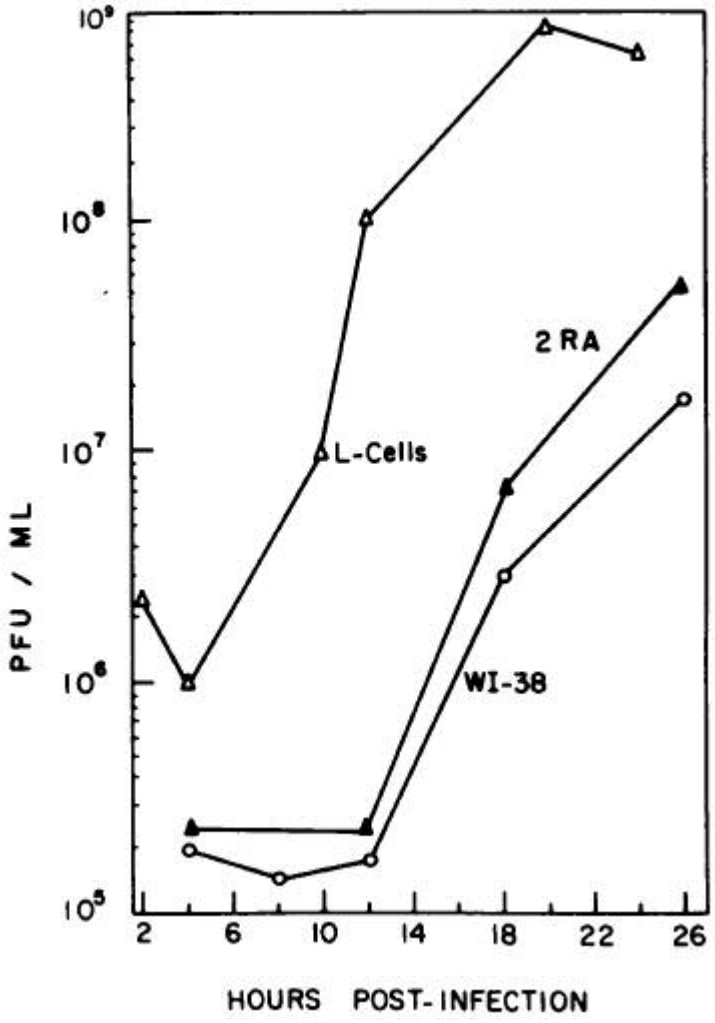

Figure 1.3 One step replication cycles of reovirus in L, 2RA and WI-38 cells.

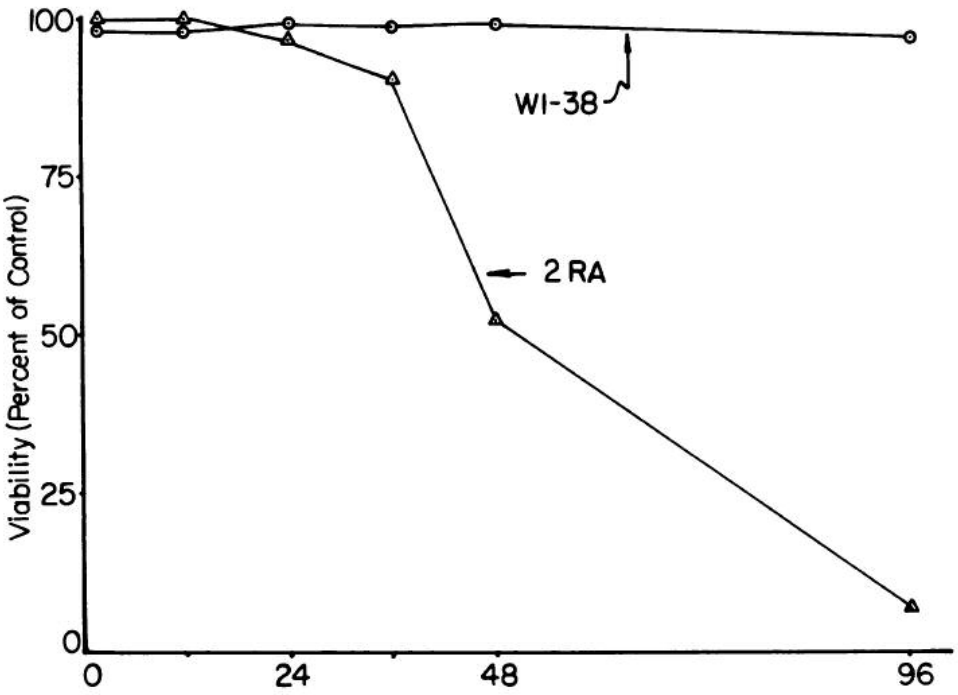

Figure 1.4 Viabiltiy of WI-38 and 2RA cells hours after infection.

In other cell lines, studies have shown that NIH-3T3 fibroblast cells with a functional epidermal growth factor receptor (EGFR) render cells permissive to reovirus infection. On the other hand, cells expressing no EGFR were less susceptible to reovirus infection (11). Subsequent studies have showed that phosphorylation of the double–stranded RNA-activated protein kinase (PKR) blocks translation initiation of viral genes in untransformed NIH-3T3 cells infected with reovirus. PKR phosphorylation inhibits viral protein synthesis because it phosphorylates the eukaryotic protein synthesis initiation factor 2 (elF-2) on it's α subunit. elF-2 α is part of the cellular initiation apparatus and its phosphorylation prevents the exchange of GDP for GTP, and there is inhibition of initiation of translation. However, when these cells were

transformed with SOS (Son of Sevenless) or Ras and then infected with reovirus, PKR is not phosphorylated because Ras or SOS release the block and therefore there is translation initiation of viral genes (10). Additional studies also showed that the inactivation of PKR by 2-aminopurine, or the deletion of the *Pkr* gene in normal cells resulted in viral mRNA translation (10). Figure 1.5 illustrates the role of PKR in translation initiation.

Reovirus lysis of Ras-transformed cells is therefore very important given the fact that more than 30% of all human cancers have activating mutations in the H-, N-, or K-ras genes (12). On the other hand, c-myc amplification has been found in approximately 30% of primary breast cancer cells (26). The c-myc gene, in conjunction with the ras gene, has been shown to enhance oncolytic properties. The Myc protein is prone to rapid degredation by the 26S proteasome and has a half life of approximately 10 minutes (27). The Ras pathway slows down the degradation process and thereby allowing the accumulation of the Myc protein. The constitutive expression of the c-myc oncogene induces a block in differentiation and in the β-globulin gene at the level of transcription (28).

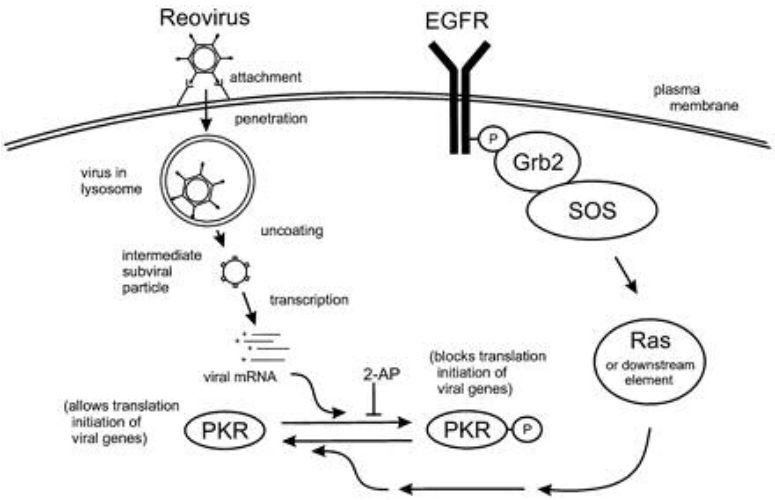

Figure 1.5 Molecular basis of reovirus lysis

Previous experiments had used only reovirus serotype three to examine differential sensitivity. Table 1.3 and figure 1.6 and demonstrate the preferential lysis of transformed cells by reovirus ST3 (unpublished observations**).** This project examines the differential sensitivity L929, WI-38, 2RA, MMEC, T1, N1, and MYC-3 to reovirus serotypes one, two, and three.

Table 1.3 Replication data for ST3

Cell line	Virus produced per cell after 72 hrs	Lysis of >50% of cells
L929 (mouse-fibroblast-chemically transformed)	**1031.18**	+
WI-38 (human-embryonic lung normal)	**1.36**	–
WI-38 VA132RA (human –embryonic lung- SV40 transformed)	**21.85**	+
MMEC (mouse –mammary epithelium –normal)	**1.48**	–
T1 (MMEC –transformed with mutant c-Ha-RAS)	**2.15**	–
N1 (MMEC –transformed with normal c-Ha-RAS)	**32.02**	+
MYC-3 (MMEC –transformed with normal c-myc)	**51.04**	+

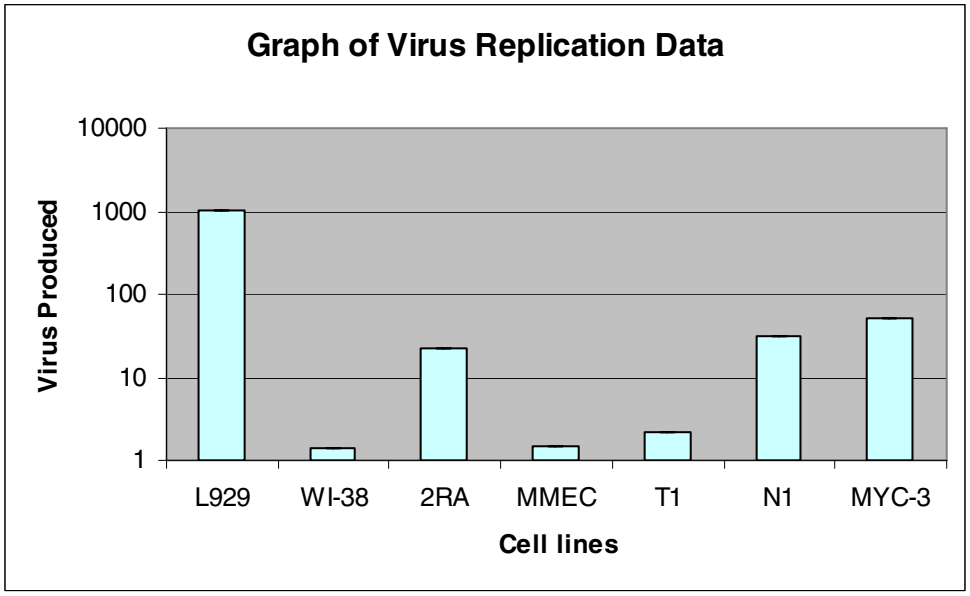

Figure 1.6 Graph of replication data of ST3

1.3 Monoreassortants

Reovirus, like influenza, is unique in that its genome is segmented and can therefore undergo recombination by a process known as reassortment. One of the ways that reassortment can occur is if cells are simultaneously infected with any 2 of the 3 serotypes. The new virus strains or reassortants, are genetic mosaics of the coinfecting strains and individual genome segments can be identified by the electrophoretic mobilities of the dsRNA genome segments during polyacrylamide gel electrophoresis (7). When 2 serotypes of reovirus infect the same cell, the probability of reassortants should be 2^{10} because each serotype contains 10 segments. Researchers that have isolated reassortants between ST1and ST3 have discovered that some reassortants were dominant (occurred in higher numbers) and that most of the segments were derived from ST1 (19,20). They have demonstrated that reassortment between reovirus serotypes is a nonrandom event and that there can be associations of parental alleles that reflect the ratio of reassortants.

Monoreaassortants contain only one genome segment from one parental strain and nine genome segments from the other parental strain. Monoreassortants can be used in phenotypic differences between individual viruses to individual genome segments. Figure 1.7 and 1.8 show the electrophoretic mobilities of the dsRNA genome segments of two sets of reovirus monoreassortants, ST3 with ST2 gene insertions, and ST1 with ST3 gene insertions, following polyacrylamide gel electrophoresis. The designations L1, L2, *et cetera* signify the monoreassortants containing the ST3 L1 genome segments and the ST3 L2 genome segments.

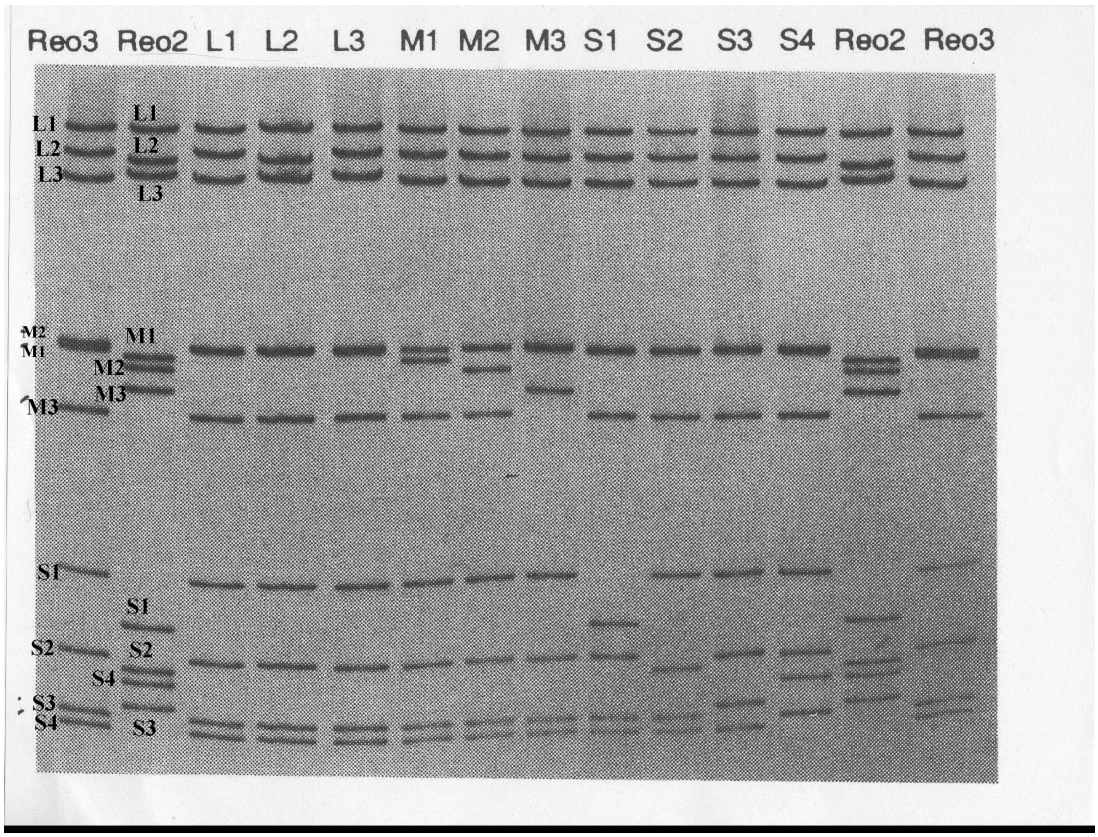

Figure 1.7 Electrophoretic mobilities of dsRNA segments of ST3, ST2, and the monoreassortants

Reo1 Reo3 L1 L2 L3 Reo1 Reo3 M1 M2 M3 S1 S2 S3 S4 Reo1 Reo3

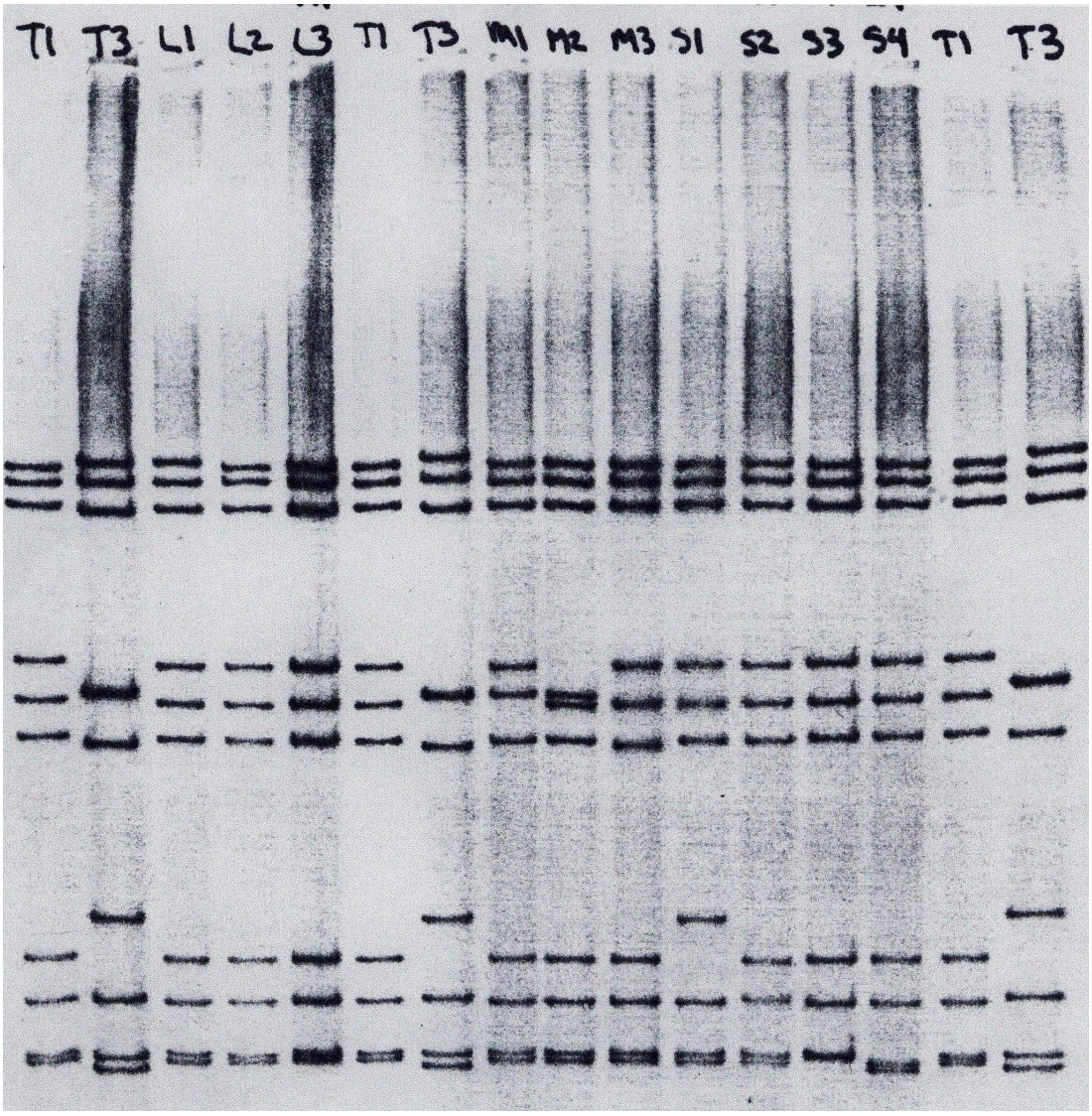

Figure 1.8 Electrophoretic mobilities of dsRNA segments of ST3, ST1, and the monoreassortants

CHAPTER 2

MATERIALS AND METHODS

2.1 Maintaining and Enumeration of Cells

2.1.1 Maintaining of Cells

All seven cells lines were maintained in cell culture flasks to allow them to anchor to a surface. Due to the fact that both transformed and normal cell lines were being used, the cells had different media requirements. L929 cells were maintained in minimal essential media (MEM) with 1% antibiotic containing penicillin and streptomycin (P/S) and 5% Bovine Calf Serum (BCS). The 2 normal cell lines WI-38 and MMEC, were maintained in MEM with 1% P/S and 10% fetal bovine serum (FBS). Since the transformed cells lines have reduced serum requirements, they were maintained in MEM with 1% P/S and 5% FBS. When cell culture was confluent, cells were split in a biological laminar flow hood using aseptic techniques. To split the cells, the media that the cells were growing in was first discarded into a container. The cells were rinsed in 1X saline sodium citrate (SSC). Trypsin is then used to release cells anchored to the bottom of cell culture flasks. The appropriate media is then added to the flasks and pipetted up and down to mix the cells and the media evenly. The dilution ratios for splitting cells were used depending on how soon the cells had to be confluent.

The media in cell culture flasks was refreshed in between splitting of cells to maintain healthy growth of cells.

2.1.2 Enumeration of Cells

A defined number of cells were needed when seeding cells onto the 6- and 24-well plates used for this work. A hemacytometer was used in the enumeration of cells. After cells had been trypsinized and the appropriate amount of media added, a 1 ml pipet was inserted into the media and then loaded onto both grooves of the hemacytometer chamber. Five large squares of one chamber were counted. The following formula was used to determine the number of cells per ml: hemacytometer count $\times 2 \times 10^3$. The following formula was then used to determine the number of cells needed to set up cells at 40% confluency: the required number of cells per well divided by the number of cells per ml.

2.1.3 Freezing of Cells

Freezing of cells was necessary to maintain a stock just in case some cell line got infected with bacteria or fungi during the maintenance of cells. In addition to that, the cell line WI-38 has a passage number of 50 and cannot be split thereafter or else the cells begin to die. To freeze cells, the cell culture was grown in a 75cm^2 flasks and allowed to be confluent. This was enough to give at least twelve 1 ml vials but only 10 vials were frozen. The media in the flasks was decanted and the cells were rinsed with SSC and trypsonized using 2 ml of trypsin. The cells were counted using a hemacytometer as previously described. Cells were then resuspended in a 50% FBS and 50% MEM solution at a concentration of 1×10^6 cells per ml. Dimethyl sulfoxide

(DMSO) was added to a final concentration of 10% DMSO. Careful attention was taken to not shake the cells too hard so as not to damage the cells. One ml was placed into each cyrogenic tube, capped, and placed in cyrogenic storage container. The container was then filled with isopropanol and placed in the -80 ˚C incubator overnight. The vials were then stored in liquid nitrogen.

2.2 Growth of Reovirus Serotype 1, 2, and 3

2.2.1 1st Passage Lysate

Twenty-five cm^2 flasks were seeded with 1 x 10^6 L929 cells with five mls of minimal essential medium (MEM) with 1% antibiotic containing penicillin and streptomycin (P/S) and 5% Bovine Calf Serum (BCS). Flasks were incubated overnight at 37 ˚C, 5% carbon dioxide (CO_2). The flasks of L929 cells were infected at a multiplicity of infection (MOI) of 0.01. Two and a half mls of MEM with 5% BCS and 1% P/S was added to each flask. Flasks were incubated at 37 ˚C, 5% CO_2 until more than 80% of cells were lysed. Cells were then sonicated and lysate was stored at -20 ˚C.

2.2.2 2nd Passage Lysate

Seventy five cm^2 flasks were seeded with 4 x 10^6 L929 cells with ten mls of MEM with 1% P/S and 5% BCS. Flasks were incubated overnight at 37 ˚C, 5% CO_2. The flasks were infected with ½ of 1st passage lysate (1.25 mls) plus two mls of MEM with 5% BCS and 1% P/S. Flasks were then incubated for 1 hour at 37 ˚C, 5% CO_2. After one hour, the volume of each flask was brought to ten mls. Flasks were then incubated at 37 ˚C, 5% CO_2 until more than 80% of cells were lysed. Cells were then sonicated and lysate was stored at -20 ˚C.

2.2.3 3rd Passage Lysate

Spinner flasks of L929 cells with 250 ml of RPMI with 1% P/S and 5% BCS were placed on a stir plate. Smaller flasks with 20 mls MEM with 1% P/S and 2.5% BCS were infected with ½ of 2nd passage lysate (five mls) and incubated for one hour. Contents of the smaller flasks were diluted into larger flasks to one hundred mls total with MEM with 1% P/S and 5% BCS. The infection was allowed to proceed until the viability dropped to 20%. Five mls were then transferred to a smaller tube while ninety five mls remained in a larger container and both were stored at -20 ˚C.

2.3 Plaque Assay to Determine Titer of Virus

Six-well plates were seeded with 5×10^5 L929 cells/ well with two mls MEM with 1% antibiotic P/S and 5% BCS. Plates were incubated overnight at 37 ˚C, 5% CO_2. After twenty four hours, noble agar was melted in the microwave and placed in the 42˚C incubator. The lysate of each serotype was sonicated to release virus trapped in cells. 10^{-1} to 10^{-6} dilutions of 3rd passage lysate were prepared. Dilution blanks contained nine hundred µl of MEM with 1% of P/S with no serum while one hundred µl of each lysate was used as the sample. Media in the 6-well plates incubated overnight was discarded by the use of a vacuum pump attached to a sterile pipet at one end to ensure sterility. Two hundred and fifty µl of each virus dilution was added in duplicate to the corresponding wells. The 6-well plates were rocked back and forth every 15 minutes while incubating (to allow virus to adsorb) for one hour at 37 ˚C, 5% CO_2. During this time, 2X MEM with 2% antibiotic P/S and 5% BCS was incubated for not more than 40 minutes before the addition of overlay agar. After incubation for an hour,

an equal amount of 2X MEM was mixed with 2% noble agar plus 1% fungicide. Two mls of overlay was gently added to each well and the agar was allowed to solidify before incubating at 37 ˚C, 5% CO_2. Plaque assays for ST1 and ST3 take five days to read and therefore overlay was again added on the third day. On the fourth day, 2% neutral red was mixed with the overlay agar before overlay was added onto the wells. Plaque assays for ST2 take ten days to read. However, they can also be read on the 9^{th} day. Overlay was therefore added on day 3, day 5, day 7, and day 8. On the eighth, 2% neutral red was added. Neutral red was added in the dark to prevent light from reacting with cells that had adsorbed neutral red and thereby causing cell death due to free radicals. The number of plaques on each well and its corresponding duplicate well were counted. The mean of the two wells for each dilution was determined. The following formula was used to determine the number of plaque forming units per milliliter (pfu/ml): plaque number × reciprocal dilution × reciprocal volume in ml.

2.4 Differential Sensitivity of Cell Lines

After determining the titer of each serotype, then L929, WI-38, 2RA, MMEC, T1, N1, and MYC-3 cells were used to seed 6-well plates. The plates were seeded with 5×10^5 cells/ well with two mls MEM with 1% antibiotic P/S and 5% FBS. Plates were incubated overnight at 37 ˚C, 5% CO_2. 1×10^6 cells per well were infected with each reovirus serotype at a multiplicity of infection (MOI) of 0.1. Media in the 6-well plates incubated overnight was discarded by the use of a vacuum pump attached to a sterile pipette at one end to ensure sterility. Virus was added in 250 µl of MEM with 1% antibiotic. The 6-well plates were rocked back and forth every fifteen minutes while

incubating (to allow virus to adsorb) for one hour at 37 ˚C, 5% CO_2. Seven hundred-fifty µl MEM with 1% antibiotic and 5% BCS was added after one hour and the plates were incubated at 37 ˚C, 5% CO_2.

2.4.1 Viability Test

After 72 hours of incubation, the media was sucked out of the wells using a pipette attached to a vacuum pressure pump. One ml of dye consisting of 0.1 ml of trypan blue and 0.9 ml of MEM with 1% P/S, was added to each well. The plates were then incubated for 5 minutes at 37 ˚C, 5% CO_2. The live cells excluded the dye while the dead cells adsorbed the dye. To determine viability, the ratio of dead to live cells was determined by counting the dead cells under a microscope. A total of 500 cells were counted in duplicate wells.

2.4.2 Harvesting of Cells

After seventy two hours, cells were harvested by scraping the bottom of the wells with the aid of a cell scrapper. The 1 ml volume was placed in a microcentrifuge tube and stored at -20 ˚C until used.

2.4.3 Plaque Assay to Determine Virus Yield

The 1 ml microcentrifuge tube that contained the harvested cells was sonicated and a plaque assay was done to determine the amount of virus produced per cell. It took five days to read plaques for ST1 and ST3 but nine days to read plaques for ST2. The procedure for performing a plaque assay has already been described in detail in section 2.2.

2.5 Monoreassortants

The results of the infection of the seven cells lines with reovirus ST1, ST2, and ST3 were examined. Cell lines that exhibited differential sensitivity to the different serotypes were selected for further testing to map the specific segment(s) responsible for the difference in lysis or persistence. The monoreassortants chosen were the ones that had nine ST1 genes and one ST3 gene.

2.5.1 1st Passage Lysate of Monoreassortants

Ten 25cm^2 flasks were seeded with 1 x 10^6 L929 cells with five mls of MEM with 1% P/S and 5% BCS. Flasks were incubated overnight at 37 ˚C, 5% CO_2. The L929 cells were then infected with one hundred µl of selected monreassortants. Five mls of MEM with 5% BCS and 1% P/S was then added to each flask. Flasks were incubated at 37 ˚C, 5% CO_2 until more than 80% of cells were lysed. Cells were then sonicated and lysate was stored at -20 ˚C.

2.5.2 2nd Passage of Monoreassortants

Ten 75cm^2 flasks were seeded with 4 x 10^6 L929 cells with 5mls of MEM with 1% P/S and 5% BCS. Flasks were incubated overnight at 37 ˚C, 5% CO_2. The 75cm^2 flasks of L929 cells were then infected with 500 µl of 1st passage lysate. Flasks were incubated for an hour and 7.5 mls of MEM with 5% BCS and 1% P/S was then added to each flask. Flasks were incubated at 37 ˚C, 5% CO_2 until more than 80% of cells were lysed. Cells were then sonicated and lysate was stored at -20 ˚C.

2.6 Plaque Assay to Determine Titer of Monoreassortants

The procedure for performing a plaque assay has already been described in detail in section 2.2. Since the monoreassortants selected were from ST1 and ST3, the plaques assay only took five days to read.

2.7 Differential Sensitivity of Monoreassortants

Once the titer of the monoreassortants had been determined L929, MMEC, and T1 cells were used to seed 24-well plates. The plates were seeded with 1×10^5 cells/ well with 1 mls MEM with 1% antibiotic P/S and 5% BCS. Plates were incubated overnight at 37 ˚C, 5% CO_2. Media in the six-well plates incubated overnight was discarded by the use of a vacuum pump attached to a sterile pipet at one end to ensure sterility. 3×10^5 cells per well were infected with the 10 monoreassortants, ST1and ST3 at a multiplicity of infection (MOI) of 0.1. Virus was added in two hundred-fifty µl of MEM with 1% antibiotic and incubated for 1 hour at 37 ˚C, 5% CO_2. Seven hundred-fifty µl MEM with 1% antibiotic and 5% BCS was added after one hour and the plates were incubated at 37 ˚C, 5% CO_2.

2.7.1 Viability Test

After 72 hours of incubation, the media was sucked out of the wells using a pipette attached to a vacuum pressure pump. One ml of dye consisting of 0.1 ml of trypan blue and 0.9 ml of MEM with 1% P/S, was added to each well. The plates were then incubated for five minutes at 37 ˚C, 5% CO_2. The live cells excluded the dye while the dead cells adsorbed the dye. To determine viability, the ratio of dead to live

cells was determined by counting the cells under a microscope. A total of five hundred cells were counted in duplicate wells.

2.7.2 Harvesting of Cells

After 72 hours, cells were harvested by scraping the bottom of the wells with the aid of a cell scrapper. The 1 ml volume was placed in a microfuge tube and stored at -20 ˚C until used.

2.7.3 Plaque Assay to Determine Virus Yield

The microcentrifuge tube containing the 1 the harvested cells of L929, MMEC, and T1 was sonicated and a plaque assay was done to determine the amount of virus produced per cell. It took five days to be able to read the plaques. The protocol for performing a plaque assay has been described in detail in section 2.2.

CHAPTER 3

RESULTS

3.1 Titer of ST1, ST2, and ST3

After the 3rd passage lysate, plaque assays for ST1, ST2, and ST3. Table 3.1 shows plaque forming units per milliliter (pfu/ml) for each serotype.

Table 3.1 Titer of ST1, ST2 and ST3

Reovirus Serotype	Titer
ST1	7.4×10^5 pfu/ml
ST2	2.0×10^6 pfu/ml
ST3	2.0×10^8 pfu/ml

3.2 Differential Sensitivity of Cells to Reovirus Infection

Once the titer of each serotype was determined, L929, WI-38, 2RA, MMEC, T1, N1, and MYC-3 cells were seeded onto 6-well plates and infected at 1×10^6 cells/well with all serotypes at an MOI of 0.1. The following calculation was used to determine the amount of virus to add to each cell line. The number of cells to be infected was multiplied by the MOI then divided by the titer of each serotype. For ST1, one hundred-thirty five µl were added to each well while for ST2 fifty µl were added to

each well. A 1:100 dilution was necessary before the addition of fifty µl of ST3 to each well.

3.2.1 Viability test and Virus Yield of ST1, ST2, and ST3

The following are tables showing the amount of virus produced per cell and the viability of each cell line after 72 hours.

Table 3.2 Reovirus ST3

Cell line	**Virus produced per cell after 72 hrs**	**Lysis of >50% of cells**
L929 (mouse-fibroblast-chemically transformed)	**2060**	**+**
WI-38 (human-embryonic lung normal)	**0.546**	**–**
WI-38 VA132RA (human –embryonic lung-SV40 transformed)	**6.88**	**+**
MMEC (mouse –mammary epithelium – normal)	**59.4**	**+**
T1 (MMEC –transformed with mutant c-Ha-RAS)	**0.074**	**–**
N1 (MMEC –transformed with normal c-Ha-RAS)	**1.24**	**+**
MYC-3 (MMEC –transformed with normal c-myc)	**0.134**	**+**

Table 3.3 Reovirus ST2

Cell line	Virus produced per cell after 72 hrs	Lysis of >50% of cells
L929 (mouse-fibroblast-chemically transformed)	**1.66**	**–**
WI-38 (human-embryonic lung normal)	**0.005**	**–**
WI-38 VA132RA (human –embryonic lung-SV40 transformed)	**0.098**	**+**
MMEC (mouse –mammary epithelium – normal)	**0.0056**	**+**
T1 (MMEC –transformed with mutant c-Ha-RAS)	**0.0196**	**+**
N1 (MMEC –transformed with normal c-Ha-RAS)	**0.3**	**+**
MYC-3 (MMEC –transformed with normal c-myc)	**0.0454**	**+**

Table 3.4 Reovirus ST1

Cell line	Virus produced per cell between after 72 hrs	Lysis of >50% of cells
L929 (mouse-fibroblast-chemically transformed)	**17.6**	**+**
WI-38 (human-embryonic lung normal)	**0.148**	**–**
WI-38 VA132RA (human –embryonic lung-SV40 transformed)	**2.52**	**+**
MMEC (mouse –mammary epithelium – normal)	**2.4**	**+**
T1 (MMEC –transformed with mutant c-Ha-RAS)	**0.112**	**+**
N1 (MMEC –transformed with normal c-Ha-RAS)	**0.8**	**+**
MYC-3 (MMEC –transformed with normal c-myc)	**0.502**	**+**

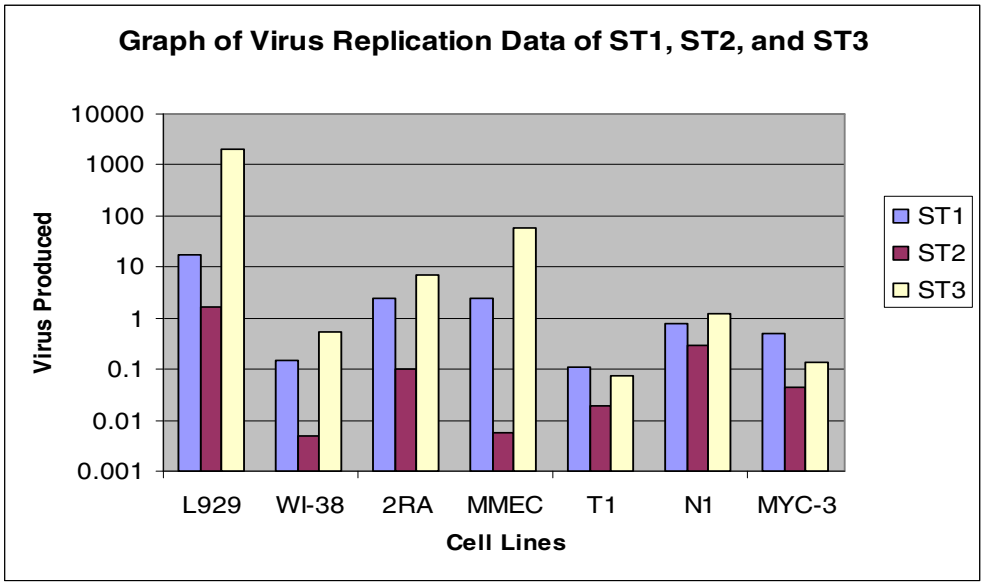

Figure 3.1 Graph of replication data of ST1 and ST3

3.3 Titer of Monoreassortants

Table 3.5 Titer of monoreassortants

ST3 SEGMENT IN ST1 VIRUS	PFU/ML
L1	2.44×10^7
L2	2.42×10^6
L3	1.44×10^5
M1	3.28×10^5
M2	1.52×10^6
M3	6.6×10^6
S1	3.62×10^6
S2	4.14×10^6
S3	1.68×10^5
S4	1.02×10^8

3.4 Differential Sensitivity of Cells to Monoreassortants

24-well plates were seeded with L929, MMEC, and T1 cells and infected at 3×10^5 cells/well with 10 monoreassortants, ST1, and ST3 at an MOI of 0.1. The following calculation was used to determine the amount of virus to add to each well. The number of cells to be infected was multiplied by the MOI then divided by the titer of the monoreassortant or serotype. Dilution of samples was necessary when the amount to be added was not feasible.

Table 3.6 Infected L929 cells with all 10 reassortants, ST1 and ST3.

ST3 SEGMENT IN ST1 VIRUS	Virus produced per cell between after 48 hrs	Lysis of >50% of cells (48hrs)
L1	22	+
L2	1	+
L3	0.86	+
M1	2.18	+
M2	11	+
M3	0.088	-
S1	0.094	-
S2	6	+
S3	1.18	+
S4	5.94	+
ST1 wildtype virus	0.156	+
ST3 wildtype virus	0.24	+

Table 3.7 Infected T1 cells with all 10 reassortants, ST1 and ST3.

ST3 SEGMENT IN ST1 VIRUS	Virus produced per cell between after 48 hrs	Lysis of >50% of cells (48hrs)
L1	1.6	+
L2	0.492	+
L3	0.144	+
M1	0.502	+
M2	0.226	+
M3	0.128	+
S1	0.033	-
S2	0.6	+
S3	0.0276	-
S4	0.0322	-

Table 3.8 Infected MMEC cells with reassortants, ST1 and ST3.

ST3 SEGMENT IN ST1 VIRUS	Virus produced per cell between after 48 hrs	Lysis of >50% of cells (48hrs)
L1	14.2	+
L2	2.28	+
L3	0.314	+
M1	1.38	+
M2	1.36	+
M3	0.0336	-
S1	0.0394	-
S2	5.2	+
S3	0.352	+
S4	1.44	+

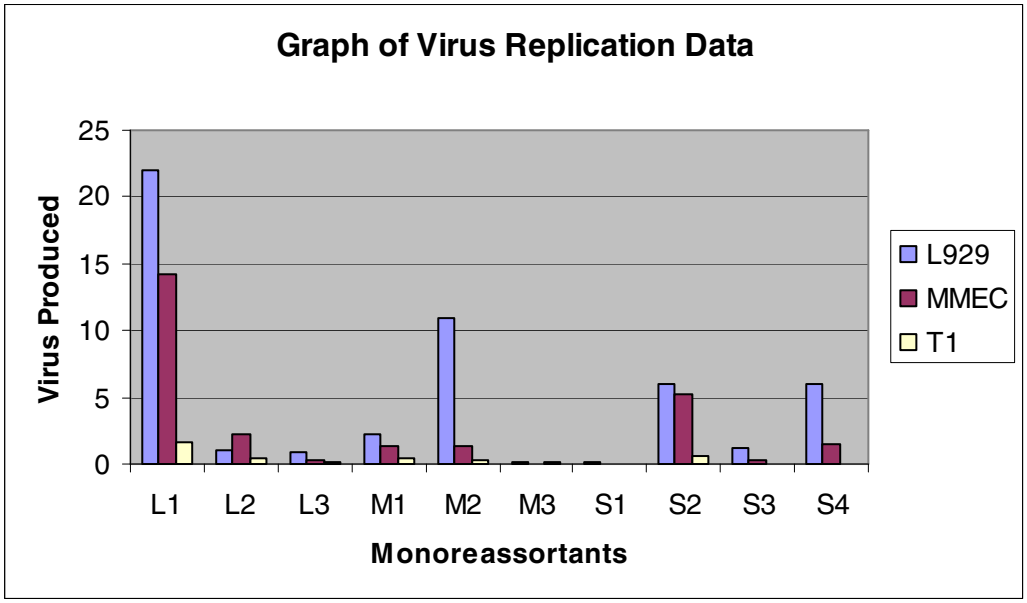

Figure 3.2 Graph of replication data for monoreassortants

CHAPTER 4

DISCUSSION AND CONCLUSION

Reovirus serotypes, ST1, ST2, and ST3, were used to infect normal or transformed cells. The transformed cell line, L929 produced the greatest amount of virus per cell when infected with ST1, ST2, or ST3. This was to be expected since L929 cells are transformed and have been the cell line of choice to grow reovirus for more than 30 years. Growth in the normal WI-38 cells was compared with its transformed counterpart 2RA cells, and the results confirmed the hypothesis that all 3 serotypes should produce more virus in the transformed 2RA cells than in normal WI-38 cells. When virus replication in the normal MMEC cells was compared to transformed T1, N1, and MYC-3 cells, all 3 serotypes produced more virus in the MMEC cells than in any of its transformed counterparts. This was unexpected and may be due to the fact that the particular MMEC stock that was used in these experiments replicated at a very fast rate compared to the transformed cell lines, most transformed cell lines replicate faster then their normal cell counterparts. When the three MMEC-derived transformed cells were compared, N1 always produced more virus than T1 and MYC-3. The N1 cells were transformed with normal c-Ha-Ras and supported more virus production per cell then the T1 cells, which were transformed with a mutant c-Ha-Ras. Reovirus replication appears to be regulated by the specific molecular mechanism

driving transformation of the virus-infected cell line. The virus production per cell in MYC-3 was always higher than T1 but lower than N1.

ST2 produced very little virus per cell when compared with the other two serotypes. This may be because ST2 takes a longer time to replicate and lyse the cells compared to ST1 and ST3. When doing plaque assays, it took up to ten days in order to be able to visualize the ST2 plaques. ST2 virus was therefore not used to study the effects of the use of monoreassortants to define reovirus lysis of transformed cells because results would have been problematic. However, in the future, ST2 lysis of transformed cells and the use of monoreassortants with ST2 genes to define reovirus lysis should be examined.

In order to identify a gene or genes involved in the differential lysis of normal and transformed cells by reovirus using reassortant viruses it was initially required that two reovirus serotypes be found that exhibited differential lysis of a target transformed and/or normal cell. For reovirus ST1 and ST3 such a difference was found upon infection of the MMEC-cells transformed with a mutant c-Ha-RAS, the T1 cell line. Upon infection of the T1 cells by reovirus ST3, the cells produced virus but were not lysed. In sharp contrast, infection of the T1 cells with ST1 virus resulted in virus production and lysis of the cells. This difference between the wildtype ST1 and wildtype ST3 viruses provided the phenotypic difference that we could exploit using the monoreassortants viruses.

The monoreassortants with nine ST1 genes and one ST3 gene were used to examine the differential oncolysis displayed by reovirus. The monoreassortants were

used to infect L929, MMEC, and T1 cells. The L929 cells were used as the control while MMEC was used because it was the normal counterpart, and the T1 cells the differential cell line. The genes that displayed differential sensitivity are the S3 and the S4 gene.

Studies using the temperature sensitive (ts) mutant tsG453 mapped the ts lesion to the S4 gene. S4 gene encodes the protein σ3, which is composed of three hundred sixty-five amino acids and has six hundred copies in the virion (6). Genetic studies have demonstrated that the σ3 protein stabilizes viral particles due to inactivation by heat and SDS and for this reason, it plays an important role in determining the survival of virions outside the cell (20). When L929 cells were infected with either ST2 or ST3, it was discovered that ST2 inhibited protein and RNA synthesis much more rapidly than ST3. Sharpe and colleagues used reassortants derived from both ST2 and ST3 to map the gene responsible for differential lysis to the S4 gene (33). The treatment of cells with interferon leads to the induction of PKR. However PKR requires dsRNA to become activated. As previously described in the introduction, nontransformed cells infected with reovirus have PKR that is phosphorylated and so there is inhibition of initiation of viral genes. Imani and colleagues found that the σ3 protein is responsible for the inhibition of initiation of translation (34). The σ3 protein inhibits translation by binding dsRNA and hence blocking the activation of the dsRNA activated protein kinase, PKR. Mutations in the S4 gene are vital in the establishment of persistent infection. The σ3 protein is located in the outer capsid and has to be degraded proteolytically before viral transcription occurs. At the restrictive temperature, viruses

with ts lesions that map to the S4 gene do not assemble outer capsids. This shows that σ3 is important in the assembly of the virus during virus replication (35).

Ramig *et al* performed studies using the temperature sensitive mutant (tsE320) and mapped that mutation to the S3 gene encoding the segment σNS (30). The gene S3 encodes the protein σNS and consists of three hundred-sixty six amino acids and has a molecular mass of 41kDA (23). Whereas tsE320 (the mutant lacking σNS) is unable to synthesize dsRNA at restrictive temperatures, σNS protein binds to ssRNA (the viral plus-strand RNAs) with high affinitiy (29, 31). In the analysis of cellular extracts during early infection, when σNS is assembled into 13-19S particles, it is the only protein bound to selected regions of viral mRNA (29, 32). This protein also has the capacity to bind RNA-DNA hybrids and gradually displaces the DNA (23). This σNS activity is very similar to some DNA binding proteins that are known to be involved in the replication of DNA. The premise therefore is that σNS has a role in the replication and packaging of plus strand RNAs of reovirus.

The idea that the S3 and S4 genes are involved in the differential lysis of T1 cells is not unexpected. Given that reovirus serotype 3 did not lyse T1 cells, the S4 gene would have been a likely culprit since it is involved in the establishment of persistent infections. In addition to the S4 gene, the S3 gene would also be involved in ST3 not lysing T1 cells. The S3 gene encodes σNS protein, which is responsible for the conversion of ssRNA to dsRNA. Therefore if the S3 gene does not function properly, there is reduced virus production. This is a possible reason why ST3 did not lyse T1 cells.

Results were problematic for the M3 and S1 gene even though there was a differential sensitivity to ST1 and ST3. The reason being that the reassortants have consistently been shown to act like their parents and lyse the transformed L929 cells after 48 hours of infection. This conflicts with data for this work that shows that the M3 and S1 gene were not lysed by ST1 reassortants containing the M3 or S1 ST3 gene segments. The virus yields for these two monoreassortants is also very low compared to the remaining 8 reassortant viruses. We will explore this finding in greater detail to determine if these genes, the M3 and the S1 are also involved in reovirus oncolysis or if these monoreassortants are unique in their interaction/replication in L929 cells.

This project has advanced the search for the reovirus gene(s) responsible for oncolysis. It has identified at least two genes involved in the lysis of transformed cells. Future studies will involve using the ST3/ST2 monoreassortant viruses and additional normal and transformed cell lines as well as targeted mutagenesis to mutant the individual reovirus genes identified to play a role in reovirus oncolysis.

Table 4.1 Summary table

Virus	Cell Line		
	L929	MMEC	T1
ST3	+	+	(-)
ST1	+	+	+
ST1/ST3 L1	+	+	+
ST1/ST3 L2	+	+	+
ST1/ST3 L3	+	+	+
ST1/ST3 M1	+	+	+
ST1/ST3 M2	+	+	+
ST1/ST3 M3	(-)	(-)	+
ST1/ST3 S1	(-)	(-)	(-)
ST1/ST3 S2	+	+	+
ST1/ST3 S3	+	+	(-)
ST1/ST3 S4	+	+	(-)

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BIOGRAPHICAL INFORMATION

Christine Mutsoli received her undergraduate degree in Biology from Texas Woman's University. Prior to graduation, she had the privilege of interning for three months as a microbiologist in a radiopharmaceutical company that manufactured cancer drugs. She mainly tested water for pharmaceutical use. After graduation, she wanted to take a break from school and worked in various odd jobs. She later found a position as a microbiologist at a pharmaceutical company in Grand Prairie.

Christine has always been interested in virology from the first she ever took a virology class. A lot of human suffering could have been avoided if more people understood the pathogenesis of viruses like HIV, influenza, ebola, and many other pathogenic viruses. After she graduates with her master's degree, she hopes to work as a researcher in the biotechnology industry or in a research lab. She then plans on going back to school and getting her PhD. Her ultimate goal is to someday work with HIV both in the lab and with HIV infected people.