uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Dion Shah
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Microbiology and Immunology)
GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Cell Tropism of a Mumps Urabe Virus

TITRE DE LA THÈSE / TITLE OF THESIS

Dr. Kathryn Wright
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. John Bell

Dr. John Pezacki

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

CELL TROPISM OF A MUMPS URABE VIRUS

by

Dion Shah

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Biochemistry, Microbiology and Immunology

The University of Ottawa

© Dion Shah, Ottawa, Canada, 2008

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-48505-7
Our file Notre référence
ISBN: 978-0-494-48505-7

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Abstract

Mumps virus is member of the paramyxovirus family of viruses. The Urabe strain of mumps was attenuated to produce a vaccine that was introduced in several countries, but withdrawn from the market when it was discovered to be insufficiently attenuated. Two vaccine-associated Urabe viruses have been shown to have different tropisms in tissue culture. Gw7, isolated directly from the vaccine and which has been shown to be attenuated in an animal model, shows limited growth in human lung epithelial cells (A549) compared to monkey kidney cell line (Vero). 1004 10/2 is a virus isolated from a patient with post-vaccination meningitis caused by the Urabe vaccine and this virus grows to high titres in both A549 and Vero cells. A limited number of genetic differences between Gw7 and 1004 10/2 have been identified that must account for the growth differences in tissue culture. Using a combination of functional assays and genetic analysis of other Urabe viruses with growth patterns similar to Gw7 or 1004 10/2, it was demonstrated that the failure of Gw7 to replicate well in A549 cells is not due to differences in the sensitivity to interferon, differences in receptor binding, the M protein, or the SH protein. The results implicated the L protein. This was further supported by the observations of reduced protein synthesis and RNA synthesis in A549 cells compared to Vero cells. This suggested that genetic differences between the viruses caused differences in the function of the L protein during transcription of mRNA, which resulted in the observed tropisms.

Table of Contents

Abstract....i

Table of Contents.... ii

Tables....v

Acknowledgements vi

Chapter One....1
- Mumps History....2
- Classification....4
- Structure 5
- Replication Cycle of Mumps Virus....9
- Pathogenesis of Mumps 18
- Mumps Vaccines....20
- Urabe Am9 26

Hypothesis 35

Objectives 35

Chapter Two 36
- Viruses:....37
- Plaque Assay: 38
- Hemagglutination Assay (HA):....38
- RNA Isolation: 38
- Reverse Transcription (RT) and Polymerase Chain Reaction (PCR): 39
- Plasmid DNA preparation:....41
- Agarose Gel Electrophoreses: 41
- Quantification of Viral RNA:....42
- Immunoprecipitation:....42
- Western Blot:....44
- SDS-PAGE Analysis:....45
- Transfection:....45
- Heme Binding Assay:....46
- β-Galactosidase Assay: 47
- Binding Assay: 47
- Interferon treatment of Vero cells: 48
- Interferon secretion: 49
- Neutralization of Interferon β: 49

Chapter Three 51
- Interferon....58

Binding 70
Viral Protein Synthesis 84

Chapter Four 96

References 113

Figures

Figure 1a and b: Mumps Virion and Mumps Virus genome. 12

Figure 2: Mumps Replication Cycle..14

Figure 3: Kinetics of replication of Gw7 in Vero and A549 cells....................................55

Figure 4: Receptor Binding Assay Comparing Gw7 HN to 1004 10/2 HN. 76

Figure 5: Effects of differeing concentrations of RBCs on receptor binding by Gw7 and 1004 10/2 HN proteins..78

Figure 6a and b: Immunoprecipitation of Gw7 protein expression in infected Vero and A549 cells. 88

Figure 7a and b: Western Blot of Gw7 protein expression in Vero and A549 cells. 90

Tables

Table 1: Mumps vaccine in use. 25

Table 2: Non-Coding Sequence Differences Between Gw7 and 1004 10/2. 31

Table 3: Coding Sequence Differences Between Gw7 and 1004 10/2....32

Table 4: Replication of Urabe Viruses in Human Cell Lines....53

Table 5: Hemagglutinating Gw7 Released from Vero and A549 cells 57

Table 6: Induction of Antiviral State in Vero cells by IFNβ....61

Table 7: Effects of IFNβ on titres of Gw7 and 1004 10/2 in Vero cells 63

Table 8: Secreted IFN by A549 Cells when infected by Gw7 and 1004 10/2 65

Table 9: Titration of Anti-IFNβ antibody using 100 and 20 U/ml of IFNβ in A549 cells68

Table 10: Titres of Urabe Mumps Virus in A549 cells in the Presence of a Neutralizing anti-IFNβ antibody 69

Table 11: Cell Associated Gw7 1004 10/2 After Binding to A549 and Vero cells 73

Table 12: Replication of Additional Urabe Viruses in A549 cells....82

Table 13: Tritiated Uridine Incorporated into Viral RNA in Vero and A549 cells....95

Acknowledgements

I would like to thank my supervisor, Dr Kathryn E. Wright, for her supervision and guidance throughout this project.

I would like to also thank Dr Ken Dimock and Dr David Stojdl for their valuable advice during my Thesis Advisory Committee meetings.

I would also like to give a special thanks to the present and past students in the Wright, Dimock, and Brown labs for their friendship.

This thesis is dedicated to the memory of my grandmother

Chapter One

Introduction

Mumps History

The earliest record of mumps or *Myxovirus Parotitidis*, named for the classical disease it causes, can be traced to a 5th century epidemic where Hippocrates observed a disease associated with swelling near the ears and of one or both testes in young males (Lamb and Kolakofsky 2001; Andrewes *et al.*, 1955). During the period of 1714 to 1859, epidemic records revealed that mumps was prevalent from Iceland to Egypt and from Alaska to Polynesia. As the industrial revolution began and areas became more populated, mumps spread rapidly, and was predominant in overcrowded areas such as prisons, orphanages, boarding schools, garrisons, and ships (Gorden and Kilham 1949). In the early 20th century, the average annual incidence of mumps in Europe was observed to be 300 per 100 000 individuals, although a greater incidence was recorded in military populations (6000 per 100 000) and in some communities in the United States (2000 per 100 000, Hillsborough County, Florida) (Gorden and Kilham 1949; Levitt *et al.*, 1970; Plotkin 2004). In unvaccinated populations, cases increased during spring/winter months with epidemic outbreaks occurring every three or seven years (Galazka *et al.*, 1999; Gorden and Kilham 1949).

Routine vaccination against mumps was introduced in North American and Europe during the 1970s and 1980s. At that time, the first licensed mumps vaccines were Jeryl Lynn and Urabe AM9, and since the introduction of these vaccines, the incidence of mumps disease has decreased (Peltola *et al.*, 2007; Galazka *et al.*, 1999). In the United States, a 99% decrease in mumps was seen from 152 209 cases reported in 1968 to 666 cases reported in 1998 (Galazka *et al.*, 1999). Scandinavian countries, which showed an average incidence of mumps of 439 cases per 100 000 individuals

during 1977-79, also experienced a 99% reduction in the incidence of mumps (3.5 per 100 000) by 1993-95 (Galazka *et al.*, 1999), as did England and Wales where the number of cases went from 20 713 reported cases to 4277 within 3 years of introducing the mumps vaccine (Balraj and Miller 1995).

Although the vaccines have proven to be very effective in decreasing the overall incidence of mumps, vaccination with mumps is not common in many countries, and mumps outbreaks have been recently reported in several countries where vaccination has been routine for many years. In Canada, 711 cases of mumps were confirmed during 2007 with 95% of the cases reported in Nova Scotia and New Brunswick (http://www.phac-aspc.gc.ca/mumps-oreillons/prof-eng.php). In the United States during 2006, 5783 cases were reported with the majority of cases found in Iowa (Reef *et al.*, 2006). It was found in Iowa that 49% of individuals had received two doses of the vaccine while 14% of individuals had received one dose (Reef *et al.*, 2006). Hence, many of the accounted cases can be attributed to some sort of vaccine failure. Either the vaccine simply failed to induce an adequate initial immune response or the immunity originally induced by the vaccine wanes with time. Due to the appearance of these types of cases, investigators have begun to question the efficacy (i.e., the ability to prevent disease) of many of the licensed live attenuated vaccines.

Another concern with vaccination with live attenuated vaccines is insufficient attenuation. For mumps vaccines, many complications such as parotitis, unilateral or bilateral deafness, diabetes mellitus, and specifically aseptic meningitis have been indications of an insufficiently attenuated vaccine (Balraj and Miller 1995). Vaccines should be continually screened to investigate the ability to prevent disease as well as

the potential to cause disease in the brain. However, the screening of the mumps virus has proven difficult to achieve since there is a lack of reliable markers to distinguish virulence, attenuation, and the level of neurovirulence.

Classification

The mumps virus is a member of the family *Paramyxoviridae*, which is a family of vertebrate viruses of the order Mononegavirales. The family is divided into two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae*, based on nucleocapsid morphology, coding potential of some genes, and the number of expressed proteins (Lamb and Kolakofsky 2001; ICTVdB - The Universal Virus Database). Likewise, the two subfamilies are divided into genera that are categorized according to size, organization of the genome, and on the similarity of encoded proteins. The subfamily *Pneumovirinae* contains two genera, Pneumovirus and Metapneumovirus, which are distinguished from one another by the number of transcriptional elements within the virus. The genus Pneumovirus, typified by respiratory syncytial virus (RSV), contains viruses with ten transcriptional elements, while the genus Metapneumovirus contains viruses such as avian pneumovirus, with eight transcriptional elements.

The *Paramyxovirinae* subfamily contains five genera that include Respirovirus, Morbillivirus, Henipavirus, Avaluvirus and Rubulavirus (ICTVdB - The Universal Virus Database; Lamb and Kolakofsky 2001). The genera in this subfamily are classified based on the expression of various non structural proteins (NS). Sendai virus (SeV), Measles virus (MV), and Hendra virus characterize the Respirovirus,

Morbillivirus, and Henipavirus genera, respectively. These viruses express the NS proteins C and V, and each genus is further distinguished based on the physical and enzyme properties of the attachment protein (Lamb and Kolakofsky 2001). Sendai virus expresses a protein (HN) with hemagglutinin and neuraminidase activity, the Measles virus attachment protein (H) lacks neuraminidase activity but has hemagglutinin activity, and the Hendra virus attachment protein (G) lacks both hemagglutination and neuraminidase activities (Lamb and Kolakofsky 2001). The other two genera in the Paramyxovirinae subfamily, Rubulavirus and Avulavirus, contain viruses that express a V protein, but no C protein, and encode HN. These genera are differentiated from each other on the basis that Avulaviruses, such as Newcastle disease virus (NDV), affect many avian species (ICTVdB - The Universal Virus Database). The mumps virus falls within the Rubulavirus genus, along with the prototype virus for this genus, parainfluenza virus 5 (PIV5), formerly known as simian virus 5 (SV5). For the mumps virus, the term strain refers to individual mumps viruses, which are generally named after the person or the location where the virus was first isolated.

Structure

Mumps viruses, like all *Paramyxoviridae,* contain a single stranded non-segmented negative sense RNA genome, although on occasion virions contain a positive sense single-stranded copy. The mumps virion is enveloped and spherical, with a diameter of 150 to 350 nm (Figure. 1a) (Lamb and Kolakofsky 2001). The

envelope is host derived and embedded with two integral membrane glycoproteins that span the membrane once: a type I C-terminal anchored fusion glycoprotein (F) and the hemagglutinin-neuraminidase (HN), a type II N-terminal anchored glycoprotein.

The HN protein is an oligomeric tetramer with a globular region supported by a long slender stalk region (Thompson 1988; Lamb and Kolakofsky 2001). The globular region of HN binds sialic acid molecules, can mediate hemagglutination, cleaves sialic acid by neuraminidase (NA) activity, and is the region recognized by the immune system; while the stalk region is involved in promotion of fusion activity (Thompson 1988; Villar 2006). Studies have shown that the mumps virus hemagglutination and neuraminidase activities are mapped to different sites on the HN protein, and fusion, mediated by the F protein, is dependent on interactions with the HN (Waxham and Aroncjwski 1988; Tanabayashi *et al.*, 1992).

The paramyxovirus fusion protein is a homotrimer that mediates viral to cellular membrane fusion at neutral pH after HN binding to sialic acid (Russell *et al.*, 1994). The fusion protein is translated as a non-active precursor protein F_0 that undergoes cellular proteolytic cleavage to become a biologically active peptide consisting of two disulfide linked subunits, F_1F_2 (Homma and Tamagawa 1973; Scheid and Choppin 1974). The F_1 portion contains the fusion peptide, a hydrophobic stretch of amino acids, which mediates viral fusion once inserted into the host membrane (Homma and Tamagawa 1973; Scheid and Choppin 1974).

Underneath the envelope, the virion is held together by the Matrix protein (M), which is the smallest of the structural proteins in the virion (Lamb and Kolakofsky 2001). M is complexed to the cytoplasmic portion of both HN and F, and to the NP

component of the ribonucleoprotein (RNP). At the center of the virion is the RNP, which is composed of the genomic viral RNA encapsidated by NP. Electron microscopy of the SeV RNP has suggested that a single NP monomer is bound to every six nucleotides of the genome (Egelman *et al.*, 1989). Subsequently, experiments with PIV5 and SeV have shown that genomes replicated more efficiently when constructed as multiple of six (Murphy and Parks 1997, Calain and Roux 1993), and Clarke et al (2000) showed replication of a mumps genome constructed as a multiple of six. Other proteins associated with the RNP are the Phosphoprotein (P) and the Large protein (L), which is the viral polymerase (Lamb and Kolakofsky, 2001). The mumps virus genome is 15200-15900 nucleotides in length, depending on the specific strain, and consists of seven open reading frames that encode 9 proteins (Figure.1b) (ICTVdB - The Universal Virus Database). The gene order for the mumps genome is 3' NP-P-M-F-SH-HN-L 5' (Elango *et al.*, 1988). The genome contains sequences of non-coding nucleotides flanking and within the genome. At the genome 3' end is the leader (50 nucleotides) and sequences at the 5'end are called the trailer (50-160 nucleotides). Within the genome, the non-coding sequences are referred to as intergenic sequences, which are essential in separating conserved transcriptional start and stop sequences between genes (Lamb and Kolakofsky 2001). These range in size from 1 to 7 nucleotides in length.

The NP is the most abundant viral protein translated, and for mumps, NP monomers form a tightly coiled filamentous flexible rod structure that resembles other paramyxoviruses nucleocapsid structures (Finch 1970). Recently, for mumps and measles virus, the assembly of NP monomers into the rod like structures was shown to

require the N-terminal but not the C-terminal region of NP (Kingston *et al.*, 2004). The L protein is considered the catalytic unit of the viral RNA polymerase (vRNAP). The protein exists as an oligomer and is known to directly bind to both P and NP. The P protein is a co-factor for the polymerase. This protein is the most phosphorylated protein in paramyxoviruses (Hsu and Kingsbury 1982), and phosphorylation of the protein has been proposed to be important in viral transcription (Mazumder and Barik 1994; Kaushik and Shaila 2004). The P protein can be expressed from edited or unedited P mRNA, depending on differences of the P gene sequence in genera within the *Paramyxovirinae* (Lamb and Kolakofsky, 2001). The mumps virus P protein is expressed from mRNA edited with the insertion of two guanine residues in the mRNA. The protein has been shown to oligomerize, and for the mumps virus, oligomerization appears to consist of homotrimers (Curran *et al.*, 1995).

All the viruses in the *Paramyxoviridae* family encode NS proteins that have been associated with regulating cellular and viral processes during infection, specifically the interferon response. For the viruses in the Pneumovirinae subfamily, the non-structural proteins include the M2, C, W, and D proteins. While in the Paramyxovirinae, such as mumps virus, the non-structural proteins include V and I. The mumps V protein is expressed from unedited mRNA transcribed from the P gene and contains a cysteine rich domain that is necessary for homo- and hetero-oligomerization (Ulane *et al.*, 2005; Takeuchi *et al.,* 1990). The I protein is also expressed from the P gene. This protein is expressed from I mRNA that is edited by the insertion of one guanine residues during transcription of the P gene. No known function has been ascribed to the protein (Paterson *et al.*, 1990).

There is one last protein encoded by the mumps virus. It is a small hydrophobic (SH) protein that has been identified as an integral membrane protein for PIV5 (Hiebert *et al.*, 1988) and RSV (Olmsted and Collins 1989). This protein has been also identified as a membrane protein for mumps (Takeuchi *et al.*, 1996) but is not known if it is expressed only in infected cells or in the virus envelope. Recently, phylogenetic investigations have categorized mumps viruses into 12 distinct genotypes (A-L) based on sequence analysis of a hyper-variable region in the SH gene (Muhlemann 2004). Researchers have used this genotyping as a means to observe the geographic distribution of mumps strains and to classify the strains of mumps vaccines (Muhlemann 2004). In addition, researchers have shown that certain genotypes are associated with increased neurovirulence although there is little difference in the ability of different genotypes to be neutralized by antibodies to mumps (Muhlemann 2004; Rubin *et al.*, 2006).

Replication Cycle of Mumps Virus

Mumps virus, like other *Paramyxoviridae* viruses, uses sialic acid as an attachment molecule to begin the replication process (Figure. 2). Sialic acid is found primarily on glycoproteins and glycolipids (sialoglycolipid or gangliosides) but little information is known about the specific receptors for any of the paramyxoviruses, or whether there are preferences for specific sialic acid linkages by any of the viruses (Villar 2006). It has recently been shown that at least one strain of a mumps virus, the Urabe strain, can bind to both 2-3α and 2-6α sialic acid linkages (Reyes-Leyva *et al.*,

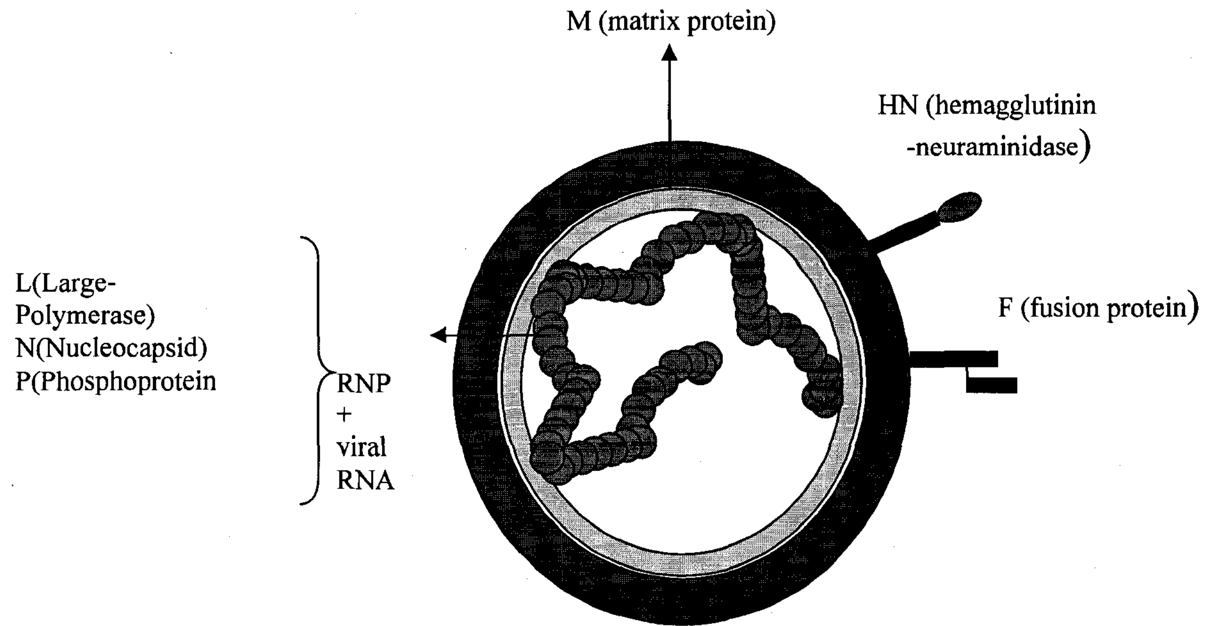
2007). The crystallized structures of the HN of NDV, human parainfluenza virus 3 (hPIV3), and PIV5 have been determined, allowing predictions about the location of potential sialic binding sites. The HN of NDV contains two binding sites, both in the globular head, with the second binding site formed after a conformational change in HN due to initial binding to sialic acid (Crennell *et al.*, 2000; Zaitsev *et al.*, 2004), while the crystal structure of hPIV3 and PIV5 suggest the presence of a single sialic acid binding site (Lawrence *et al.*, 2004; Yuan *et al.*, 2005). For the mumps virus, the number of sialic acid binding sites has not been determined, but is assumed to be the same as PIV5. Binding of HN to sialic acid induces a conformation change and, possibly through the stalk region of the protein, activates the F protein for fusion of the viral and cellular membrane (Wang *et al.*, 2004; Corey and Iorio 2007). Once F is activated, the hydrophobic fusion peptide is exposed and inserts into the host membrane, which brings the two membranes into proximity for fusion at neutral pH. The mechanism of activation of the F protein by HN is not entirely clear (Morrison 2003). One suggestion is that the fusion peptide is released once HN attaches to sialic acid, and then HN interacts with F and induces the conformation change in the F protein. Another idea proposes that HN and F are complexed prior to HN attachment, and F undergoes a conformation change after being released from the complex during HN receptor interaction.

Once the viral and cell membrane have fused, the ribonucleoprotein is released into the cytoplasm and viral transcription begins. The promoter region of the paramyxoviruses is bipartite and includes nucleotides of the leader and elements of the untranslated region (UTR) 3' of NP or the UTR 5' of L (Kolakofsky *et al.*, 2005). At

the 3'end, these elements are a sequence of repeats that consist of $[n^1n^2n^3n^4G^5C^6]_3$ for Rubulaviruses like mumps (Murphy and Parks 1999). This promoter region is called the genomic promoter (G/Pr) and is complementary to the antigenome promoter region (AG/Pr). The G/Pr is involved in replication and transcription, while the AG/Pr directs only replication.

Figure 1a and b: Mumps Virion and Mumps Virus genome.
1a. The mumps virion is composed of two glycoproteins (HN and F) within the viral envelope. Underneath the envelope is the M protein that maintains the virion structure. At the centre of the virion is the ribonucleocapsid (RNP) core that consists of the L, P, and NP proteins and RNA.
1b. The mumps virus genome consists of seven genes flanked by a leader and trailer sequence at the 3' and 5' ends respectably. Between the genes are intergenic sequences that contain gene start and stop sequences

a.



b.

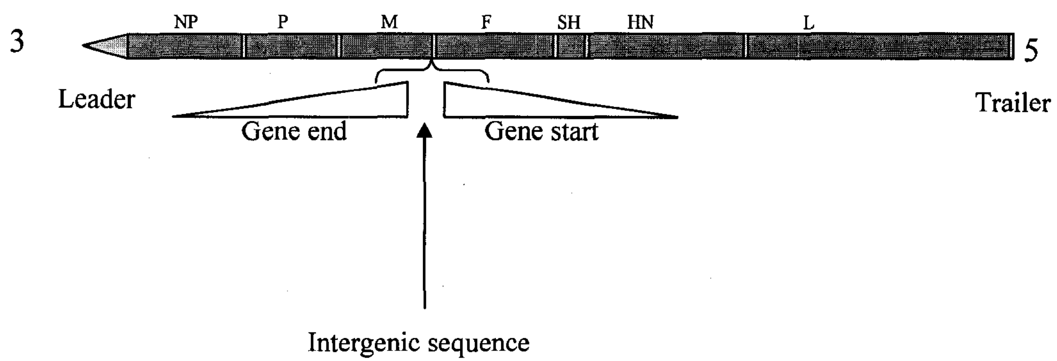
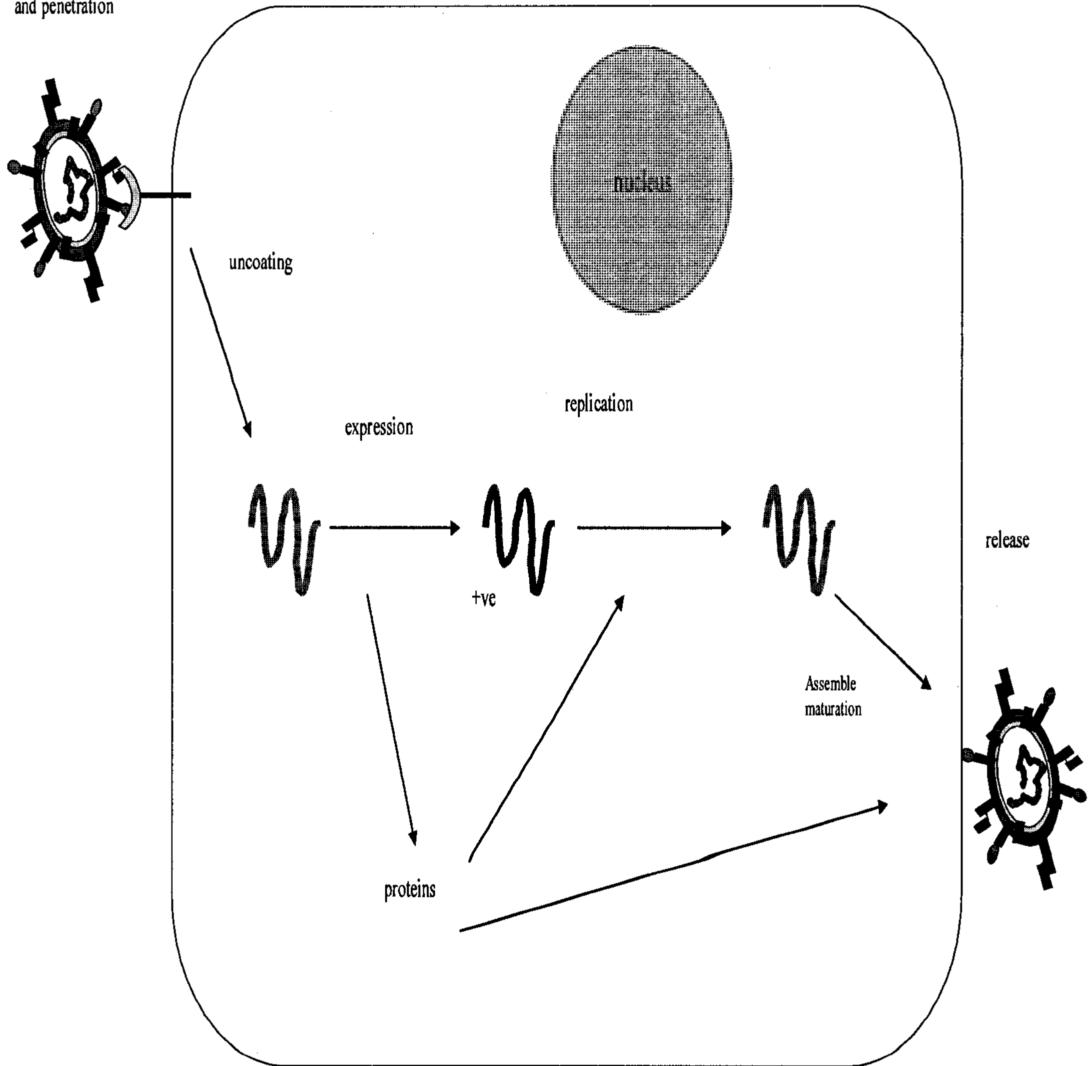


Figure 2: Mumps Replication Cycle

The attachment and penetration phase of the lifecyle consists of the mumps virus attaching to the host receptor and the fusing of the viral envelope with the host cell membrane. The viral nucleocapsid is uncoated or in other words released into the cytoplasm. Genomic RNA (-ve sense strand) within the nucleocapsid undergoes transcription into mRNA and replication for antigenomic RNA (+ve sense strand), which is used as a template for synthesis of genomic RNA. Translation of mRNA into viral proteins occurs and viral protein is used in the replication and is transported to the cell membrane. The virion of mumps is assembled at the cell membrane and then released.


Attachment
and penetration
nucleus
uncoating
replication
expression
+ve
release
Assemble
maturation
proteins

It has been shown for SeV that the presence of tubulin positively regulates transcription (Moyer *et al.*, 1986) and this may occur through binding of tubulin to M releasing it from the RNP thus allowing transcription to proceed (Ogino *et al.*, 2003).

For transcription and replication, the phosphorylation of P (Huntley *et al.*, 1997; Mazumder and Barik 1994; Kaushik and Shaila 2004) and the interaction of P with L (Horikami *et al.*, 1992) are critical for efficient RNA synthesis. Transcription of the genome starts from the G/Pr at the 3' end of the genome in the leader sequence. After transcription of the leader, the vRNAP begins transcription of the genes starting at the NP gene. The emerging NP mRNA is capped and polyadenylated by L when transcription terminates at the stop sequence (Lamb and Kolakofsky 2001, Ogino 2005). The vRNAP then releases the newly transcribed NP mRNA and scans the genome for the next gene start sequence, which is the P gene (Fearns 1999, Lamb and Kolakofsky 2001). Transcription of the P gene involves mRNA editing, where there is an editing sequence present in the transcript. This is a stutter site that causes the polymerase to backtrack and insert one or two G residues (Kolakofsky *et al.*, 2005). For Rubulaviruses, the unedited P gene codes for V mRNA and editing of the P gene codes for P (2 Gs added) and I (1 G added). The polymerase then falls off the genome and reinitiates for all the following genes to produce mRNA for M, F, SH, HN, and L. During the process of stopping and reinitiating in the gap junction, the vRNAP at times disregards the stop sequences and continues to transcribe the next gene, which leads to the production of bi- and polycistronic mRNAs. In addition, when the vRNAP falls off, it sometimes fails to reinitiate at the subsequent gene, and will restart transcription

at the beginning of the NP gene. This process results in an accumulation and gradient of mRNA from the 3'end of the genome than the 5'end (Lamb and Kolakofsky 2001).

Translation occurs simultaneously with transcription, and the accumulation of viral proteins, particularly NP, initiates the switch from transcription of short mRNAs to replication of the full length genome, although the mechanism of how this occurs is unknown. The glycoproteins, HN and F, are glycosylated in the RER, then are transported through the Golgi to the cell surface. Cleavage of F_0 into the 2 segments, F1 and F2, occurs in the transGolgi during transport by a host cell protease furin (Carbone and Wolinsky 2001). Other viral proteins are transported to the cytoplasm for the formation of the ribonucleoprotein complex.

During replication, the vRNAP recognizes the G/Pr sequence and ignores the start and stop sequences of genes to synthesize a complementary copy (positive strand or antigenome) of the genome (Hwang 1999). At the same time during synthesis, free NP monomers are chaperoned by the P protein to encapsidate the antigenome to yield the antigenome nucleocapsid (Curran *et al.*, 1995). After completion, the antigenome undergoes synthesis in the same manner, with initiation from the AG/Pr to generate genomic RNA that is also encapsidated by NP monomers (Lamb and Kolakofsky 2001).

The M protein is assumed to play a major role in virion assembly as studies have shown M to be attached to the cytoplasmic portions of the HN and F glycoproteins proteins and to the ribonucleoprotein complex (Sanderson *et al.*, 1994; Yoshida *et al.*, 1979). The viral glycoproteins are inserted into the membrane and the M protein associates with the viral glycoproteins present in the plasma membrane

(Sanderson *et al.*, 1994). Once assembled, the ribonucleoprotein complex associates with regions of the membranes containing the M protein and the virion is formed by the process of budding from the host membrane (Yoshida *et al.*, 1979).

Pathogenesis of Mumps

Mumps is a contagious disease that is spread by droplets from the mouth and nose. The natural route of invasion seems to be through mucosal surfaces, with an average incubation period of 18 days (Gordon and Kilham, 1949). The virus is shed in saliva up to 7 days after the appearance of symptoms (Plotkin 2004). Having gained entry to the host, mumps, like any other virus, must initiate an infection by entering a susceptible cell. This primary infection determines whether the infection will remain localized at the site of entry or spread to become a systemic infection. For mumps, primary infection occurs in the epithelial cells of respiratory tract or the conjunctivae. The virus multiplies then spreads systemically by viremia (Gordon and Kilham, 1949). The initial symptoms of mumps are prodromal symptoms, which include headache, fever, anorexia, and malaise (Carbone and Wolinsky 2001). However, once the virus spreads systemically, specific symptoms related to secondary infection in tissues appear. Classically, mumps is characterized by enlargement of the parotid gland due to inflammation, but the virus has been shown to replicate in the salivary glands, testes, central nervous system (CNS), epididymis, prostate, ovary, liver, pancreas, spleen, thyroid, kidneys, labyrinth, eyes, thymus, heart, mammary glands, lungs, bone marrow, and joints (Carbone and Wolinsky 2001).

Mumps virus spread to the CNS is common and 50% of such cases occur without parotid gland association (Carbone and Wolinsky 2001). Mumps invasion of the CNS is thought to occur from the blood by mumps virus crossing the choroid plexus. The choroid plexus is the barrier between the blood and CNS and consists of choroid epithelial and ependymal cells, which produce cerebral spinal fluid (CFS), surrounding vascularized stroma (Strazielle *et al.*, 2000). In addition to epithelial cells, the mumps virus can infect mononuclear cells, T lymphocytes, and possibly B lymphocytes (Fleischer and Kreth 1982). It is generally thought that the virus can use these cells as a vehicle to cross through pores in the endothelium of the capillary and infect the choroid epithelial cells, but the virus is also assumed to cross the endothelium and infect choroid epithelial cells (Carbone and Wolinsky 2001). Once the choroid epithelial cells are infected, mumps replicates and the released virions are distributed throughout the CNS. Within the CNS, mumps commonly infects the meninges (Carbone and Wolinsky 2001). Meningitis occurs in 10% of individuals infected with mumps and is characterized by the onset of fever, neck stiffness, and photophobia (Galazka *et al.*, 1999). The virus also infects ependymal cells lining the brain ventricles which allow infection of the brain parenchyma cells leading to inflammation and thus encephalitis. However, encephalitis is quite rare and occurs only in 0.02 to 0.3% of individuals infected with the virus (Carbone and Wolinsky 2001). Symptoms associated with infection of cells within the CNS range from unconsciousness to coma, emotional lability, irritability, and behavioural impairments that are caused by lesions in areas of the central nervous system (Galazka *et al.*, 1999). Infection of the CNS often resolves without complications; however, unilateral or

bilateral deafness, aqueductal stenosis, hydrocephalus, myelitis, and cerebellar ataxia have been reported (Viola *et al.,* 1998; Carbone and Wolinsky 2001).

The humoral immune response to the virus is first detectable during the onset of symptoms (Franková and Sixtová 1987; Ukkonen *et al.*, 1981; Friedman 1982). Specifically, IgM and IgG are released early during infection, with IgM reaching maximum levels within seven days (Franková and Sixtová 1987) and IgG peaking during the third or fourth week in serum samples (Ukkonen *et al.*, 1981). IgA antibodies have been shown to appear one day after the onset of disease in saliva samples and reach a maximum during the first and second week of symptoms (Friedman 1982). Antibodies to structural proteins of the mumps virus exist, however only antibodies to the two glycoproteins, F (Houard *et al.*, 1995) and HN (Orvel 1984), are known to neutralize mumps virus infections in animal models, and in tissue culture only the HN antibody has shown to neutralize mumps. The cell mediated immune response has been shown to peak at four weeks during vaccination studies and clearance of virus coincides with the increase in serum neutralizing antibodies (Carbone and Wolinsky 2001).

Mumps Vaccines

The immunity induced by mumps vaccines in individuals is based on the knowledge that antibodies raised against one strain of mumps protect against infection from other stains of mumps i.e., the mumps virus exists as a single serotype. Rubin

(2006) has recently confirmed this by showing that human sera against several genotypes of the mumps virus neutralized two different genotypes of mumps.

Development of the first mumps vaccine can be traced to John Enders in 1945. His group isolated viruses from the parotid glands of infected *M. mulatta* monkeys and prepared two vaccines by inactivating the viruses with formalin (formalized vaccine) or alum-precipitated formalin (alum-precipitated formalized vaccine) (Enders *et al.*, 1945). The vaccines were initially tested on monkeys and both induced a low humoral immune response in 60% of the primates, as measured by complement-fixation (Enders *et al.*, 1945). A human clinical trial of these inactivated vaccines resulted in only a slight humoral response in only 50% of the individuals tested, and because inactivated substances generally induce immunity of short duration, the vaccines were considered ineffective (Enders *et al.*, 1945). At the same time that these studies were published, Karl Habel successfully propagated the mumps virus in developing chick embryo and prepared a vaccine by inactivating viruses using ultraviolet radiation or ether (Gorden and Kilham 1949). Evaluation of the vaccine in primates showed an adequate humoral immune response and protection when challenged with virus isolated from an infected monkey's parotid gland (Habel 1951a). In a clinical trial and at the start of a mumps epidemic in 1945, this inactivated vaccine resulted in a decrease in the incidence of disease; however, the vaccine was not routinely used in children due to the lack of a long term protective effect (Habel 1951a; Habel 1951b; Buynak 1966).

Due to the limited span of immunity induced by inactivated vaccines, the use of these types of vaccines has declined in favour of attenuated live vaccines (Weibel *et*

al., 1970; Henle *et al.,* 1951; Carbone and Wolinsky 2001). The first known live attenuated mumps vaccine was prepared by Enders and colleagues (Enders *et al.*, 1946). Enders attenuated a virus by continued passage within eggs and demonstrated that the virus still displayed an immunogenic response in primates. Despite this, the vaccine failed to induce a reaction in a small human trial. Further analysis by Enders and colleagues revealed that the vaccine was over-attenuated, based on an immunological response in a child receiving a virus after a single passage in eggs, Enders suggested that an earlier number of passages in eggs might produce a satisfactory attenuated vaccine (Enders *et al.*, 1946).

The Jeryl Lynn vaccine was the first licensed mumps vaccine, and originally consisted of lots of the Jeryl Lynn strain (JL) of mumps virus prepared by numerous passages through chick embryo eggs and chick embryo cell cultures (Buynak and Hilleman 1966). Candidates for vaccine preparation were chosen based on the passage number and serological tests in animal models (Buynak and Hilleman 1966). Two lots of Jeryl Lynn were designated A and B and the extent of attenuation was tested in a clinical trial involving children (Buynak and Hilleman 1966). Jeryl Lynn B was chosen for the vaccine, as findings demonstrated that this virus was more attenuated than Jeryl Lynn A, which caused parotitis in some of the children and mumps virus was excreted in the children's urine (Buynak and Hilleman 1966).

With the success of the JL vaccine, research on mumps virus slowed, and so reliable markers for virulence or attenuation of mumps viruses have not been identified (Plotkin 2004). Consequently, vaccines have often been too virulent and copied symptoms of disease very closely, or over-attenuated and unable to provide any

protection from the disease (Plotkin 2004). Because there is no clear marker of virulence or attenuation, mumps vaccines continue to be licensed for usage based on the finding that the vaccines show sufficient attenuation in clinical settings (Plotkin 2004).

More than 10 live attenuated vaccine strains are currently in use (Table 1). The vaccines are available as monovalent, bivalent measles-mumps, and trivalent measles-mumps-rubella (MMR), with Urabe AM9 and Jeryl-Lynn being the most common mumps strains used in the MMR, at least up until the late 1980's (Galazka *et al.*, 1999; Carbone and Wolinsky 2001).The Jeryl Lynn vaccine is associated with the lowest incidence of aseptic meningitis, with less than 1 case out of 100 000 doses administered, although the ability to prevent disease (efficacy) varies from 60-94% during case studies (reviewed Bonnet *et al.*, 2006). The Rubini vaccine, derived from passage of a virus isolated in a human diploid cell line in Europe (Gluck *et al.*, 1986), demonstrated sufficient attenuation in clinical trials, in that it failed to cause disease, and a seroconversion rate of 95% in vaccinees (Peltola *et al.*, 2007). However, after outbreaks where Rubini was the mumps component in the MMR, the vaccine showed no clinical effectiveness in preventing mumps disease (Goh 1999; Ong *et al.*, 2005). The Leningrad-3 vaccine, a guinea pig kidney and quail egg derived strain, was demonstrated to have a protection efficacy of 94% by Smorodintev and colleagues (Smorodintev *et al.*, 1963) and out of 85 vaccinated children only 2.3% became ill compared to 38.8% out of 100 unvaccinated children during an outbreak. However, the vaccine was later shown to be insufficiently attenuated, with a high number of aseptic meningitis cases reported in clinical studies in Germany (Tischer and Gerike 2000).

The L-Zagreb strain, a further attenuated form of the Leninigrad-3, is now used in developing countries. This vaccine causes aseptic meningitis at a rate of 1 case per 19,247 doses; however, the association of aseptic meningitis with this vaccine is debatable, as no mumps vaccine viruses were identified from vaccinees showing symptoms (Peltola *et al.*, 2007).

Table 1: Mumps vaccine in use.

STRAIN	MANUFACTURER	MAIN AREA OF DISTRIBUTION
Jeryl Lynn	Merck	Worldwide
RIT 4385	GlaxoSmithKline	Worldwide
Urabe	Aventis Pasteur	Worldwide
	Biken	Japan
Hoshino	Kitasato Institute	Japan
Rubini	Swiss Serum Institute	Europe
Leningrad-3	Bacterial Medicine Institute, Moscow	Russia
Leningrad-Zagreb	Institute of Immunology of Zagreb	Yugoslavia
	Serum Institute of India	Worldwide
Miyahara	Chem-Sero Therapeutic Research Institute	Japan
Torii	Takeda Chemicals	Japan
NK M-46	Chiba	Japan
S-12	Razi State Serum and Vaccine Institute	Iran

Reproduced from Plotkin SA. Mumps Vaccine Chapter 20: In Vaccines 2004

Urabe Am9

The Urabe Am9 vaccine was first licensed in Japan and many other countries such as the United Kingdom, Canada, Belgium, France, and Italy during the 1970s and 1980s (Galazka *et al.*, 1999). Urabe Am9 is considered very immunogenic, showing a seroconversion rate of 95% in vaccinated children and displaying a protection efficacy of approximately 75% in outbreak conditions (Peltola *et al.*, 2007). However, due to unacceptable or relatively high rates of meningitis the vaccine was removed from these countries' vaccination programs (Peltola *et al.*, 2007).

Urabe Am9 was originally manufactured by Biken, a Japanese company, during 1967. The virus was isolated from the saliva of a patient (Yamanishi *et al.*, 1973), and then attenuated by passages in chicken amniotic cavities and quail embryonic fibroblast cell cultures (Yamanishi *et al.*, 1970; Yamanishi *et al.*, 1973). At the end of these passages, six clones were chosen and tested in children (Yamanishi *et al.*, 1970). Among the six clones, the Am9 clone (ninth plaque purified clone) exhibited the highest seroconversion rate for neutralizing antibody and displayed no clinical side effects (Yamanishi *et al.*, 1970; Yamanishi *et al.*, 1973). The Am9 clone was then subjected to additional passages in chicken amniotic cavity and used for preparation of the Urabe vaccine (Yamanishi *et al.*, 1970; Yamanishi *et al.*, 1973). In 1986, the Urabe Am9 vaccine was included as part of Trivirix measles, mumps, and rubella (MMR) vaccine used in Canada (Furesz and Contreras 1990). Cases of aseptic meningitis were reported after 4 weeks of administration of the MMR Trivirix in British Columbia, Ontario, and Quebec (Hockin and Furesz 1988; Champagne and Thomas 1987). Post vaccination aseptic meningitis associated with Urabe AM9

containing vaccines was also reported in the United Kingdom, Australia, Belgium, France, Germany, Ireland, and Japan, with rates reported as high as 1 case per 900 doses in Japan, 1 case per 62 000 doses in Canada and 1 case per 120 000 dose in France (Gray and Burns 1989a, Murray and Lewis 1989, Gray and Burns 1989b, Forsey *et al.*, 1992, Yawata 1994). Partial sequencing analysis of the F, SH, P, and HN genes from CSF mumps isolates confirmed the relationship of the illnesses to the Urabe vaccine and provided evidence for withdrawal of the vaccine from use in Canadian, Japan, and the United Kingdom (Turner *et al.*, 1991; Yamada *et al.*, 1990; Brown *et al.*, 1991, Forsey *et al.*, 1990).

While investigating the failure of the Urabe Am9 vaccine, several groups identified that the vaccine was a mixture of viruses distinguished by an adenosine (A) or a guanine (G) at hemagglutinin-neuraminidase nucleotide (nt) 1081, and included additional subpopulations of 'A' viruses with other changes in the HN gene (Brown *et al.*, 1996; Mori *et al.*, 1997; Afzal *et al.*, 1998; Wright *et al.*, 2000). Viruses with an 'A' at nt position 1081 possessed a lysine at amino acid (aa) residue 335, which represents the sequence from most known mumps strains and viruses isolated from individuals suffering post vaccinated illnesses (Brown *et al.*, 1996; Mori *et al.*, 1997). Viruses with 'G' at nt 1081 possessed glutamic acid at aa 335 and viruses with this sequence were not associated with post vaccination disease (Wright *et al.*, 2000; Brown *et al.*, 1996; Mori *et al.*, 1997, Afzal *et al.*, 1998). These observations suggested that viruses with the 'G' nt at this position were fully attenuated as they have never been associated with symptoms.

Wright et al, (2000) identified 6 unique Urabe viruses in the vaccine while investigating HN gene sequence, plaque morphology, fusion activity, temperature sensitivity and viral growth in Vero cells of Urabe variants purified from the vaccine and viruses associated with post-vaccinated illness. Specifically, viral populations associated with post-vaccination meningitis but not parotitis displayed additional nt differences in the HN gene sequence affecting aa 464. Additionally, viruses from the vaccine were isolated with a guanine to adenosine substitution at nt 1480 of HN (Glu to Lys, aa468); while two viruses associated with post-vaccination parotitis displayed neither of these changes. Analysis of two Urabe vaccine 1081G variants displayed no further changes in the sequence of HN (Wright *et al.*, 2000). However, Afzal (1998) identified differences at nt 1476 and nt 1570 in 1081G variants isolated from a SKB vaccine and from an individual without post vaccination illness.

An 'A' virus, 1004, isolated from a patient with post vaccination meningitis from Urabe and a 'G' virus, Gw7, isolated directly from the Urabe vaccine, were tested for virulence in the monkey neurovirulence test (MNVT). The MNVT is assigned by WHO (World Health Organization) to evaluate the safety of many vaccines, including mumps vaccines. However, the validity of this test for distinguishing attenuation and virulence of mumps virus was not established. When Urabe mumps viruses were tested in the MNVT, Gw7 showed less neurovirulence than 1004 (Rubin *et al.*, 1999). However, it was shown in this study that this test did not correlate the amount of disease in the brain of monkeys to the extent of attenuation or virulence of mumps strains, nor can it ensure the ability of attenuated virus not to cause disease (Rubin *et al.*, 1996; Maximova *et al.*, 1996). A rat neurovirulence test (RNVT) was developed

which shows promise for distinguishing attenuated and virulent mumps viruses (Rubin *et al.*, 1999; Rubin *et al.*, 2000). Recently, the RNVT was used to compare the virulence of Gw7 and 1004 10/2, a plaque purified isolate from the stock of 1004 post vaccination virus, and in this rat assay, Gw7 was significantly less neurovirulent than 1004 10/2 (unpublished Shah *et al.*, 2007). Additionally, Gw7 scored as attenuated as JL, thus confirming the proposal that 'G' viruses are more attenuated than some 'A' viruses.

The study of the differences in virulence of the two viruses has been of primary interest in the Wright laboratory. For this reason, the genomes of Gw7 and 1004 10/2 were sequenced to determine which gene(s) might be responsible for differences in virulence. In addition to the change in the HN gene at aa 335 and aa 464 (Wright *et al.*, 2000), 18 differences were observed between Gw7 and 1004 10/2 (Tables 2 and 3). Five were non-coding changes, which included nt 1387 in the NP gene, nt 1880 in the intergenic region between NP and P, nt 7573 in HN, nt 10529 in L, and nt 15328 in the trailer region (Table 2) (unpublished Shah *et al.*, 2007). The other differences resulted in coding differences (Table 3) and were located in P, M, F, SH, HN, and L genes. The change in the P gene resulted in a coding change in P, but not in the other two proteins expressed from this gene. In P, M, and SH, single coding differences occurred at aa274, aa53, and aa40 respectively. In HN, the differences at aa 335 and aa 464 were accompanied by an additional difference at aa 526. In F, two differences were at aa 91 and aa120, while L displayed differences at aa 163, 320, 512, 1085, and 1871. When the sequences of Gw7 and 1004 10/2 were compared to published Urabe sequences, it was found that Gw7 was unique at aa274, aa53M, aa120F, aa335HN, aa163L, and

aa320L. For 1004 10/2, unique differences were found at aa91F and aa526HN. The sequences at aa40SH, aa512L, aa1085L, and aa1871L were shared between 1004 10/2 and the published sequence of 87-1005, another virus isolated from a case of post-vaccination meningitis in Canada. Although it is unknown which genetic changes affect the virulence of these viruses, the change in virulence of other strains of mumps has been associated with mutations in multiple genes. The change in virulence of a mumps 88-1961 strain was localized to a single mutation in each of HN and L (Rubin *et al.*, 2003). In a mumps Kilham strain, neurovirulence in a hamster model was narrowed to a single mutation in HN (Kovamees *et al.*, 1990), and in another Kilham strain, virulence was mapped to multiple mutations in F (Lemon *et al.*, 2007).

Based on the observed differences in virulence and in the genome, the two viruses were assessed in a human lung cell-line to investigate possible growth variations in cells representing normal targets for mumps replication in humans. Previous work had shown that 'G' viruses replicated to titres 1 log lower than viruses associated with post-vaccination meningitis in Vero cells (Wright et al 2000). Assessment of growth of Gw7 and 1004-10/2 in A549 cells, a human epithelial lung line, revealed that 1004-10/2 replicated as well in these cells as in Vero cells, to titres of at least 10^7/ml. On the other hand, for Gw7 the amount of infectious virus produced in A549 cells was reduced by 3 logs compared to titres in Vero cells. This reduced growth was also confirmed in other human cells such as Hela T4 cells. The observations suggested a difference in cell tropism between the two viruses for tissue culture cells of human origin, and may correlate with the differences in virulence *in vivo.*

Tropism is the specificity of a virus for infecting a particular cell or tissue type, and the difference in tropism of Gw7 and 1004 10/2 must be due to the observed genetic differences between the two viruses. Mumps has shown a tropism for the respiratory tract of ferrets (Gordon *et al.*, 1955) and human glandular, lymphoid and nervous tissues (Carbone and Wolinsky 2001). Recently, an Urabe 'A' virus displayed tropism for neuroblastoma cells after three passages in these cells, whereas, Urabe 'G' virus showed minimal replication after the same number of passages in the cell line (Santo-Leyes *et al.*, 2006).

Table 2: Non-Coding Sequence Differences Between Gw7 and 1004 10/2.

Genes	Sequence Difference	Gw7	1004 10/2	Consensus
NP	nt 1387	A	G	G
NP-P	nt 1880	C	T	C
HN	nt 7573	T	C	T
L	nt 10529	C	T	C
Untranslated region	nt 15328	A	G	A

Table 3: Coding Sequence Differences Between Gw7 and 1004 10/2.

Genes	Sequence Differences	Gw7	1004 10/2	Consensus
NP, V, I	-	-	-	-
P	nt 2797 aa274	G Val	A Lys	A Lys
M	nt 3571 aa 53	T Leu	A Gln	A Gln
F	nt 4817 aa 91	C Ala	T Val	C Ala
	nt 4903 aa 120	G Gln	C Gln	G Gln
SH	nt 6385 aa 40	C His	A Asn	C His
HN	nt 7616 aa 335	G Gln	A Lys	A Lys
	nt 8005 aa 464	C Asn	A Lys	C Asn
	nt 8191 aa 526	A Lys	C Asn	A Lys
L	nt 8925 aa 163	A Asn	C Thr	C Thr
	nt 9397 aa 320	G Met	A Ile	A Ile
	nt 9972 aa 512	C Ser	T Phe	C Ser
	nt 11692 aa 1085	G Leu	T Phe	G Leu
	nt 14049 aa 1871	C Ala	T Val	C Ala

The difference in cell tropism of these two viruses may have arisen if the ability to attach and enter the cell or to utilize cellular factors for expression and replication of the genome is altered by these genetic differences (Tyler and Fields 2001). Such altered tropism because of genetic changes in the genome has been reported for the porcine parvovirus (PPV). The Kresse strain of PPV displayed growth in primary bovine testis cells (TV), but another strain of PPV, NADL-2, failed to grow in the same cells (Bergeron et al., 1996). The sequencing of the genome of both viruses mapped tropism to genetic differences within the coding region of the VP2 protein, which is a viral capsid structural protein (Bergeron et al., 1996). Another determinant of tropism has shown to be interferon (IFN) in a study with the Mahoney strain of polivirus (Hosonuma et al., 2005). In this study, it was shown that the Mahoney strain of poliovirus caused more disease in transgenic mice unresponsive to IFN than transgenetic mice tissues responsive to IFN (Hosonuma et al., 2005). In addition, Hosonuma et al (2005) observed low viral titres of poliovirus in mouse tissue that displayed a high expression of interferon stimulated genes (ISGs) compared to high viral titres in mouse tissue with a low expression of ISGs. Other studies have shown that genes involved in the replication and assembly such as the L and M genes can be a determinant of tropism. The differences in these genes contributed to the growth differences of two measles strain in numerous cell lines (Tahara *et al.*, 2005). All of these studies demonstrate that any stage of the replication cycle from binding to RNA replication can control tropism for specific cells. So, any or all of the genetic differences found between the viruses could account for the cell specific difference in growth. Since the same group of genetic differences controlling tropism in vitro also

controls attenuation/virulence *in vivo*, it is possible that these two phenotypes are correlated, but even if they are not, understanding the basis of the reduced growth of Gw7 in A549 cells will shed light on virus/host interactions at the cellular level.

Hypothesis

The hypothesis is that one or more of the coding differences between Gw7 and 1004-10/2 are responsible for the growth differences between the viruses in A549 cells.

Objectives

To distinguish attenuated and virulent mumps strains the study has three objectives, it is important to understand the basis of the differences in tropism between the attenuated and virulent viruses. The objectives for this project are:

1. To confirm that reduced growth of Gw7 in A549 cells is not due to a shift in growth kinetics
2. To examine a role for interferon in the growth differences between Gw7 and 1004-10/2 in A549 cells and Vero cells.
3. To determine which viral gene(s) are responsible for the observed growth differences of Gw7 in Vero and A549 cells, and between Gw7 and 1004-10/2.
4. To confirm the gene mapping with a functional assay(s).

Chapter Two

Materials and Methods

Cell lines and Culture:

Vero cells (African green monkey kidney) and A549 cells (Human lung epithelial carcinoma) were originally obtained from ATCC. Hela T4 cells (Human cervical epithelial stably transfected with human CD4) were from the NIH Repository or AIDS Research and Reference Resource Program. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Wisent) supplemented with 1% (v/v) L-glutamine (Gibco/BRL life Technologies), 1% (v/v) Penicillin-Streptomycin, and 7% (v/v) of heat inactivated fetal calf serum (FBS) (Wisent). Cells were grown in 25 cm^2 and 75 cm^2 tissue culture flasks (Sarstedt) in a 37°C humidified 5% CO_2 incubator. Cells were split by trypsinization and recultured in fresh medium as needed.

Viruses:

Stocks of all Urabe mumps strains used in this study had previously been prepared in the Wright lab. All were prepared in Vero cells by inoculation at a MOI of 0.1. After 1 hour incubation, the inoculum was removed and replaced with DMEM supplemented with 7% FBS and supernatants were harvested at 3 or 4 days post infection (p.i.). Original stocks of 1004 and 1005 were received from the Bureau of Biologics, Health Canada. Other Urabe viruses had been isolated directly from vaccine lots as described in Wright et al. (2000). Vaccinia virus expressing T7 polymerase was received from Dr. B. Moss. A stock of VSV was obtained from Dr. E. Brown, University of Ottawa.

Plaque Assay:

To quantify infectious virus, 10 fold serial dilutions of samples were made in sterile phosphate buffer saline (PBS) +0.1% gelatin (Sigma). Monolayers of Vero cells in a six well plate were inoculated with 300ul of diluted virus and incubated 1hour. The inoculum was then removed and cells were overlaid with 3ml of a mixture of a 1:1 ratio of 2x199 media (Gibco) supplemented with 10% FBS and 1.2% agarose in H_2O. Cells were incubated for 96 hours, then fixed with Carnoy's fixative (20% (v/v) ethanol and 7.5% (v/v) acetic acid) prior to staining with 0.1% crystal violet in H_2O.

Hemagglutination Assay (HA):

To quantify total virus, triplicate samples of approximately 1.0ml were pooled and ultracentrifuged at 50000 rpm (Sorvall RC70, SW50) for 1 hr at 4°C. The supernatant was removed and the pellet was resuspended in 100ul PBS. Two fold serial dilutions of samples were prepared in 50ul of PBS in a 96 well plate and an equal volume of 5% Chicken red blood cells was added. The plate was incubated at room temperature for 45 minutes to allow hemagglutination. The titre of virus was taken to be the last dilution showing complete hemagglutination activity.

RNA Isolation:

Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies) from Vero cells infected 4 days previously at an MOI of 0.1. Briefly, media was

removed from $3.0x10^6$ cells infected Vero cells. Cells were washed once with PBS (Gibco), and lysed in 1ml of Trizol reagent per $1.5x10^6$ cells at room temperature. Cell lysates were passed several times through a pipette and incubated for 5 minutes at room temperature, then 200ul of chloroform per 1 ml of Trizol was added to the cell lysates, which were shaken for 15 seconds, and incubated for 3 minutes at room temperature. Samples were centrifuged at 6000 rpm for 15 minutes at 4°C to separate into a lower red phenol-chloroform phase and an upper colourless RNA aqueous phase. To precipitate RNA, 500ul of ice cold isopropanol was added to the aqueous phase from 1 ml Trizol treated sample and samples were incubated at room temperature for 10 minutes. The RNA was pelleted by centrifugation at 6000rpm for 10 minutes at 4°C, washed with 1 ml 75% ethanol per 1 ml of Trizol reagent used and centrifuged again at 3000 rpm for 5 minutes at 4°C. The RNA pellet was vacuum dried for 5 minutes at medium heat, dissolved in 50ul RNAse free H_20 and incubated for 10 minutes at 55-60°C. If a RT reaction did not immediately follow afterwards, RNA extraction was halted after RNA precipitation and the sample was stored at -80°C.

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR):

To generate viral cDNA, 10ul of total RNA was used as the template for a 50ul RT reaction containing 80ng of each primer, 0.4ul of 25mM dNTP stock (Amersham-Pharmacia), 10ul 5x buffer (Invitrogen), 5ul DTT (Invitrogen), 1ul Rnase inhibitor (Amersham-Pharmacia), 21ul RNAse free ddH2O and 1ul of Superscript II reverse transcriptase (Invitrogen). The reaction was incubated for 10 minutes at RT, 1 hour at 42°C and 30 minutes at 50°C as suggested by the manufacturer.

For PCR amplification, the 50ul PCR reaction contained 5ul cDNA, 0.8ul of 100ng stock of each primer, 4ul of a solution of 50mM $MgCl_2$ (Fermentas), 5ul of a solution 10x buffer (Fermentas), 0.4ul of 25mM dNTP, 33ul ddH_2O and 1ul Taq polymerase (Fermentas). Depending on the expected sizes of the PCR product, fragments were analyzed using agarose gel electrophoresis with either 1.2% or 0.8% (w/v) agarose (Wisent) in 1x Tris acetate Buffer (TAE, 10mM Tris acetate, 1mM EDTA) and purified from the agarose gel using the GFX PCR DNA and Gel band purification PCR kit (Amersham Biosciences).

All sequences for primers were from taken from the Genbank sequence of Urabe 87-1004 (accession AF314560). For amplification of the M gene ORF, the primers were M3-HA3'(5'GGCATAATCCGGCACATCATAAGGGTATAGGTTGCTCATTGAGGC AGACTT 3') and URM-4401 (5'CTGGCTGCAGGATGAAATTCTCATAGGGTTG C'3). The thermoprofile for the reaction consisted of 30 cycles at 94°C for 30 seconds, 51°C for 30 seconds, and 72°C for 30 seconds. For the SH gene ORF the primers were UR SH +ve (5'AGAATGAATCTCCTGGGGTC3') and UR SH-ve (5'CTTTCTAGAGTGAGTGATCAAAACT3') and the thermoprofile consisted of 30 cycles at 95°C for 30 seconds, 45°C for 30 seconds, and 72°C for 30 seconds.

Sequencing of M and SH purified PCR products was performed by the DNA Sequencing Facility Ontario Genomics Innovation Centre Ottawa Health Research Institute using the same primers that were used in the PCR reactions.

Plasmid DNA preparation:

Plasmids containing the HN gene from Gw7 and 1004 10/2 Urabe viruses had previously been cloned in the lab and were in pDrive (Qiagen) under the control of the T7 promoter. Plasmid DNA for use in hemadsorption studies were prepared from cultures of *E.coli* transformed with pDrive containing the HN genes, which was grown in 100 ml LB media plus 0.1 mg/ml ampicillin overnight at 37°C. Extraction of HN plasmids was done with the Promega Wizard Plus Midi prep DNA purification system according to the company's protocol (Promega). DNA preps were subjected to agarose gel electrophoresis, and quantified by spectrophotometry.

Agarose Gel Electrophoreses:

DNA plasmids and PCR products were analyzed by gel electrophoresis through a 0.8% (w/v) agarose (Wisent) gel in 1x Tris acetate Buffer (TAE, 10mM Tris acetate, 1mM EDTA) and for smaller PCR fragments through a 1.2% (w/v) agarose in TAE. Samples were mixed with 6x DNA gel loading buffer (60mM Tris-HCL, 6mM EDTA 0.25% (w/v) xylene cyanol, 0.25% (v/v) bromophenol blue, 30%(v/v) glycerol (Sigma), and an appropriate volume of water. Electrophoresis was carried out for approximately 45 minutes at 85-100V, and then the gel was stained with ethidium bromide (1.6ug/ml) and visualized under a shortwave ultraviolet light in the Mulitimage Light Cabinet (Alpha Innotech Corp., Dan Leandro, CA).

Quantification of Viral RNA:

At various times after inoculation with Gw7 at MOI 0.1, Vero and A549 cells were incubated with 1 ml DMEM 7%FBS containing 5ug/ml actinomycin D and 25 μCi or 50μCi of tritiated uridine (Amersham-Pharmacia) then incubated for 8 or 12 hours. RNA was isolated as previously stated and subjected to electrophoresis, or scintillation counting. RNA was analyzed by gel electrophoresis with a 1.5 % (w/v) agarose gel in H_2O mixed with 20% (v/v) 5X gel running buffer (100mM MOPS, 40mM NaAc, 5mM EDTA pH 8), and 6.5% (v/v) Formaldehyde 37%. Total RNA was mixed with 2ul 5X gel running buffer, 3.5ul Formaldehyde 37%, and 10ul formamide. The samples were denatured at 65°C for 15 minutes, chilled on ice, and mixed with 1ul Ethidium Bromide (1μg/μl diluted stock) and 2ul sample loading buffer (50% glycerol, 1 mM EDTA pH 8, 0.25% Bromophenol blue). Electrophoresis was carried out for approximately 45 minutes at 85-100V. After visualization under a shortwave ultraviolet light, the gels were fixed in 20% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min at room temperature, incubated in Amplify solution (Bioscience) for 30min at room temperature, dried, and exposed to Kodak BioMax Light Film (Kodak).

Immunoprecipitation:

Vero and A549 cells in 60 mm dishes were infected at an MOI of 0.1 with Gw7, and at various time points from 6 to 96 hrs p.i. cells were metabolically labeled with ^{35}S-methionine. Briefly, cells were washed twice with Minimal Essential Media

(MEM) without Met (Met-free MEM) and then incubated in 2ml of Met-free MEM for 1hour at 37°C. The Met free MEM was then removed and replaced with 60uCi of ^{35}S-Cell Labeling Mix (Amersham Bioscience) in a volume of 2ml Met free MEM. After 1hour at 37°C, cells were washed once with cold PBS (Gibco), washed once with cold Immunoprecipitation Rinse buffer #1 (20mM Tris, 137mM $NaCl_2$, 1mM $CaCl_2$, and 0.5mM $MgCl_2$) then lysed with 100ul of cold Immunoprecipitation (IP) lysis buffer (Rinse buffer #1 with 1% v/v NP-40 and 10% v/v glycerol) supplemented with 5ul of the Protease Inhibitor Cocktail (Sigma) to prevent degradation of viral proteins. Radiolabeled cell lysates were centrifuged at 13000rpm for 10 minutes at 4°C, and then supernatants were incubated with Protein A Sepharose beads (Sigma) to reduce non specific binding. For each sample, 50ul of a solution of 6% (w/v) protein A beads were washed 3 times in IP Rinse buffer. After resuspension in the starting volume, 50ul of Protein A beads were added to each tube of lysate. After incubation for 30 minutes at 4°C, beads were pelleted and lysates were transferred to a clean tube. A polyclonal human sera with known antibody to mumps virus (obtained from Dr. E. Brown University of Ottawa) was added to each radiolabeled lysate at a final dilution of 1/50 and incubated for 1 hour at room temperature. Immunoprecipitates were then formed by adding 100ul of freshly washed Protein A beads to the samples and incubating for 30 minutes at 4°C. The radiolabeled immunoprecipitates were then washed five times in Lysis Wash Buffer (100mM Tris and 500 mM LiCl). After the last wash, pelleted beads were resuspended in 30ul of 2X reducing sample buffer (2% (v/v) SDS, 2% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, and 125mM Tris pH 6.8, 5% (v/v) bromophenol blue). The radiolabelled immunoprecipitates were freed from

Protein A beads by heating at 95°C for 5-10 minutes and the samples were then subjected to SDS-PAGE for analysis.

Western Blot:

Unlabelled proteins from mock and infected cells were prepared at various times after infection, and after separation by SDS- PAGE were transferred to an Immobilon-P membrane (Millipore) using a Bio-Rad Trans-Blot Semi Dry Transfer Cell at 15mV for 1 hour. Larger SDS gels were transferred with a wet transfer apparatus using transfer buffer (25 mM Tris, 190mM Glycine, 20% (v/v) Methanol) and subjected to 25mV at 4°C overnight. The Immobilon membrane was incubated in blocking buffer (5% skim milk in 0.1% Tween-20 + TBS 10 mM Tris-Cl, pH 7.5, 150 mM NaCl) for 1.5 hours at room temperature or at 4°C overnight. The membrane was washed 3 times with TBST (10 mM Tris-Cl, pH 7.5, 150 mM NaCl and 0.1% Tween-20) 10 minutes for each wash. The membrane was incubated overnight at 4°C or 2 hours at room temperature in a 1/1000 of a mouse anti-mumps polyclonal serum diluted in blocking buffer. This antibody has known neutralizing activity against Urabe mumps virus (Wright *et al.*, 2000). After 3 washes in TBST, the membrane was incubated for 1 hour at room temperature in a 1/2000 dilution of peroxidase conjugated goat anti-mouse (Tago) diluted in blocking buffer. The viral proteins were visualized using the Western Lightning Chemiluminscence Reagent Plus Kit (Perkin Elmer) as per the manufacturer's protocol. After 1 minute at room temperature, excess chemiluminscence reagent was removed by draining, and the membrane was placed in plastic wrap and exposed to Kodak BioMax Light Film.

SDS-PAGE Analysis:

For SDS-PAGE analysis, samples were mixed with an equal volume of 2X reducing sample buffer (2% (v/v) SDS, 2% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, and 125mM Tris pH 6.8, 5% (v/v) bromophenol blue) and heated for 5-10 minutes at 95°C. For radiolabeled immunoprecipitates, 30ul of each sample, representing cell equivalents, were loaded per well, and for Western blots, 50-200ug of unlabelled lysates were loaded per well. SDS-PAGE analysis was conducted as per Laemmli (1970). Briefly, samples were resolved electrophoretically on a 10% acrylamide separating gel (1.5 M Tris-HCL pH 8.8, acrylamide/bis, 10% SDS v/v, 10% Ammonium Persulfate, TEMED) with a 4% acrylamide stacking gel (0.5 M Tris-HCL pH 6.8, acrylamide/bis, 10% SDS v/v, 10% Ammonium Persulfate, TEMED) and the PageRuler ladder (Fermentas) was used for determination of molecular weight. Radiolabeled SDS gels were fixed in 20% (v/v) methanol and 7.5% (v/v) acetic acid overnight, dried for 1 hour and exposed to Kodak BioMax Light Film at -80°C, while non-radiolabeled SDS gels were subjected to western blot analysis.

Transfection:

Plasmids containing genes from Gw7 and 1004 10/2 were transfected into Hela T4 cells with Lipofectin reagent (Invitrogen) using Optimem medium (Gibco). For each sample to be transfected, 1ul of Lipofectin and 25ul of Optimem were incubated for 30 minutes at room temperature. A mixture of 20ul of Optimem, 500ng of plasmid with

either Gw7 hemagglutinin-neuraminidase (pHN1) or 1004 10/2 hemagglutinin-neuraminidase (pHN39) were added to the Lipofectin/Optimem mixture. As a control for normalization of transfection efficiency, 500ng of a β-galactosidase containing plasmid under control of the T7 promoter was included in all samples. The DNA/Lipofectin/Optimem mixture was incubated for 30 minutes at room temperature after which $2.5x10^5$ pfu of Vaccinia virus T7 (VVT7) and 250ul of Optimem were added. Hela T4 cells at 60-80% confluency were washed once with PBS, once with Optimem and treated with 300ul of the DNA/lipofectin/VVT7/optimem mixture per well of a 12 well plate. The cells and mixture were then incubated for 5 hours at 37°C, at which time, 300ul of DMEM supplemented with 7%FBS plus ArabinosideC (AraC) 40ug/ml was added to the cells and cells were incubated at 37°C for 18-24 hours.

Heme Binding Assay:

To assay the amount of red blood cell binding to the virus HN, cells expressing transfected HN genes were washed twice with PBS and treated for an hour at 4°C with 1mM of the neuraminidase inhibitor Zanamivir in PBS supplemented with 0.1% $MgCl_2$. As per Iorio (2001), hemagglutination was initiated by adding chicken red blood cells at various concentrations in PBS supplemented with 0.1% $MgCl_2$. After incubation for 20 minutes at 4°C, cells were washed three times with PBS at room temperature with rocking and lysed with 400ul of IP lysis buffer per well to release heme. Lysates were centrifuged at 4°C for 5 minutes and the amount of heme released from the absorbed red cells was detected by spectrophotometry at wavelength of 545

nm. Negative control wells for the assay were all treated with Lipofectin and consisted of cells without the HN genes but infected with vvT7, and uninfected cells.

β-Galactosidase Assay:

To initiate the assay, 30ul of the lysate prepared for the Heme Binding assay were mixed with 3ul 100x Mg solution (0.1 M MgCl2, 4.5 M β-mercaptoethanol), 66ul 1x ONPG (o-nitrophenyl-β-D-galactopyranoside), and 201ul 0.1 M sodium phosphate (pH 7.5) (0.2 M Na_2HO_4, 0.2 M $NaH_2PO_4 \cdot 2H_2O$). The reaction mix was incubated at 37°C for 30 minutes or until a yellow colour change was observed and the reaction was halted with 500ul 1 M Na_2CO_3. Optical density was read at a wavelength of 420 nm.

Binding Assay:

To quantify infectious virus binding to Vero and A549 cells, $2x10^5$ cells in suspension were incubated with either Gw7 or 1004 10/2 at a MOI of 2.5. Cells were incubated on ice for 1 hour to allow virus binding, then washed four times with PBS and resuspended in 50ul of PBS. Cells were lysed by freezing and thawing, and infectious virus remaining with lysed cells was quantified by standard plaque assay.

Interferon treatment of Vero cells:

Concentrations of IFN that showed protection against Vesicular Stomatis Virus (VSV) in a biological assay were established. Briefly, Hela T4 cells were treated with 2 fold dilutions of IFNβ from an initial concentration of 500U/ml for 24 hrs at 37°C. The cells were then challenged with VSV at $2.0x10^5$pfu/well. Two concentrations of IFNβ, 100U/ml and 20U/ml, were chosen for further experiments. The protective effect was determined by scoring cytopathic effect (CPE) from 0 (no CPE) to ++++ (most CPE) and comparing the CPE to wells infected with VSV but without IFNβ. After scoring CPE, the Karber Method was used to determine the virus endpoint for the dilution that would cause 50% of the tissue culture to die (TCD_{50}) the samples in the absence and presence of IFNβ. The Karber Method is indicated by the following equation: Log TCD50= D- Δ(S-0.5), where D is the log of the lowest dilution, Δ is the difference between dilutions, and S is the sum of the proportion of CPE at all dilutions. To further clarify the calculation of S, the proportion of CPE for a given dilution would be as follows: a CPE of +++ out of ++++ is 0.75 CPE, and the addition of the proportion of CPE at all dilutions would give S.

Vero cells were pretreated with various concentrations of human interferonβ (IFNβ) (Sigma-Aldrich) prior to infection. Briefly, Vero cells in a 12 well plate were treated with 100U/ml and 20U/ml of IFNβ in DMEM supplemented with 7%FBS for 24 hours at 37°C. Cells were then inoculated with Gw7 and 1004 10/2 at MOI of 0.1 and supernatants were harvested at 2 days p.i. The amount of infectious virus in IFN treated samples was quantified by standard plaque assay and compared to the amount of virus in cells without IFNβ treatment.

Interferon secretion:

To test interferon secretion in A549 cells after infection, A549 cells were inoculated at MOI of 0.1 with Gw7 or 1004 10/2 at 37°C. At 24 hours p.i., 500ul of supernatants were harvested, acidified with 23ul of 2M HCL overnight at 4°C to remove infectious virus and then neutralized with 16.6ul of 2M NaOH. Plaque assays were performed on viral media to confirm elimination of mumps viruses, and then interferon levels were determined using the Interferon biological assay. Briefly, HelaT4 cells in 96 well plates were treated with 2 fold serial dilutions of samples and 2 fold serial dilutions of IFNβ (Sigma) as a positive control starting at 1000U/ml. After 24hrs incubation, samples were removed and cells were challenged with $2.0x10^5$pfu of VSV in 100ul. The biological assays were incubated at 37°C for 3 days and cells were fixed in Carnoy's fixative and stained with 0.1 %(w/v) crystal violet. The amount of IFN in samples was determined by scoring cytopathic effect (CPE) from ++++ (most CPE) to 0 (no CPE) and comparing the amount of CPE to wells containing known amounts of IFNβ.

Neutralization of Interferon β:

Prior to use, an antibody directed to IFNβ (National Institutes of Health) was titrated to determine concentrations of antibody required to reduce IFNβ activity as measured in the VSV biological assay. Dilutions of 1/1000, 1/2000, and 1/4000 of antibody were incubated with known amounts of IFNβ from 20U/ml to 100U/ml on monolayers of A549 cells at 37°C. After 24hours, cells were inoculated with 5 fold

serial dilutions of VSV starting at 2.0×10^5pfu/ml. The assay was incubated at 37°C for 3 days and cells were fixed in Carnoy's fixative and stained with 0.1 %(w/v) crystal violet to determine any protective effect of samples. CPE was scored from 0 (no CPE) to ++++ (most CPE) and the dilutions of antibody that were able to allow VSV replication in the presence of 100U/ml and 20U/ml of IFN were chosen for use in further experiments.

A549 cells were inoculated with Gw7 and 1004 10/2 at MOI of 0.1, and after a 1 hour incubation at 37°C, 1/1000, 1/2000, and 1/4000 dilutions of a neutralizing antibody to IFNβ (National Institutes of Health) were added to culture media. At 48 and 96 hours p.i, supernatants were harvested and assayed for infectious virus by plaque assay. Titres of virus in the presence of neutralizing anti-IFNβ antibody were compared to titres in the absence of antibody.

Chapter Three

Results

Originally, it was observed that the Urabe vaccines consisted of a mixture of viruses that could be distinguished by a single change at nt 1081 of the HN gene. A plaque purified 'G' virus, Gw7, and a plaque purified post-vaccination meningitis isolate, 1004 10/2, with an A at nt 1081, were assessed in the neonatal rat model for neurovirulence. The investigation revealed that the 1004 10/2 virus was more virulent than the Gw7 virus, and the Gw7 virus was as attenuated as Jeryl Lynn (unpublished Shah *et al*., 2007). Sequencing analysis of the two viruses revealed coding differences in six of the seven viral genes and one difference in an intergenic sequence. Further characterization of the viruses showed that 1004 10/2 replicated well in human cells, such as A549 cells, when compared to Vero cells, but Gw7 displayed a 3 $\log_{10}$ growth reduction in A549 cells compared to Vero cells (Table 4) (K. Wright, unpublished results). The genetic differences between Gw7 and 1004 10/2 affected tropism in cultured cells in addition to virulence/attenuation *in vivo*.

The general goal of this thesis was to understand the growth differences of Gw7 in the two cell lines. It was uncertain whether the reduced growth of Gw7 in A549 cells was due to a change in the kinetics of viral replication, and whether the viral reduction was a reflection of a decrease in the amount of total virus or only infectious Gw7. To address this, an experiment investigating the growth of Gw7 in A549 cells at several time points was conducted. Triplicate wells of Vero and A549 cells were inoculated with Gw7 at a MOI of 0.1. At 24, 48, 72, and 96 hrs p.i., supernatants were collected and assayed for infectious virus by a standard plaque assay and for total virus by a hemagglutination assay. These time points were chosen based on kinetics of growth of mumps in Vero cells by Wright *et al* (2000).

Table 4: Replication of Urabe Viruses in Human Cell Lines

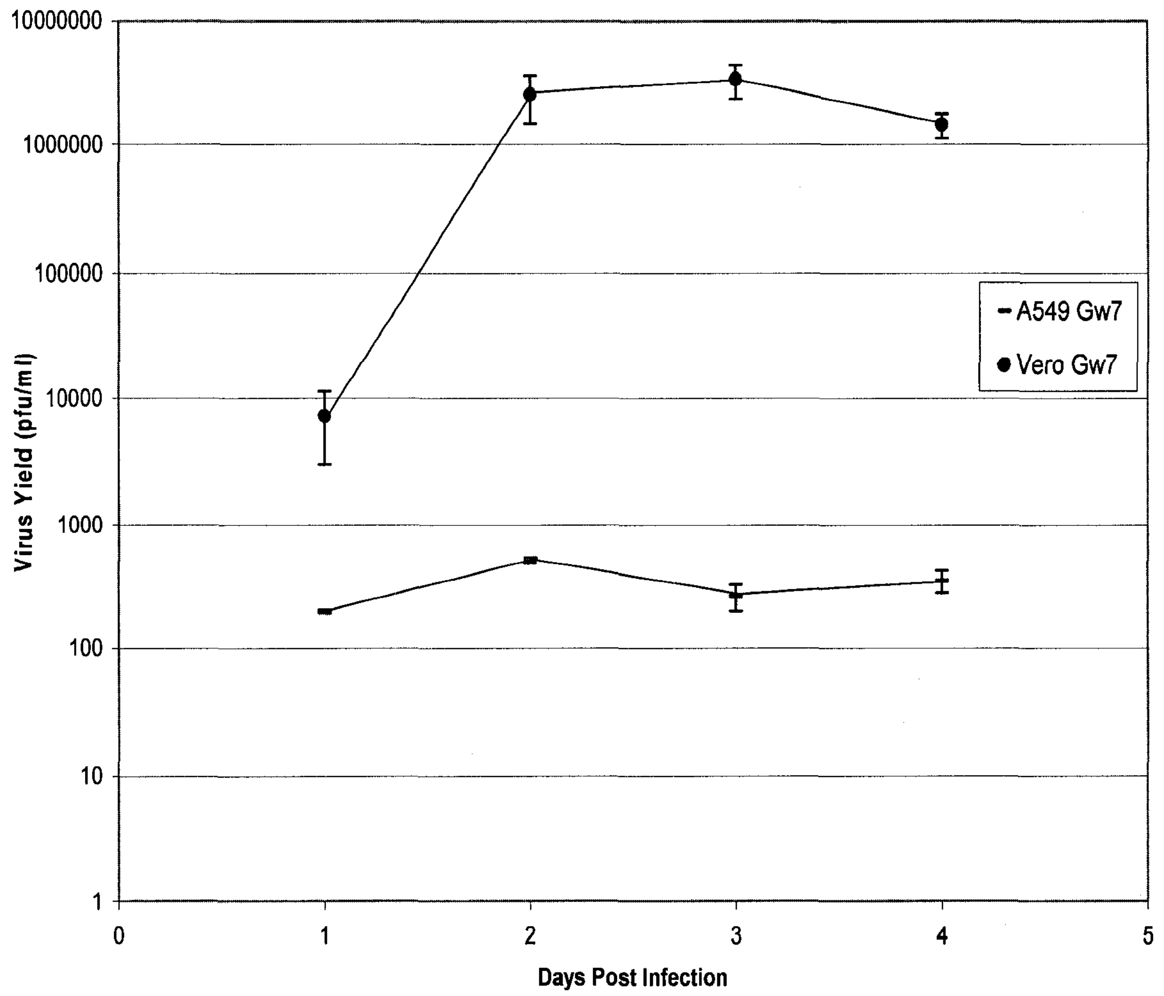
Virus	Vero	A549
Gw7	**$1.96x10^6$** $\pm$ $0.89x10^6$ (n=5)	**$1.43x10^3$** $\pm$ $0.56x10^3$ (n=6)
1004 10/2	**$3.67x10^7$** $\pm$ $1.97x10^7$ (n=6)	**$1.5x10^7$** $\pm$ $0.78x10^7$ (n=5)

Confluent monolayers of cells were infected at MOI=0.1. Supernatants were harvested at 4 days post infection and infectious virus was measured by standard plaque assay. Samples are averages of total number of wells in 2 assays.

Time points later than 96 hrs p.i. were not tested because even uninfected A549 cells did not survive past this time. At all time points, the amount of infectious Gw7 from A549 cells was reduced when compared to Vero cells, with a 3 $\log_{10}$ difference being displayed from 48 to 96 hrs p.i. (Figure 3). The titres in the two cell lines were closest at 24hrs p.i., where there was a difference of approximately 2 $\log_{10}$.

These results were confirmed with the hemagglutination assay, which reflects total virus particles released from cells. Supernatants from triplicate wells of Vero and A549 cells inoculated as before were harvested at 24, 48, 72, and 96 hrs time points were pooled, concentrated by ultracentrifugation, and resuspended in equal volumes of PBS to result in a 10 fold concentration. Resuspended samples were then diluted in 2-fold dilutions with PBS to give a range from undiluted to 1:128, and incubated with 0.5% chicken red blood cells for 45 minutes at room temperature. Wells that displayed agglutination were assigned a unit of HA activity based on the last dilution showing positive activity i.e., agglutination at 1:2 but not 1:4 was assigned a value of 2 HA units. The HA units per ml in the undiluted samples were calculated by dividing HA units by the volume of PBS used to resuspend the viral pellet and then multiplying by the 2-fold dilution factor. Supernatants from Vero cells displayed hemagglutination activity of 40, 160, and 320 HAunits/ml over 48, 72, to 96 hrs, whereas no detectable hemagglutination was shown for Gw7 grown in A549 cells at any time point (Table 5).

Figure 3: Kinetics of replication of Gw7 in Vero and A549 cells.
Triplicate wells of Vero and A549 cells were inoculated with Gw7 at MOI of 0.1. At 24, 48, 72, and 96 hrs p.i., supernatants were collected and assayed for infectious virus by standard plaque assay. Each point is the mean of the triplicate wells ±SD.



10000000
1000000
100000
10000
1000
100
10
1
0
1
2
3
4
5
Virus Yield (pfu/ml)
Days Post Infection
A549 Gw7
Vero Gw7

Table 5: Hemagglutinating Gw7 Released from Vero and A549 cells

Days p.i.	**Gw7 in Vero**	**Gw7 in A549**
1	Undetectable	Undetectable
2	**40HA Units/ml**	Undetectable
3	**160HA Units/ml**	Undetectable
4	**320HA Units/ml**	Undetectable

Concentrated virus from Vero and A549 cells inoculated at MOI of 0.1 was incubated with 5% chicken red blood cells. A HA unit was defined as the amount of virus in the last well to show red blood cell agglutination and the titre of total virus (HA Units/ml) was determine by the volume of the virus used multiplied by the HA unit.

It was surprising that no hemagglutination was detected in the A549 supernatants since some infectious virus was detected in the plaque assay, and the amount of total virus is expected to be higher than the amount of infectious virus. This might be because the ultracentrifugation step did not actually pellet the virus, or because the pellet was resuspended in too large a volume prior to making dilutions. The amount of virus needed to cause visible agglutination based on calculations with influenza has been determined to be 10^4 viral particles for a single HA unit (Wagner and Hewlett 1999). The Vero samples that displayed agglutination had infectious titres over 10^6pfu/ml or $1.5x10^6$ total pfu in a volume of 1.5 ml while the Vero samples that did not show agglutination, those at 24 hr p.i., had an average titre of approximately 10^4pfu/ml or $1.5x10^4$ total pfu/well. The A549 samples displayed infectious titres in the range of 10^3pfu/ml or total $1.5x10^3$pfu/well. This suggests that a HA unit for mumps virus may be greater than 10^4 particles. These results measuring growth over 24, 48, 72, and 96 hrs p.i. confirmed the initial observation of restricted growth of Gw7in A549 cells, and showed that reduced titre at 4 days p.i. was not due to a faster replication cycle.

Interferon

Because it is well known that Vero cells do not produce interferon (Emeny and Morgan 1979), the differences in growth of Gw7 in Vero cells compared to A549 cells could be due to the presence of interferon in A549 cultures. If this were the case then the fact that 1004 10/2 grows well in A549 cells would imply that Gw7 is more

sensitive to IFN than is 1004 10/2. The interferon (IFN) response in non immune cells includes production of type I interferon. Type I IFN, which includes IFNα and IFNβ, is induced in cells by viral dsRNA (double stranded RNA) (Gotoh *et al.*, 2002). Investigations involving A549 cells have shown that the cell line is capable of producing IFN after infection with viruses from the *Paramyxoviridae* family (Wansley and Parks 2002).

The suggestion that IFN is the factor accounting for the difference in 1004 10/2 and Gw7 growth in A549 cells was doubtful due to the following previous observations made in the Wright lab. Firstly, sequencing of the P gene, which encodes the V protein, from both viruses displayed no nucleotide differences that would result in changes to V (unpublished Shah *et al.*, 2007). This protein is known to be responsible for circumventing the IFN response of cells infected with the mumps virus (Kubota *et al.*, 2001, 2005). Secondly, growth of Gw7 in CV-1 cells, a monkey kidney cell line that produces IFN (Zhirnov *et al.*, 2007), yielded titres of the virus identical to those in Vero cells (K. Wright, unpublished observations). In addition to these observations, studies have shown that closely related *Paramyxovirinae* viruses, such as PIV5 limit the amount of IFN produced during infection (Wansley *et al.*, 2005). However, to confirm the lack of a role for the cytokine in the different growth patterns of 1004 10/2 and Gw7, it was decided to do further experiments.

The first set of experiments was to determine the effects of IFNβ treatment of Vero cells on titres of the two Urabe viruses. Doses of IFNβ were initially chosen based on a titration of IFNβ in a biological assay for IFN using vesicular stomatitis virus (VSV) (Friedman and Maheshwari 1983). Hela T4 cells were treated for 24 hrs

with 2-fold dilutions of IFNβ starting at a concentration of 500U/ml. The cells were then challenged with VSV at $2.0x10^5$pfu/well. Hela T4 cells treated with IFN alone displayed no CPE, and it was found that preincubation of cells with IFN units of 500 to 15.6 U/ml showed complete protection of Hela T4 from VSV (results not shown).

Two concentrations of IFN, 100 and 20 U/ml, were chosen and tested for effects on VSV virus growth in Vero cells. Duplicate wells of Vero cells were treated with 100U/ml and 20U/ml of IFNβ for 24hrs and then the cells were challenged with 5-fold dilutions of VSV starting at $2.0x10^5$pfu/well. The cells were scored for CPE (0 to ++++), and the amount of CPE in cells treated with IFNβ was compared to CPE in cells without IFNβ across all dilutions of virus (Table 6). Vero cells treated with IFNβ alone displayed no CPE. The values for CPE from duplicate sets of wells were averaged and used in the Karber equation to calculate the dilution of VSV at which 50% of the tissue culture dies ($TCID_{50}$). At the highest dose of virus, the amount of CPE in wells receiving 100U/ml and 20U/ml of IFNβ was equal. At all other doses of virus, wells with 100U/ml of IFNβ showed less CPE than wells with 20U/ml of IFNβ. The amount of virus in wells without IFN causing 50% CPE was $10^{1.07}$, while in the presence of 100U/ml the amount of virus needed was $10^{3.07}$ and for 20U/ml, the amount of virus needed was $10^{2.07}$. Thus 100U/ml IFNβ reduced the effect of VSV by a factor of 100, while 20U/ml was less effective and reduced the amount of virus 10-fold. The Vero cells were less sensitive to the effects of IFNβ than Hela T4 cells, but these two concentrations of IFNβ did reduce virus titres, so were chosen for experiments with Gw7 and 1004 10/2.

Table 6: Induction of Antiviral State in Vero cells by IFNβ

VSV pfu/well	O units IFNβ U/ml	100 Units IFNβ U/ml	20 Units IFNβ U/ml
$2x10^5$	++++	++++	++++
$4x10^4$	++++	+++	++++
$8x10^3$	++++	++	+++
$1.6x10^3$	+++	++	++
$3.2x10^2$	+++	+	++
$6.4x10^1$	+++	+	++

Vero cells were untreated or treated with 100U/ml or 20U/ml of IFNβ for 24hrs, and cells were challenged with VSV starting at $2.0x10^5$pfu/well. Three days p.i., cells were fixed and stained with crystal violet. The protective effect was determined by comparing cytopathic effect (CPE) to wells infected with VSV without treatment with of IFNβ. CPE was scored from zero to ++++.

To determine whether IFNβ treatment of Vero cells would reduce the replication of the two Urabe mumps viruses, Vero cells were treated with 100 and 20 U/ml of IFNβ for 24hrs and then the cells were inoculated with Gw7 and 1004 10/2 at a MOI of 0.1. At 96 hrs p.i., supernatants were harvested and infectious virus was determined by a standard plaque assay. As shown in Table 7, the titres observed in cells treated with 100U/ml were only slightly lower than untreated cells and the differences were not significant. Thus, this result shows that neither Gw7 nor 1004 10/2 was sensitive to the effects of IFNβ, at least at the doses used, and that there were no significant differences in sensitivity to IFNβ between the viruses, as measured by titres at 96 hours. Recent studies by Carlos (2005) and Rosas-Murrieta (2007) have shown that much higher doses of IFN, 1000U/ml and 2000U/ml, can have an effect on the early cell-to-cell spread of mumps virus (Carlos *et al.*, 2005), and the titres of virus early in infection in Hela cells, but a reduction in titre of only less than a log was observed after 48 hours (Rosas-Murrieta *et al.*, 2007). This effect of high doses of IFN was also reported with PIV5 where the spread of the virus to neigbouring cells in a tumour cell line was delayed, but there was no reduction in the final titre of the virus at 96 hours p.i. (Wansley *et al.*, 2005). So, even when high amounts of IFN are used, there is not the drastic effect on viral titres observed for Gw7 in A549 cells.

Although it seemed unlikely that high amounts of IFN were responsible for reduced growth of Gw7 in A549 cells, it was important to determine whether infection of A549 cells with the Urabe mumps viruses would induce IFN. As noted above, paramyxoviruses such as PIV5 generally block IFN production (Wansley *et al.*, 2005; Wansley and Parks 2002). Based on this information, it was suspected that if the Urabe

Table 7: Effects of IFNβ on titres of Gw7 and 1004 10/2 in Vero cells

IFNβ Units/ml	Gw7 (pfu/ml)	1004 10/2 (pfu/ml)
0	**$9.97x10^5$** ± $4.97\ x10^5$ n=3	**$5.82x10^6$** ± $2.90x10^6$ n=3
20	**$1.36x10^6$** ± $0.808x10^5$ n=3	**$2.56x10^6$** ± $0.196x10^6$ n=3
100	**$6.07x10^5$** ± $3.47x10^5$ n=3	**$2.67x10^6$** ± $0.750x10^6$ n=3

Confluent monolayers of cells were treated with 100 u/ml and 20 u/ml of IFNβ for 24hrs. After 24hrs, the cells were infected at MOI=0.1 with Gw7 and 1004 10/2 for 96hrs p.i. Supernatants were then removed and assayed with a standard plaque assay. Results are the means of triplicate wells ± standard deviation.

viruses did induce IFN, it would be at low levels. To measure the amount of IFN secreted by A549 cells when infected with the two Urabe viruses, cells were inoculated with Gw7 and 1004 10/2 at a MOI of 0.1 and supernatants from triplicate wells were harvested at 24, 48, 72, and 96 hrs p.i. Samples for each time point were pooled and underwent a process of acidification and neutralization with HCL and NaOH to inactivate viruses prior to assaying for IFN. Hela T4 cells were treated with 2-fold serial dilutions of neutralized samples. After 24hrs incubation, samples were removed and cells were challenged with $2.0x10^5$pfu of VSV. A standard curve was included where known amounts of IFNβ from 1000 U/ml to 10.93 U/ml were used to pretreat cells. The amount of IFN in test samples was determined by extrapolating from the CPE in wells treated with unknown samples to the standard curve, and then to the amount of IFNβ. The values were then multiplied by the dilution factor of samples. Although supernatants were treated with acid to inactivate virus, all wells containing undiluted samples showed total loss of the monolayer. It was suspected that not all of the mumps virus was inactivated by the acidification or the samples were still too acidic, and so killed the cells. According to the remaining dilutions, Gw7 was shown to induce 35U/ml of IFN and 1004 10/2 induced an amount of 147 U/ml IFN (Table 8). The results show that some IFN is produced by A549 cells when infected with both Gw7 and 1004 10/2, and that 1004-10/2 induced higher amount of IFN than Gw7. This result validates the choice of IFN concentrations used to induce an antiviral state in Vero cells (Table 6), as the amount of IFN measured in the Gw7 infected A549 cultures was between 20 and 100 U/ml, while the amount of IFN induced by 1004-10/2 was only slightly higher than 100 U/ml. The amount of IFN detected was not at levels

shown to affect spread of mumps virus by others (Carlos *et al.*, 2005). As previously mentioned, much higher amounts of IFN did not significantly affect the titre of mumps when grown in Hela T4 cells (Rosas-Murrieta *et al.*, 2007); thus, the amount of IFN in the A549 cultures infected with Gw7 would be unlikely to account for the 3 log reduction in titre.

Table 8: Secreted IFN by A549 Cells when infected by Gw7 and 1004 10/2

Virus	IFN measured (U/ml)
Gw7	**35**
1004 10/2	**147**

A549 cells were inoculated at a MOI of 0.1 with Gw7 and 1004 10/2. After 4 days p.i., supernatants were harvested and viruses were neutralized. CPE was scored from zero to ++++. Secreted IFN for each virus was determined by a standard curve from CPE with known amounts of IFN-β.

This experiment was conducted only once, but it was decided to use a second approach to prove that IFN in the A549 cultures was not affecting growth of Gw7. This approach was to neutralize IFN in the cultures of A549 cells with an antibody to human IFNβ. Prior to conducting this experiment, the antibody was titrated to determine the dilutions that were effective in neutralizing known amounts of IFNβ. Wells of A549 cells were treated with 100 and 20 U/ml of IFN-β, and either no antibody or 1/4000, 1/2000, and 1/1000 dilutions of the neutralizing Ab. After 24hrs, the cells were then challenged with 5 fold dilutions of VSV starting at $2.0x10^5$pfu/ml and cultured in the presence of antibody for 3 days. Like the other biological assays, the amount of CPE was scored from 0 (no CPE) to ++++ (most CPE). Because a CPE score of ++++ was shown at all doses of VSV in the absence of IFNβ, a reduction in virus titre in the presence of 100 and 20 U/ml of IFNβ could not be determined. Instead, the lowest amount of virus showing a CPE score of ++++ was determined visually for wells receiving 100 and 20 U/ml of IFNβ (Table 9). For 100 U/ml, the lowest amount of virus causing maximum CPE was $2x10^3$ pfu/well, and for 20 U/ml, the lowest amount of virus was $2x10^2$. When the neutralizing antibody was added to the cultures, the lowest amount of virus showing ++++ CPE at 100 U/ml was 0.02 pfu/well of VSV for all antibody dilutions. For 20 U/ml, the lowest amount of virus showing ++++ CPE was 0.2 pfu for 1/1000 dilution of Ab and for the remaining Ab dilutions, titres of 0.02 pfu/well. These results showed that at all three dilutions, the antibody was able to neutralize the effects of IFNβ at concentrations up to 100 U/ml, allowing increased virus growth of up to 5 logs.

Using the 1/4000, 1/2000, and 1/1000 antibody dilutions, the investigation of growth of Gw7 and 1004 10/2 in A549 cells in the presence of IFN neutralizing antibody was studied. Triplicate wells of A549 cells were inoculated with Gw7 and 1004 10/2 at a MOI of 0.1 and immediately incubated with 1/4000, 1/2000, and 1/1000 dilutions of the neutralizing antibody. At 48 and 96 hrs p.i., supernatants were harvested and assayed for infectious virus. The results in Table 10 show that the presence of antibody in the media had no effect on the growth of Gw7 and 1004 10/2 at 48 and 96 hrs p.i. compared to control wells cultured in the absence of the anti-IFNβ antibody. These results confirm the findings of the previous experiments.

The overall results of the IFN experiments suggest that Gw7 and 1004 10/2 induce low amounts of IFN in A549 cells, the viruses are not highly sensitive to IFNβ, and that Gw7 is not more sensitive to IFNβ than 1004 10/2. The results confirm that IFN is not the reason for the growth differences between the viruses in A549 cells and the growth restriction of Gw7 in the human cell line compared to Vero cells.

Table 9: Titration of Anti-IFNβ antibody using 100 and 20 U/ml of IFNβ in A549 cells

Ab	**IFN-β**	
	100 U/ml	20 U/ml
	Lowest Amount of virus showing ++++ CPE	
0	**$2x10^3$ pfu**	**$2x10^2$ pfu**
1/1000	**0.02 pfu**	**0.2 pfu**
1/2000	**0.02 pfu**	**0.02 pfu**
1/4000	**0.02 pfu**	**0.02 pfu**

A549 cells were treated with 100 u/ml and 20 u/ml of IFNβ, and 1/4000, 1/2000, and 1/1000 dilutions of the neutralizing Ab. After 24hrs, cells were challenged with VSV starting at $2.0x10^5$pfu/well for 3 days p.i. Cells were then fixed and stained crystal violet and CPE was scored from zero to ++++.

Table 10: Titres of Urabe Mumps Virus in A549 cells in the Presence of a Neutralizing anti-IFNβ antibody

Antibody dilution	Gw7 (pfu/ml)		1004 10/2 (pfu/ml)	
	48 hrs	96 hrs	48 hrs	96 hrs
0	**$8.73x10^2$** ± $5.04x10^2$ n=2	**$7.53x10^2$** ± $6.26x10^2$ n=3	**$1.32x10^7$** ± $0.58x10^7$ n=3	**$2.67x10^6$** ± $1.01x10^6$ n=3
1/1000	**$1.57x10^3$** ± $0.41x10^3$ n=3	**$2.04x10^3$** ± $0.64x10^3$ n=3	**$2.15x10^7$** ± $0.74x10^7$ n=3	**$7.61x10^6$** ±$5.45x10^6$ n=3
1/2000	**$1.30 x 10^3$** ±$0.16x10^3$ n=3	**$2.47x10^3$** ± $1.44x10^3$ n=3	**$1.68x10^7$** ± $0.99x10^7$ n=2	**$5.59x10^6$** ± $0.82x10^6$ n=2
1/4000	**$1.02x10^3$** ±$0.88 x 10^2$ n=3	**$7.72x10^2$** ±$1.54x10^2$ n=3	**$1.76x10^7$** ± $0.87x10^7$ n=3	**$4.11x10^6$** ±$0.45x10^6$ n=3

Monolayers of A549 cells were inoculated with Gw7 and 1004 10/2 at a MOI of 0.1 and at the same treated with 1/1000, 1/2000, and 1/4000 dilutions of anti-IFNβ antibody. After 48 and 96 hrs p.i., supernatants from triplicate wells were harvested and assayed for infectious virus with a standard plaque assay.

Binding

Having eliminated a role for IFN, the next logical step was to look at early stages in the replication cycle: virus binding to cellular receptors and fusion. As previously described, there are three amino acid (aa) differences between the HN proteins of the two viruses at residues 335, 464, and 526, and two differences in F, at aa 91 and 120. The differences in the aa sequence may affect the multiple functions of the HN protein, which are sialic acid binding, neuraminidase activity, and promoting fusion, and the function of the F protein, which is to cause viral and cellular membrane fusion. The ability of HN and F from both viruses to cause fusion in Hela T4 cell membranes was previously investigated in the lab and it was demonstrated that Gw7 HN/F mediated higher fusion than the HN/F pair from 1004 10/2 (K. Wright unpublished observations). These results showed that low growth of Gw7 in A549 cells was not due to failure of the virus to fuse, thus, eliminating a role of the aa differences in F, or in the fusion promoting function of HN. The neuraminidase function of HN plays a role in releasing virus after budding, but early experiments showed that there was no more infectious virus associated with A549 lysates at 48 and 96 hrs p.i. than was being released (K. Wright, unpublished results). The next sets of experiments were completed to examine the HN binding activities.

Two of the three differences in HN were originally used as markers to differentiate Gw7 and 1004 10/2. Previous work by others has shown that HN335 is within an antigenic region recognized by antibodies (Yates *et al.*, 1996) and 1004 10/2 was selected for a change at HN464 (Afzal *et al.*, 1998; Wright et al., 2000), which is a potential N-linked glycosylation site. Recently this virus was shown to contain an

additional change at amino acid 526 (unpublished Shah *et al.*, 2007). The locations of these aa residues on the HN molecule were determined by placing these sites on the known crystal structure of the Newcastle disease virus HN protein using the Protein Homology/analogY Recognition Engine (Phyre) program (www.sbg.bio.ic.ac.uk/phyre/). Phyre predicts the three dimensional (3D) structures of proteins, and then uses an algorithm to align the query amino acid sequences with known 3D structures. Based on the alignment, the program copies the coordinates and relabels the residues according to the query amino acid sequence. After the 3D structure of Gw7 HN was predicted, the 3D structure was placed into the Vector alignment search tool (VAST) in the NCBI website (www.ncbi.nlm.nih.gov/) that allows direct comparisons of the newly determined structures by superimposing the query structure with homologous protein structures in the database. It was found that aa 335, 464, and 526 are predicted to reside on the globular head of HN and not within the sialic acid binding sites or hydrophobic areas involved in dimer formation and fusion promotion, as determined from the crystallized structure of the Newcastle disease virus HN protein (Crennell *et al.*, 2000; Takimoto *et al.*, 2000, 2002).

Although the differences do not lie within sialic acid binding sites, the differences could still either enhance 1004 10/2 binding or reduce Gw7 attachment. Experiments were designed to compare Gw7 binding to A549 and Vero cells, to compare the ability of the two viruses to bind to each cell line, and to compare the attachment of the HN protein from each virus to sialic acid.

The first experiment examined the binding of Gw7 and 1004 10/2 to A549 cells compared to Vero cells. Equal numbers of A549 and Vero cells were pelleted and

infected at a MOI of 2.5 with either Gw7 or 1004 10/2. After incubation on ice for 1 hour and several washes, the cells were lysed and cell associated infectious virus was assayed by plaque assay. As shown in Table 11, Gw7 displayed slightly reduced binding to A549 cells compared to Vero cells although the values were not statistically different (p=0.3877, Student's t-test). Furthermore, Gw7 bound as well or better to both cell types than 1004 10/2, where the difference in binding between the two viruses was statistically significant for Vero cells (p=0.0126, Student's t-test) and A549 cells (p=0.0002, Student's t-test). In addition, slightly higher binding was observed for 1004 10/2 to Vero cells than A549 cells and statistically, this also was significant (p=0.0377, Student's t-test). A second experiment was conducted comparing only binding of the two viruses to A549 cells, and the results support the the first experiment, showing a statistical difference between the viruses in binding (p=0.0001, Student's t-test) (data not shown).

The results of these experiments suggested that lack of binding is not responsible for the restriction in growth of Gw7 in A549 cells. To examine HN binding in more detail, it was decided to investigate the binding of the protein to sialic acid. For this experiment, Hela T4 cells were transfected with 500ng of plasmids that contained either the HN gene from Gw7 or 1004 10/2 under the control of the T7 promoter, then infected with VVT7 for expression of T7 polymerase (Fuerst *et al.*, 1986). Hela T4 cells were used as they have been found to be somewhat resistant to the CPE caused by the vaccinia virus, and are cells used routinely for expressing viral glycoproteins.

Table 11: Cell Associated Gw7 1004 10/2 After Binding to A549 and Vero cells

	A549 (pfu/ml)	**Vero (pfu/ml)**
Gw7	$\mathbf{6.00x10^3} \pm 0.402x10^3$ n=3	$\mathbf{7.00x10^3} \pm 1.74x10^3$ n=3
1004 10/2	$\mathbf{1.70x10^3} \pm 0.355x10^3$ n=3	$\mathbf{2.59x10^3} \pm 0.357x10^3$ n=3

Results are the means of triplicate samples ± SD. Equal numbers of cells were infected at MOI=2.5. Cells were incubated for 1hr on ice, washed and then lysed. Infectious virus was measured by standard plaque assay. Results are the means of triplicate samples.

The transfection efficiency of each plasmid preparation had previously been assessed by another student in the lab using indirect immunofluorescence , and the results showed an equal number of cells expressed HN from Gw7 and 1004 10/2 (Personal communication, S. Ramzan). However, for these experiments cells were co-transfected with a β-gal plasmid, also expressed from the T7 promoter, to control for transfection efficiency. The negative controls in the study consisted of cells transfected with β-gal and infected with VVT7 (β-gal cells) without the HN plasmid and untreated cells. After 24hrs, the cells were incubated at 4°C for 20 minutes with 0.5 % chicken red blood cells (RBCs) (Iorio *et al.*, 2001) and a neuraminidase inhibitor Zanamivir at a concentration of 1mM. This dose chosen was based on the use of Zanamivir in hemadsorption assays with influenza virus (Personal communication, L. Keeta). Next, the cells were gently washed, lysed, and heme was quantified at a wavelength of 545nm. The first hemadsorption assays attempted showed no binding in cells expressing Gw7 and 1004 10/2 HN proteins compared to the background levels in the negative controls (results not shown). To ensure these hemadsorption values were representative of equal transfection of Gw7 and 1004 10/2 HN proteins, the experiments were repeated with the β-gal plasmid to normalize hemadsorption values to β-gal activity in the β-gal control. Two experiments were conducted. The first experiment showed that Gw7 HN binding was lower than 1004 10/2 HN binding, whereas in contrast, the second experiment binding was equal for both HN proteins (Figure 7).

In an effort to improve the level of hemadsorption, the assays were repeated with 5%, 2%, and 0.5% chicken RBCs. When the values for HN were normalized to β-

gal activity, 5% and 2% RBC displayed higher hemadsorption and better reproducibility between experiments than experiments conducted with 0.5% RBCs. In all these experiments, Gw7 HN demonstrated increased levels of attachment to sialic acid compared to 1004 10/2, with the 5% RBC experiment showing a 1.7-fold increase and 2% RBC experiment displaying a 2-fold increase. Although a statistically significant difference was shown only for 2% RBCs ($p=0.00753$, Student's t-test) and not for 5% ($p=0.0731$, Student's t-test) and 0.5% ($p=0.628$, Student's t-test) (Figure 5). For 2% RBC, Gw7 HN showed binding values of 0.42 ± 0.016 and 1004 10/2 displayed values of 0.19 ± 0.022, while with 5% RBC, values of 0.24 ± 0.028 for Gw7 HN and 0.15 ± 0.0078 for 1004 10/2 were observed. These results demonstrate that binding of sialic acid by the HN of Gw7 is not reduced compared to activity of the HN from 1004-10/2, and in fact Gw7 HN may have a higher binding activity which supports the previous cell associated binding experiment.

Overall, the cell associated binding experiment and the Hemadsorption assays show that the amino acid differences between the Gw7 and 1004 10/2 HN proteins do not result in reduced binding of Gw7 to sialic acid or to A549 cells compared to 1004-10/2. Thus, the results show that the reduced growth of Gw7 in the A549 human cell line is not due to changes in the function of HN having to do with receptor binding. Although not important for the phenotype of low growth in A549 cells, there were differences in the binding ability of the viruses observed in these assays. Specifically, Gw7 displayed higher binding to both Vero and A549 cells, and also higher Hemadsorption activity compared to 1004 10/2 HN.

Figure 4: Receptor Binding Assay Comparing Gw7 HN to 1004 10/2 HN.
Chart represents units of hemadsorption activity for Gw7 and 1004 10/2 HN expressed in Hela T4 cells. Values are normalized to transfection efficiency as determined by β-galactosidase activity, and background levels of RBC binding to cells infected with VVT7 alone have been subtracted. Values shown are two independent experiments each performed in duplicate (n=2).

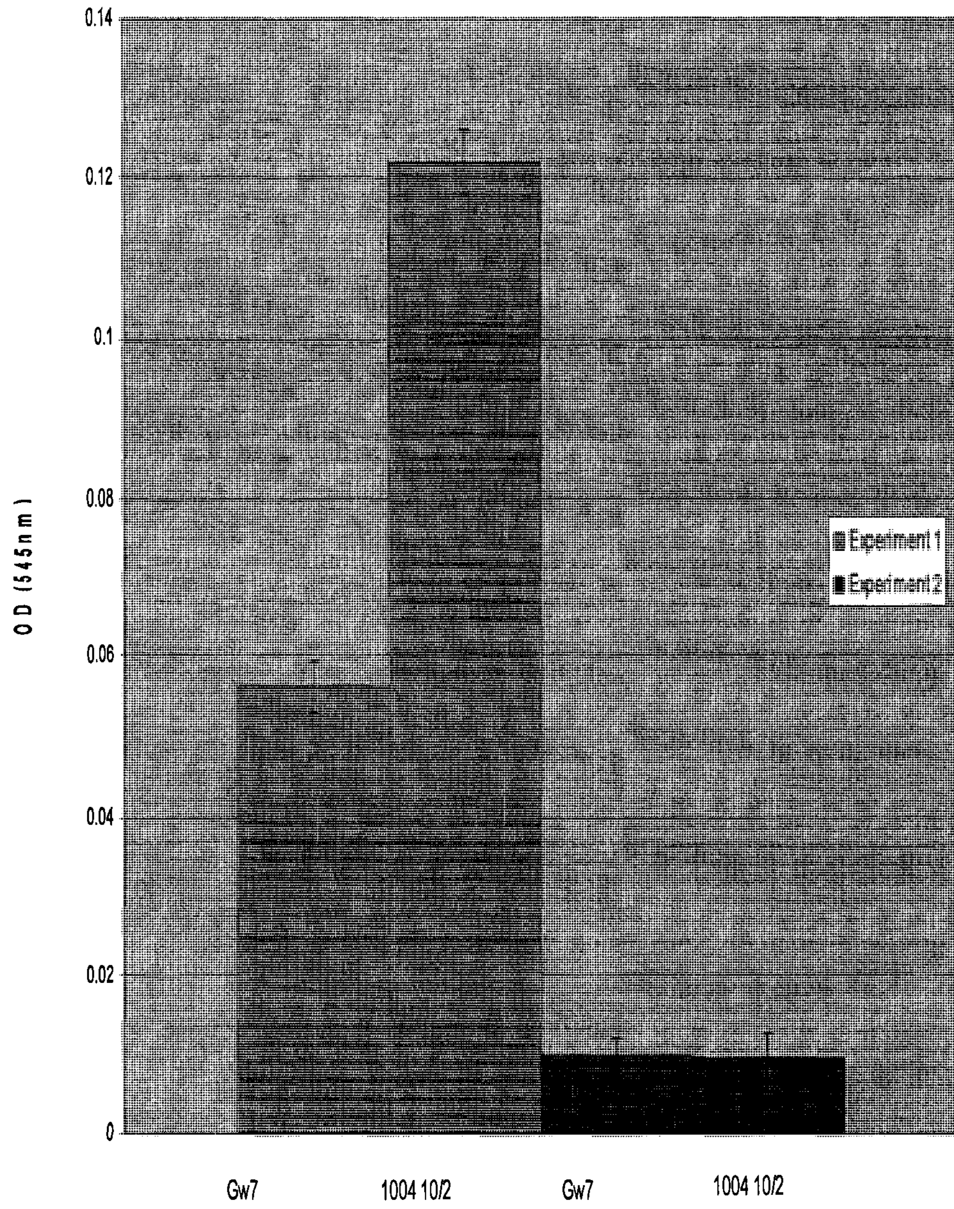
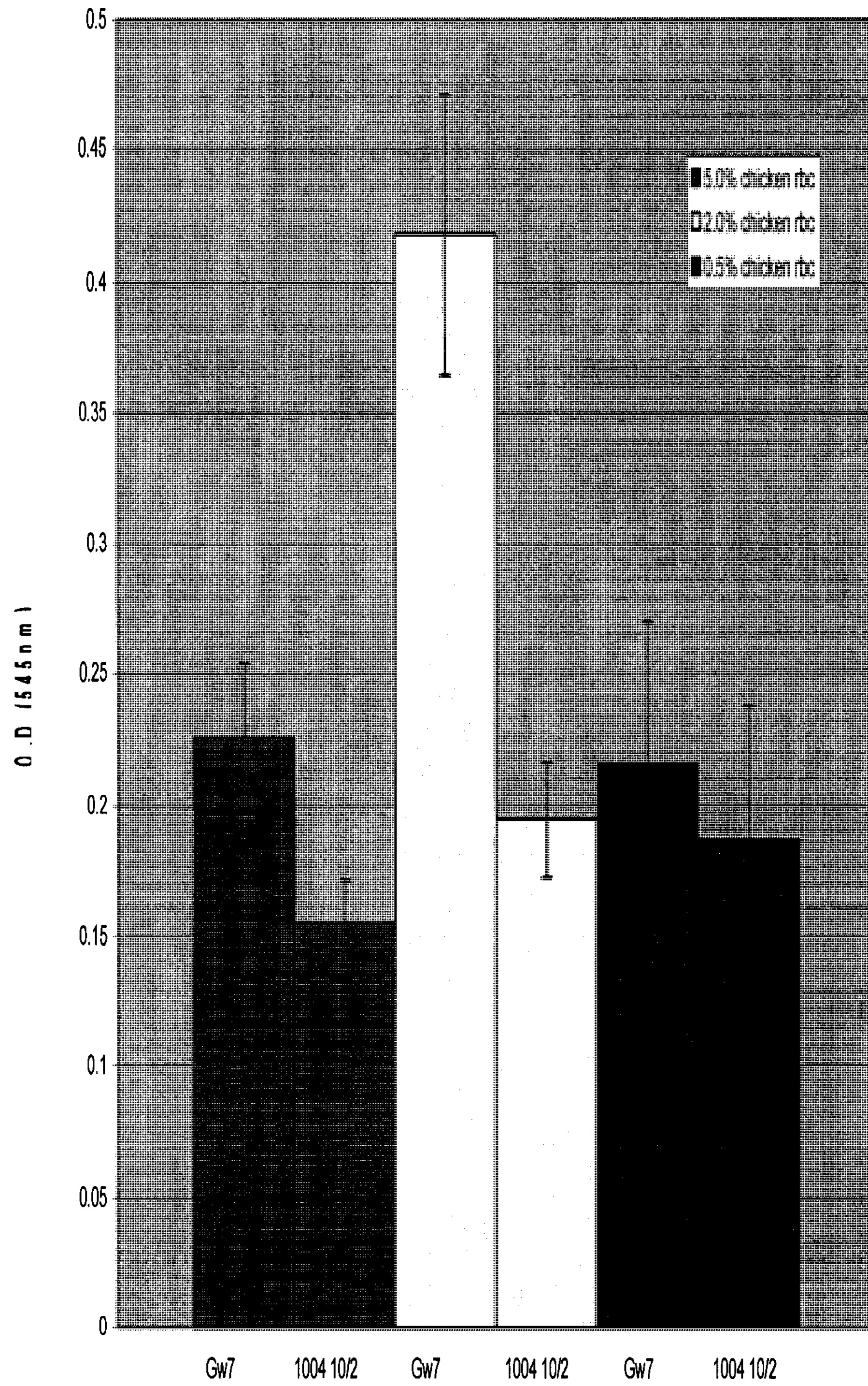

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
O D (5 4 5 n m)
Experiment 1
Experiment 2
Gw7
1004 10/2
Gw7
1004 10/2

Figure 5: Effects of differeing concentrations of RBCs on receptor binding by Gw7 and 1004 10/2 HN proteins.
Chart represents hemagglutination activity normalized to transfection efficiency. Background levels have been subtracted from levels of RBC binding to cells infected with VVT7 and are the mean result of two independent experiments preformed in duplicate (n=4).



0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
O.D (545nm)
5.0% chicken rbc
2.0% chicken rbc
0.5% chicken rbc
Gw7
1004 10/2
Gw7
1004 10/2
Gw7
1004 10/2

Growth of Additional Urabe Viruses

Having eliminated IFN, reduced binding (HN) and/or fusion (F), it appeared that the inability of Gw7 to grow in A549 cells must be due to differences in the remaining genes where Gw7 and 1004 10/2 differed – M, SH, P and L. Hence, two approaches were undertaken to map the growth differences to the genetic differences between the two viruses.

The first approach looked at the growth pattern and sequence of specific genes of the following additional Urabe viruses: A5, 1004 4/1, 1005, and a temperature sensitive virus A9. The A5 and A9 viruses are isolates plaque purified directly from the Urabe vaccine and are both 'A' viruses with a lysine at amino acid 335. A9 has a unique difference at amino acid 468 of the HN gene (Wright *et al.*, 2000). The 1005 virus is associated with post-vaccination meningitis and the published sequence of the L and SH genes is identical to 1004 10/2 (Genbank accession AF314562). The 1004 4/1 virus was plaque purified from the same stock of 1004 as 1004 10/2 but has the same sequence in HN as A5 (Wright *et al.*, 2000). The second approach to mapping the genes responsible for reduced growth in A549 cells was a functional approach to determine where in the replication cycle Gw7 growth is restricted in the A549 human cell line.

For the first approach, growth studies of the additional Urabe viruses were conducted to see if the growth of these viruses in A549 cells was similar to either Gw7 or 1004 10/2. The growth of A9 had already been conducted by undergraduate students in the lab (D. Wong, S. Ramzan). This virus had the same growth pattern as Gw7, with a reduction of 3 $\log_{10}$ growth in A549 cells compared to Vero cells. For A5, 1004 4/1

and 1005 growth, triplicate wells of Vero and A549 cells were inoculated at a MOI of 0.1 and after 96hrs p.i supernatants were harvested and titrated by plaque assay. For all viruses, at least three experiments were conducted each with triplicate wells and the results are presented in Table 8. A5 displayed similar growth to Gw7 and A9, with a reduction in titres of at least 3 $\log_{10}$ in A549 cells compared to Vero cells. The virus 1004 4/1 showed growth similar to 1004 10/2 and displayed at the most a log difference in titre between A549 and Vero cells. The 1005 virus growth pattern in A549 cells varied from experiment to experiment. This virus sometimes failed to grow in A549 cells and at other times grew well in A549 cells. Because of the inconsistent growth of 1005, further investigations were not conducted with this virus.

Having established the growth of the Urabe viruses in Vero and A549 cells, sequencing of the M and SH genes was carried out for each virus. The original sequencing had found that the M and SH genes of Gw7 and 1004 10/2 contained variations. The M gene had a nucleotide difference at position 3571 of the mumps genome, resulting in a coding difference at amino acid 53 specific to Gw7, while the SH gene contained a difference at nucleotide 6385 for a coding change at amino acid 40, which was specific to 1004 10/2.

The M gene encodes a protein that is the most abundant protein in the mumps virion and lies between the ribonucleocapsid and the viral envelope within the virion (Lamb and Kolakofsky 2001). The protein has shown to be vital in viral assembly, budding, and transcription (Lamb and Kolakofsky 2001). The M protein down regulates transcription, and interaction of M with cellular tubulin is necessary to allow the progression of transcription for Sendai virus (Ogino *et al.*, 2003). Hence, a

difference in M could affect either transcription or assembly in a cell specific manner. The function of the protein from the SH gene was undetermined until recently. The protein is now thought to be involved in preventing TNF-α induced apoptosis (Wilson *et al.*, 2006). Although SH is not essential for virus growth in Hela cells (Takeuchi *et al.*, 1996), differences in the SH gene of Gw7 and 1004 10/2 could be a factor for in growth differences between the viruses in A549 cells.

Table 12: Replication of Additional Urabe Viruses in A549 cells

Virus	Vero	A549	Log Difference
A5	**1.87×10^7** $\pm 2.12 \times 10^7$ n=15	**1.47×10^3** $\pm 2.16 \times 10^3$ n=15	4
1004 4/1	**1.35×10^8** $\pm 1.20 \times 10^8$ n=11	**7.57×10^7** $\pm 11.6 \times 10^7$ n=11	<1
1005	**1.60×10^6** $\pm 0.908 \times 10^6$ n=3 **6.20×10^7** $\pm 3.25 \times 10^7$ n=6	**<500** n=3 **3.39×10^7** $\pm 6.85 \times 10^6$ n=6	5 <1

Confluent monolayers of Vero and A549 cells were inoculated at MOI of 0.1. At 96 hrs p.i., supernatants were collected and assayed for infectious virus by standard plaque assay. The log difference represents the growth difference of each virus between cell lines. For 1005, the two sets of data in each cell line represent two experiments in a single set, and display conflicting growth pattern for the A549 cell line.

To confirm the sequence of the M gene of Gw7, and to sequence the M and SH genes of A9, A5, and 1004 4/1, Vero cells were infected at a MOI of 0.1 and after 96hrs p.i , total RNA was extracted, used as template for a RT reaction to produce cDNA, and copies of the M and SH genes were produced from the cDNA in a PCR reaction. When the PCR products of the M gene of all viruses were sequenced, no difference was shown at nucleotide position 3571 for any of the viruses, including Gw7, when compared to 1004 10/2. An independent RT-PCR reaction and direct sequencing of the PCR product confirmed the lack of a change at nucleotide 3571 in M of Gw7. This showed that the mutation in M was not confirmed, so could not account for low replication of some viruses in A549 cells.

A5 and A9 shared the phenotype of limited growth in A549 cells like Gw7 and had the same SH sequence as this virus. Sequencing of the SH gene for all viruses confirmed that the change at amino acid 40 was unique to 1004 10/2, and that 1004 4/1, which replicated well in A549 cells, did not share this mutation in SH. Also 1005, which has the same SH sequence as 1004 10/2, does not appear to replicate like 1004 10/2, although this needs to be verified. Hence, the mutation at aa40 of SH was not essential for high growth in A549 cells.

Viral Protein Synthesis

The results thus far have suggested that the differences in growth of Gw7 and 1004 10/2 in A549 cells are not due to IFN, nor to differences in HN, F, SH, and M genes. Hence, the results point to the differences in the L and P proteins. Recently, the P gene was cloned and sequenced by another student (N. Nasheri) to confirm the differences originally observed between the viruses, and her results revealed that the sequences of the P gene were identical for both viruses. This then indicated that differences in growth likely map to L, which is responsible for both transcription and replication. To determine where in the replication cycle the block in growth occurred, Vero and A549 cells were inoculated with Gw7 at a MOI of 0.1 and incubated at 37°C for 6, 12, 24, 48, and 72 hrs p.i. At these time points, the cells were labeled with ^{35}S for approximately 1 hour. Viral proteins were visualized after immunoprecipitation of lysates from equivalent numbers of cells using a polyclonal human serum positive for mumps antibody. The serum precipitated a single polypeptide in the range of 75Kda in both infected cell lines, which was absent in mock infected cells. The migration of the polypeptide is consistent with it being either the HN protein (79-75 kDa) or the Nucleocapsid protein (NP) (73-72 kDa) (Elango 1988; Orvell 1978) (Figure 6). As only a single band was observed, it is difficult to distinguish whether the polypeptide was HN or NP, although it is likely to be the NP protein because this protein is considered the most translated protein from mRNA in cells infected with paramyxoviruses (Lamb and Kolakofsky 2001). Due to the high background, it was difficult to analyse the results, and so densitometry was not performed. However, the results do show that viral protein could be detected at 24 hrs after infection of both cell

lines, and that in Vero cells, there was more viral protein synthesis at 24hrs and 72hrs than in A549 cells. In contrast to 24hrs and 72 hrs, viral protein synthesis in Vero cells at 48hrs was lower than in A549 cells and it is possible that this was a loading error or due to cell loss during infection rather than a reflection of reduced Gw7 protein synthesis at this time point.

A second immunoprecipitation was conducted, looking at synthesis of viral proteins only from 24 to 96 hrs p.i., since viral proteins were not detected at earlier time points under the conditions used for infection. In this experiment, the amount of viral protein in Vero cells peaked at 48 hrs p.i. and decreased afterwards, and in A549 cells the amount of protein seemed to remain constant at all time points and was less than the amount observed for Vero cells only at 48 hrs p.i (Figure 6b). Hence, these experiments failed to demonstrate clear differences in Gw7 protein synthesis in A549 and Vero cells despite the 3 log difference in production of infectious virus. The low amount of viral protein detected in Vero cells could have been due to the method of preparation of the lysates for immunoprecipitation. Each infected sample was prepared in an equal amount of lysis buffer, and it was assumed that each sample contained equivalent number of cells; however, cytotoxicity of the cells was observed at the 72hr and 96hr time points and the reduced protein might be due to cell loss during infection. In addition, the synthesis of viral protein was observed for only a 1hr period and longer labeling periods might have shown a difference in the viral protein synthesis between the two cell lines.

Because these results were difficult to interpret, it was decided to investigate the accumulation of viral proteins within both cells by Western Blot. Vero and A549

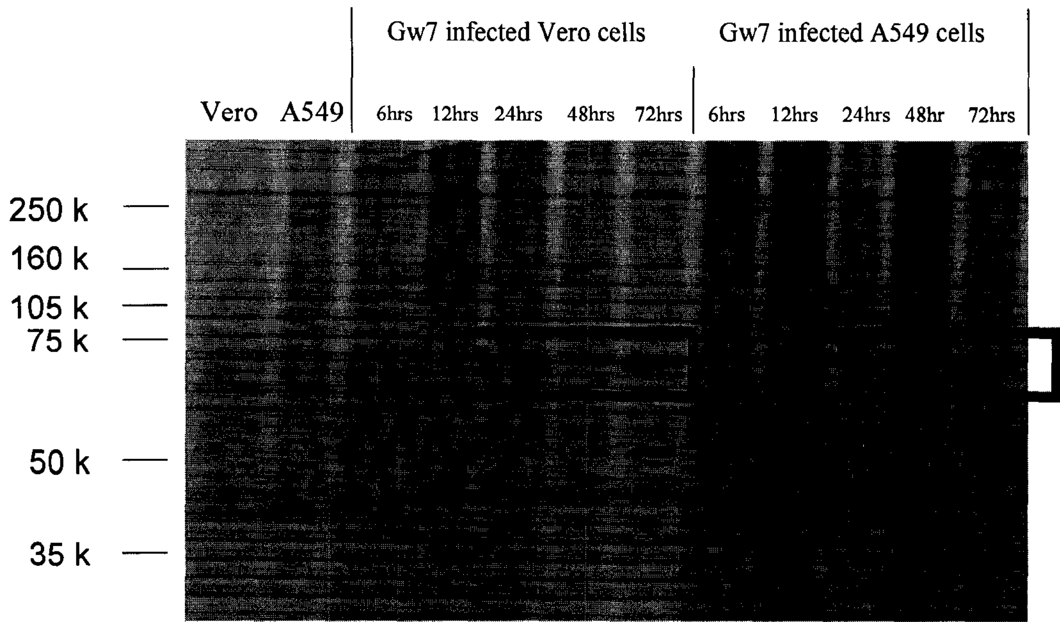
cells were inoculated with Gw7 at a MOI of 0.1. At 24, 48, 72, and 96hrs p.i., cells were lysed and equal amounts of protein were loaded and separated by SDS-PAGE. Then, the gel was analyzed by Western Blotting using a mouse anti-Urabe serum with known neutralizing activity (Wright *et al.*, 2000). Unlike the polyclonal human serum, the anti-Urabe mouse sera reacted highly to two polypeptides in the 72 kDA range in the Vero cells, with the larger protein being expressed more than smaller protein at the 24hr time point (Figure 7a). As shown in previous studies, the heavier protein should be the HN protein and the lighter protein is NP (Elango 1988; Orvell 1978). In the Vero cells, the amount of viral proteins increased from 24hrs to 96hrs but in A549 cells no viral proteins were visible at any time point (Figure 7a). For the Vero cells, the protein profile was very similar to the previous Western Blot. Viral protein increased over time and two polypeptides were visible, although it was difficult to distinguish discrete polypeptide bands due to the high amount of protein present. Because the immunoprecipitation experiment had detected viral protein in infected A549 cells, it was assumed that some viral protein was present in these samples but was undetectable at the concentration of protein loaded on the gels. The Western blot was repeated loading double the amount of the same A549 lysate to increase the amount of antigen. As with the previous experiment, no proteins were visible for the A549 samples (results not shown) demonstrating significantly reduced viral protein synthesis in A549 cells. To confirm this observation, a second experiment was conducted, again using double the amount of protein for A549 samples than for Vero samples. In this case, viral protein was detected in lysates from both cell lines at 24hrs. Substantially more viral protein was present in infected Vero cells than A549 cells at later time points

even when two times as much A549 lysate was loaded. A single viral protein was detected in the A549 samples, and it is presumed that this protein is NP (Figure 7b).

The results of the Western blots suggest that Gw7 viral protein synthesis is reduced from an early stage of infection of A549 cells, either at 24hrs or between 24 and 48 hrs. If L is the key protein in limited growth, the reduced protein synthesis could be due to limited transcription or reduced translation because transcribed mRNA is not correctly modified (i.e., capped).

Figure 6a and b: Immunoprecipitation of Gw7 protein expression in infected Vero and A549 cells.
Protein lysates were prepared from cells infected at MOI of 0.1 and labeled metabolically for 1 hour with ^{35}S-Met at indicated times p.i. Cell equivalents were immunoprecipitated and resolved by 10% SDS PAGE. Mock lanes contain lysates from uninfected cells and molecular size standards are shown on the left side of the SDS gels. The protein visualized in the 75-52 kda range may be HN or NP. a). Immunoprecipitation of Vero and A549 infected from 6hrs p.i. to 72 hrs p.i. b). Immunoprecipitation of Vero and A549 infected from 24hrs p.i. to 96 hrs p.i.

a.



b.

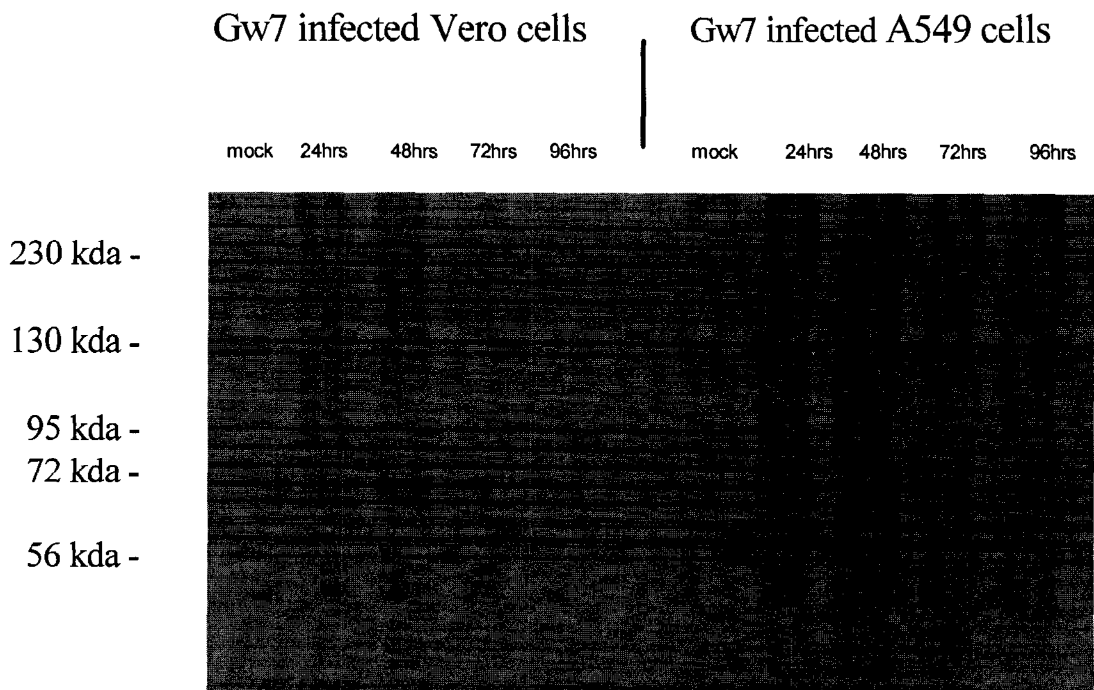
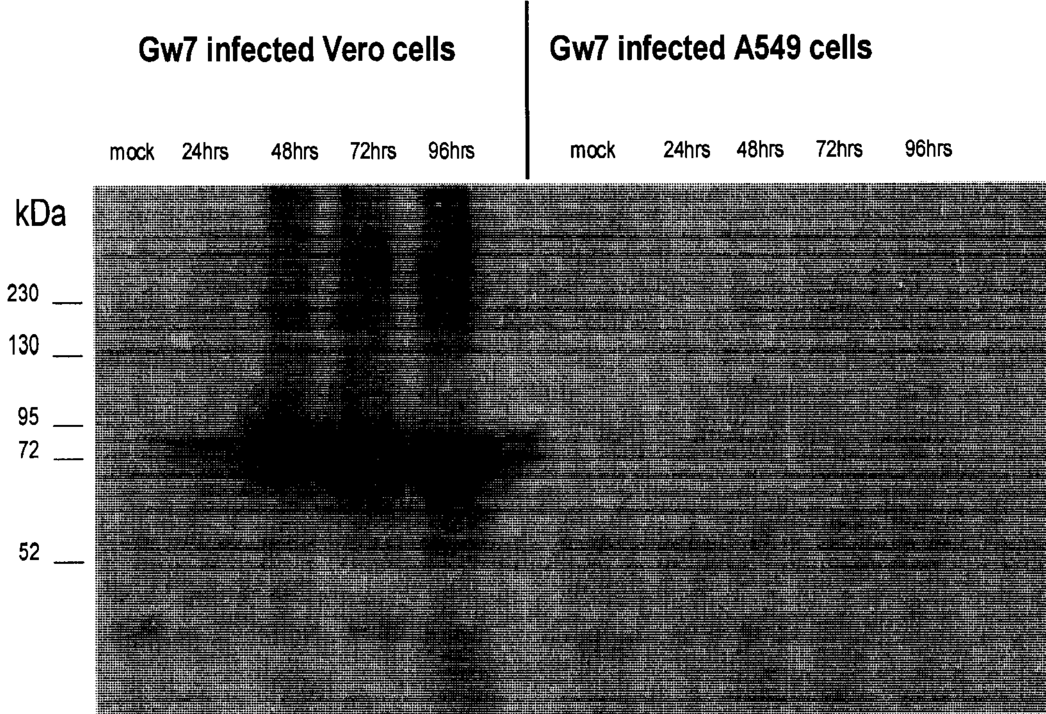
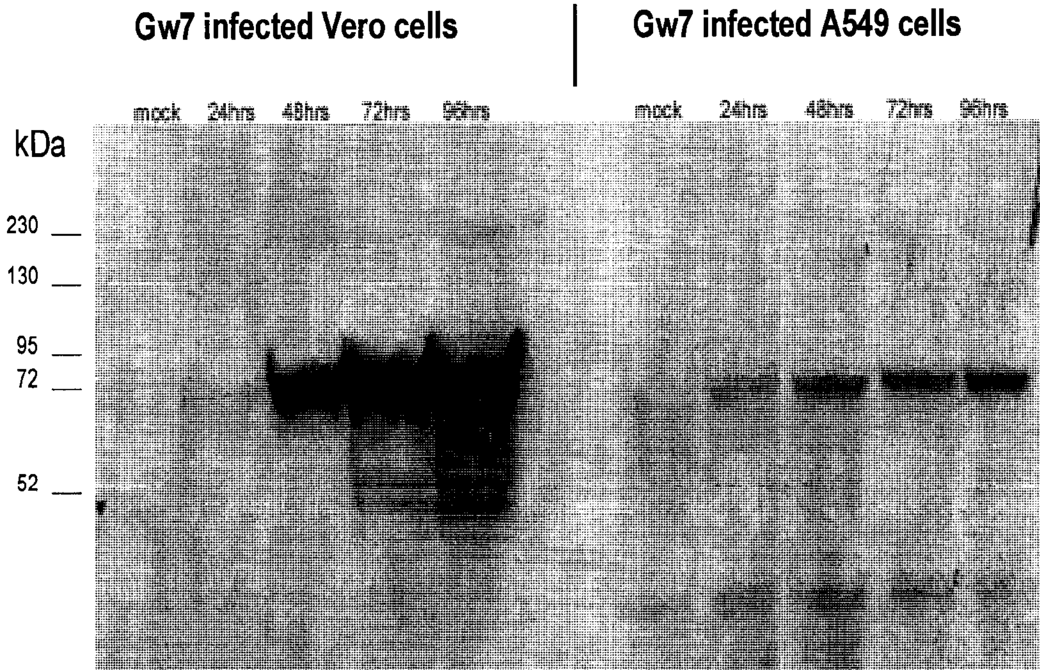


Figure 7a and b: Western Blot of Gw7 protein expression in Vero and A549 cells. Protein lysates of cells infected with a MOI of 0.1 were resolved by 10% SDS PAGE and analyzed by Western Blot. Mock lanes contain lysates from uninfected cells and the sizes of the molecular size standards are shown on the left side of the Western Blots. The larger and smaller proteins visualized in the 72-52 kda range are considered to be HN for the larger band and NP for the smaller band a). 75ug of protein were loaded for Vero and A549 samples with HN and NP visualized at 24hrs p.i. for Vero samples. b). 75ug of protein loaded for Vero samples and 150ug protein loaded for A549 samples with HN or NP visualized at 24hrs p.i. for both Vero and A549 samples.

a.



b.



Viral RNA Synthesis

Since the differential in growth of Gw7 and 1004 10/2 in A549 cells was mapped to the L gene, and the synthesis of Gw7 proteins was affected, it was suspected that mutations in the L of Gw7 might affect synthesis of viral RNA. One way to examine viral RNA synthesis is to detect the amount of tritiated uridine incorporated into newly synthesized RNA and to visualize labeled viral RNA after gel electrophoresis. For this, Vero and A549 cells were inoculated with Gw7 at a MOI of 0.1 for 24, 48, and 72hrs. Twelve hours prior to harvesting lysates, cells were labeled with 50mCi/ml tritiated uridine, and incubated with 5ug/ml actinomycin D to prevent transcription of cellular RNA. Controls for the experiment consisted of two wells of mock cells that labeled with tritiated uridine, and one of these two wells was treated with actinomycin D. At the time points indicated, RNA was extracted and equal volumes (cell equivalents) of RNA from Vero and A549 cells were counted in a liquid scintillation counter. Vero and A549 mock infected cells incubated in the absence of actinomycin D displayed high levels of label, which represented cellular RNA synthesis. In the presence of the drug, the amount of incorporated label was reduced by 50 times for Vero cells and 30 times A549 cells. This indicated that the actinomycin D was inhibiting cellular RNA synthesis. Unfortunately, the amount of tritiated uridine measured in either cell line infected with Gw7 was not above the amount in mock cells in the presence of actinomycin D (results not shown). As well, viral RNA was not visible when isolated RNA was subjected to eletrophoresis.

It was decided that infecting the cells at a higher MOI might allow the detection of viral RNA. Vero and A549 cells were then inoculated at a MOI of 5.0. These

infected cells were treated as before with 5ug/ml actinocmycin D and after 24 hrs timepoints, labeled with 25mCi/ml tritiated uridine for 12 hours. Once again at all time points in Table 9a, RNA counts detected in infected cells at any time point did not exceed the counts of mock infected cells in the presence of actinomycin D and no RNA was visualized on the formaldehyde gel.

Since viral RNA was not observed at time points after 24hrs, it was decided to try to detect viral RNA synthesis at earlier time points of 8, 16, and 24hrs. For this experiment, Vero and A549 cells were infected at a MOI of 1.0 rather than a MOI of 5.0, because of a limited amount of stock of Gw7 stock. After 8 hrs p.i., cells were labeled with tritiated uridine and treated with actinomycin D as in the previous experiments. Lysates from equivalent numbers of infected cells were prepared and equal volumes of lysate were counted. As shown in Table 9b, the amount of tritiated uridine incorporated into RNA in infected cell lines at 8hrs was lower than the mock cells with actinomycin D (background). At 16 hrs the counts were equivalent to background; however, at 24 hrs, the counts in both cell lines were almost twice the background. The results suggest that, at least at MOI of 1.0, viral RNA synthesis begins after 16hrs. The experiment showed a small difference in the amount of tritiated uridine incorporated into viral RNA between the cell lines. Vero cells infected with Gw7 displayed 1.9 times higher counts than A549 cells at 24 hrs. Interestingly, the Vero mock cells infected showed a higher amount of tritiated uridine incorporation than A549 mock cells, suggesting a better ability of Vero cells to synthesize cellular RNA. Another interesting observation is that the amount of label at 8 hrs is reduced in Gw7 infected cells in the presence of actinomycin D compared to mock cell in the

presence of the same drug. This observation is more evident in the infected Vero cells than A549 cells and suggests in the Vero cells that actinomycin D inhibition of cellular RNA synthesis is enhanced in the presence of Gw7 or that viral infection also inhibits cellular RNA synthesis.

The RNA synthesis experiment was not optimized, and infecting cells at a higher MOI might have demonstrated better viral RNA synthesis; also, including time points between 16 and 24 hrs may have provided a better observation of RNA synthesis. The failure to observe viral RNA on the formaldehyde gel indicated that viral RNA was degraded and may explain the low counts observed for all the RNA synthesis experiments. Although the RNA synthesis experiments need to be optimized and repeated, the experiment at MOI of 1.0 for 8, 16, and 24 hrs p.i. do display that Gw7 RNA synthesis in Vero cells is approximately 2X more than in A549 cells.

Table 13: Tritiated Uridine Incorporated into Viral RNA in Vero and A549 cells.

a.

Experiment 1. MOI of 5.0		
	Vero cells	**A549 cells**
Mock	148123.	23576
Mock/Actinomycin D	3670	423
Gw7/ Actinomycin D 36 hrs	1551	248
Gw7/ Actinomycin D 60 hrs	224	418
Gw7/ Actinomycin D 84 hrs	199	128

b.

Experiment 2. MOI of 1.0		
	Vero cells	**A549 cells**
Mock	56422	24507
Mock/Actinomycin D	1063	692
Gw7/ Actinomycin D 8 hrs	537	586
Gw7/ Actinomycin D 16 hrs	1161	776
Gw7/ Actinomycin D 24hrs	1922	1029

Experiment 1 was conducted at a MOI of 5.0 for 32-80 hrs. Experiment 2 was conducted at a MOI of 1 for 8-24 hrs. Time points represent the time after labeling with tritiated uridine. Actinomycin D was used to prevent synthesis of cellular RNA and was included when cells when inoculated with Gw7. Total RNA was extracted from cells, scintillated and counted with a liquid scintillation counter.

Chapter Four

Discussion

The original observation that led to this investigation was the large differential in growth of an attenuated Urabe mumps virus, Gw7, in a human lung epithelial cell line (A549) compared to growth in monkey kidney cells (Vero). Specifically, growth of this virus in A549 cells was reduced by at least 3 $\log_{10}$ compared to growth in Vero cells. In contrast, a more virulent Urabe virus associated with meningitis, 1004 -10/2, replicated as well in the human cell lines as in the Vero cells. The genomes of both viruses had been sequenced and revealed coding differences in 6 of the 9 viral genes, and 2 non coding differences in untranslated regions. Some or all of these genetic differences must cause the difference in tropism between Gw7 and 1004 10/2. This study focused on three aspects of growth in A549 and Vero cells. Firstly, the possible role of interferon on the growth of the two viruses; secondly, determination of where in the replication cycle is Gw7 was interrupted in A549 cells; thirdly, narrowing Gw7 and 1004 10/2 growth differences to specific genes.

The investigation of a role for interferon (IFN) was based on published results, which show that IFN is produced by A549 cells (Wansley and Parks 2002), whereas Vero cells do not produce IFN after viral infection (Emeny and Morgan 1979). Hence, it was logical to speculate that the differences in growth of Gw7 in A549 cells might be due to sensitivity of Gw7 to IFN, while 1004 10/2 would be less sensitive to IFN and thus replicate well in the A549 cells. Mumps virus, like other paramyxoviruses, expresses a V protein that inhibits IFN response in two ways. Firstly, the V protein limits the amount of IFN produced by inhibiting melanoma differentiation associated 5 (mda-5) protein from binding to viral RNA, which results in transcription of IFNβ (Poole *et al.*, 2002). Secondly, the V protein can prevent the signaling through the IFN

receptor after IFN binding, by targeting STAT proteins for ubiquitination (Ulane *et al.*, 2003; Kubota *et al.*, 2001, 2005). The V protein is expressed from the same open reading frame (ORF) as the P protein and the proteins possess the same amino (N) terminus but have different carboxyl (C) terminus regions. A recent publication has shown that a PIV5 virus deficient in V but expressing P still caused a reduction of IFN levels in A549 cultures (Dillion and Parks 2007).

The original sequencing of the genomes of Gw7 and 1004-10/2 showed that the P genes of Gw7 and 1004 10/2 had a single change that affected the P protein, but not the sequence of V. Re-cloning and sequencing of the P gene revealed the gene to be identical for both viruses (Personal communication, N. Nasheri); however, to confirm that IFN did not play a role in growth differences, three types of experiments were completed. All three experiments demonstrated that IFN was unlikely to account for the restricted growth of Gw7 in A549 cells. Firstly, only low levels of IFN were measured in cultures of A549 cells infected with either Gw7 or 1004-10/2. Secondly, treatment of Vero cells with IFNβ at the levels observed in A549 cells did not reduce titres of Gw7 compared to untreated Vero cells. Thirdly, the presence of an antibody with neutralizing activity against IFNβ in the media had no effect on the growth of Gw7 and 1004 10/2-compared to viral growth in the absence of the anti-IFNβ antibody.

Higher doses of IFN have been shown to affect the spread of mumps and PIV5 to neighbouring cells (Carlos et al., 2005; Wansley *et al.*, 2005). With PIV5, it was demonstrated that after 96hrs, the virus overcame these early effects of IFN and was able to spread as efficiently as the virus in the absence of IFNβ (Wansley *et al.*, 2005). Even if higher amounts of IFN had been present in the A549 cultures, other studies

have shown that doses much higher than those used here have little effect on the replication of mumps and other rubulaviruses. Rosas-Murieta *et al.* (2007) showed that 2000U/ml added to cultures of Hela cells only reduced the titres of mumps by less than a log. Similarly, the addition of 1000U/ml and 5000U/ml in cultures of A549 cells did not affect the titre of PIV5 when compared to the viral growth in the absence of IFNβ (Wansley *et al.*, 2005).

My results showed that both Urabe viruses can induce IFN, like other paramyxoviruses (Fleischmann and Simon 1974; Wansley and Parks 2002; Ito and Hosaka 1983). Although IFN was only measured once in A549 cultures, the low amounts of IFN found are consistent with the published literature demonstrating that mumps and most paramyxoviruses limit the production of IFN (Poole *et al.*, 2002; Childs *et al.*, 2007; Wansley and Parks 2002). The amount of IFN induced by Gw7 in the A549 cells was much lower than the amount shown for 1004 10/2, coinciding with the reduced replication of this virus in these cells. Neutralization of the IFN in the A549 cultures made no difference in titres of either virus. This showed that neither Gw7 nor 1004 10/2 were sensitive to the effects of IFN secreted by A549 cells. These results support the findings from the experiment where Vero cells were exposed to amounts of IFN consistent with those found in the A549 cultures, and again no effect on virus titres were observed. Overall, these experiments demonstrate that IFN was not a factor in the difference in growth between these viruses.

Having eliminated a role for IFN, the next logical step was to look at early stages in the replication cycle, which might be affected by the proteins where differences between the viruses were observed. The first protein investigated was the

HN protein, as binding of this protein to sialic acid containing cellular receptors initiates the process of viral entry. The HN proteins of Gw7 and 1004 10/2 differ from each other at 3 amino acid residues, HN335, HN464, and HN526, which may result in differences in binding to cells. Two experiments were conducted to observe whether there were differences in the binding of the two viruses, which would account for differences in the ability of the viruses to infect A549 cells. The first experiment compared the ability of Gw7 to bind to Vero and A549 cells and as well compared binding between Gw7 and 1004 10/2 to each of the cell lines. The second experiment was a heme binding assay that looked at the binding of sialic acid in red blood cells (RBCs) to the HN proteins from Gw7 and 1004 10/2.

No difference was observed in the binding of Gw7 to Vero and A549 cells, indicating that reduced growth of this virus in A549 cells was not due to inability to bind. However, the binding assay may have missed small differences in binding of Gw7 to the two cell types. An alternate approach would be to measure radiolabeled virus binding to each of the cell lines. This technique would have allowed a sensitive quantification of total virus particles binding to each of the cell lines. Not only was binding of Gw7 to A549 cells not reduced, but Gw7 bound better to each of the cell lines than 1004 10/2 and this was statistically significant. Hence, the differences between the viruses at HN335, HN464, and HN526, resulted in differences in binding, although the experiments did not distinguish the effect of each individual difference. It is possible that all three amino acids control the differences in binding between both viruses, but it is also possible that only one of the amino acids might be responsible. Of the three amino acids, only HN335 has been shown to play a role in the binding

preference of Urabe viruses to certain sialic acid linkages (Reyes-Leyva *et al.*, 2006). Recently, it was shown, using an oligosaccharide competition assay, that the change from lysine ('A' viruses) to glutamic acid ('G' viruses) at aa 335 resulted in a more marked binding to α2, 3-linked sialic acid , while 'A' viruses preferred α2, 6 linked-sialic acids (Reyes-Leyva *et al.*, 2007). This preference for specific sialic acid linkages supported the observation that a Urabe 'G' virus replicated less efficiently than an 'A' virus in a neuroblastoma cell line which expressed more α(2,6)- than α(2,3)- linked sialic acid (Santos-Lopez *et al.*, 2006). My results showing higher binding of Gw7 to Vero and A549 cells is consistent with the preference for α(2,3)-linked sialic acids reported for 'G' Urabe viruses, as both Vero cells and type II alveolar cells, like A549 cells , express largely α(2,3)-linked sialic acids (Govorkova *et al.*, 2006, Ibricevic *et al.*, 2006). Reyes-Leyva and colleagues (2006) did not present the HN sequences for the viruses used in their study, so there may be other differences contributing to the preference of sialic acid linkages. There is a possibility that HN464 may affect binding, as the change from asparagines to lysine at this site in 1004-10/2 eliminates a potential N-linked glycosylation site (Yates *et al.*, 1996), and a study on protein structures has shown removal of N-linked glycosylation alters protein conformation (Imperiali and Rickert 1995). Interestingly, this site is close to HN466, which has been reported to affect the binding of the HN protein from 86-1961 mumps virus to RBCs in a heme binding assay (Malik *et al.*, 2007). Although the binding assay used in my experiments showed a significant difference between Gw7 and 1004 10/2 in binding to A549 and Vero cells, this assay only measured binding of infectious virus and it is unknown whether the binding observed for infectious viral particles is a small or large

percentage of total viral particles present in the samples. It is possible that the amount of binding shown for infectious virus is an accurate indicator of total virus particles; however, this is only true if the total viral particle number to pfu of infectious viral is very similar or equal for both viruses.

Chicken RBCs are known to express both $\alpha(2,3)$- and $\alpha(2,6)$-linked sialic acids (Schultze *et al.*, 1992), so any differences in binding to these cells may not reflect preferences for the types of linkages. The heme binding assay confirmed that the ability of Gw7 HN to bind to RBC was not reduced compared to binding of HN of 1004 10/2, and in fact showed slightly higher binding than 1004 10/2 consistent with the other binding experiment. However, the differences between Gw7 and 1004 10/2 were only significant in one of these experiments. One problem with this assay was that the amount of RBC adsorption to the cells was quite low and this may be due to the low number of cells expressing the HN protein. It has been shown in the lab that the number of cells expressing the HN protein after transfection was at maximum 30% of the cells (Personal communications, Sarah Ramzan). Another reason for low hemadsorption may be because I did not use a high enough dose of Zanamivir. This drug was used to inhibit the neuraminidase activity of the HN protein from influencing binding activity by releasing bound RBCs rapidly enough to reduce hemadsorption. The dose used in the assay was based on an influenza binding assay, but titration may have revealed a dose which would better inhibit neuraminidase activity from the HN protein of mumps, and this may have allowed better heme binding in samples over controls. Another concern was the fact that I did not normalize to the absorption of RBCs to the amount of HN expressed on the surface of transfected cells, but rather

controlled for transfection efficiency. However, the HN plasmids for the two viruses were constructed identically and controlled by the same promoter, so it was expected that the two HN proteins should be expressed at similar levels for both viruses. Since my studies were completed, experiments completed by others in the lab showed that the HNs from the two viruses are expressed at equal levels on the cell surface (Personal communications, K. Wright).

An aspect of the HN protein function that was not examined, but which may affect titres of virus, is the neuraminidase activity of the two viruses. Previous experiments in the lab did show that at 48 and 96 hrs, cell associated Gw7 was as reduced as virus in the supernatant after infection of A549 cells, suggesting that low virus yield of Gw7 was not because of a failure of virus to be released from cells via neuraminidase activity (K. Wright, unpublished results). There may be other functions of neuraminidase which could affect growth other than release at the end of the replication cycle. It has been observed with studies of various strains of mumps virus that low or no neuraminidase activity was associated with extensive cell to cell fusion, while strains with high neuraminidase activity showed less cell to cell fusion (Merz and Wolinsky 1981; Waxham and Aroncjwski 1988; Waxham and Wolinsky 1986). It has been suggested by Merz and Wolinsky (1981) that low neuraminidase activity of HN allows longer periods of binding to sialic acid containing membranes and thus, increases the greater chance of fusion, whereas in the case of high neuraminidase activity, high neuraminidase reduces the time associated with sialic acid containing membranes and reduces the probability of fusion. In contrast to these studies, a recent study demonstrated that an attenuated mumps strain displayed low neuraminidase, as

well as low fusion activity when compared to the wild type mumps strain (Malik *et al.*, 2007). Although I did not examine neuraminidase activity mediated by the HN of Gw7 and 1004 10/2, recent work by others showed that neuraminidase activity of an Urabe 'A' virus is higher than a Urabe 'G' virus and this activity was more effective on α(2,3)-linkages of sialic acid (Reyes Leyva *et al.*, 2007). Thus, we can predict that Gw7 might have reduced neuraminidase activity compared to 1004 10/2.

The results of the binding experiments, together with experiments completed by others in the lab on fusion, pointed to differences in the M, SH and L genes of Gw7 and 1004 10/2 as accounting for growth differences. By examining the growth properties of additional Urabe viruses in A549 cells and sequencing the M and SH genes, I demonstrated that the ability to grow in A549 cells was independent of the sequence of SH and M genes. The conflicting growth results for 1005 were surprising. The published sequence for this virus is identical to the sequence determined for 1004 10/2, thus, this virus was predicted to grow similarly to 1004 10/2. Two different stocks of 1005 were used in these growth experiments, and one stock failed to grow in A549, while the second stock did grow. During the course of my experiments, I observed that A549 cells which had been passaged for several months were suddenly able to support growth of Gw7, while newly thawed A549 cells behaved as before – only 1004 10/2 grew well and Gw7 did not. This raised the possibility that something like mycoplasma contamination might have altered the cells. In order to ensure that high growth of 1005 was not occurring because of a similar change in the A549 cells, Gw7 was included in growth experiments as a control. Gw7 grew to expected titres in these experiments, thus the low and high growth of 1005 was not due to some change

in the cells. During the same time, growth of the two stocks of 1005 in Vero cells was as expected, with titres of about 1 x 10^7 pfu/ml. The newer stock of 1005 had undergone at least one additional passage in tissue culture compared to the older stock. This suggests that the newer stock of 1005 may have acquired mutations during passage which caused the difference in growth in A549 cells.

All the experimental evidence points to the L gene as controlling the difference in replication of Gw7 in A549 and Vero cells. The L protein, as shown by sequence comparisons from a number of non-segmented negative strand RNA viruses, consists of six conserved domains, which are separated by a series of variable regions (Poch et al, 1990, Sidhu *et al.*, 1993). Gw7 has one unique mutation located upstream of Domain I (L163) and a second mutation within Domain I (L320), whereas 1004 10/2 is different from other Urabe viruses at sites between Domain I and II (L512), in Domain IV (L1085 and nt 10529), and upstream of Domain V (1871). None of these differences are within the RNA-binding motif found in Domain II, a GDNQ template recognition/phosphodiester bond-forming motif found in Domain III, and a purine nucleotide binding motif found in Domain VI (Sidhu *et al.*, 1993). The high growth of 1004 4/1 in A549 cells indicated that the ability of 1004 10/2 to grow in this cell line does not map to the mutations observed in 1004 10/2 L because these are absent in 1004 4/1, if it is assumed that the sequence of this virus is identical to the published sequence 87-1004 (accession AF314560). Although the sequences of HN, M and SH of 1004-4/1 were verified in the Wright laboratory, and are identical to the sequence for 87-1004, the L has not been sequenced. There is a possibility then, that 1004-4/1 may share some mutations with 1004-10/2 in L. Otherwise, mutations L163 and L320

of L from Gw7 must be controlling tropism for A549 cells. Both mutations lie in regions shown to be important for L and P interactions in PIV5 (Parks 1994) and Sendai virus (Cevik *et al.*, 2007). In addition, the L163 mutation lies in one of two domains important in oligomerization of L in Sendai virus (Cevik *et al.*, 2007). There is a possibility that L163 and L320 affect formation and/or stability of the polymerase complex in A549 cells but not Vero cells. If this is the case, the structure of the polymerase could be unstable and the catalytic ability of the polymerase to transcribe, replicate, and modify mRNA would be impaired.

Studies with other viruses have already identified that mutations in the L gene can affect viral growth in specific cell types. Poor growth of a measles strain in lymphoid B95a cells compared to Vero cells was due to mutations in the N-terminus of the L gene, within a region of amino acids from L149 to L788 (Tahara *et al.*, 2005). The involvement of cellular factors regulating viral transcription has been well documented in negative stranded viruses. The RNA binding protein LA has been shown to be a regulator of viral RNA synthesis, as the protein enhanced transcription/replication of a Rinderpest virus minigenome through binding to the leader sequence of the virus (Raha *et al.*, 2004). For human parainfluenza virus 3 (hPIV3), transcription-was initiated by the addition of actin to ribonucleoprotein (RNP) complexes (De *et al.*, 1993). Tubulin has been shown to positively regulate transcription in vesicular stomatitis virus and other paramyxoviruses such as Sendai and measles viruses (Moyer *et al.*, 1986, 1990), and it has been shown that tubulin acts by binding the M protein and releasing it from RNP, thus allowing transcription to proceed (Ogino *et al.*, 2003). In addition, heat shock proteins (Hsps) Hsp72 and Hsp90

have been identified to be involved in the function of viral polymerases. Transcription of canine distemper virus (CDV) was prevented by antibodies against Hsp72 (Oglesbee *et al.*, 1996), and Hsp72 was shown to enhance transcription of a measles virus minigenome and the ability of the measles virus to replicate in various cell lines and tissues (Parks *et al.*, 1999; Carsillo et al. 2006a, 2006b). In this instance, increased transcription was linked to the association of Hsp72 with the viral NP protein, not L (Oglesbee *et al.*, 1996), and a measles virus selected to be less responsive to Hsp72 had mutations in NP (Carsillo et al. 2006). Hsp90 has been identified to stabilize the L protein from PIV5, Sv41, and hPIV2 and 3 (Connor *et al.*, 2007), as replication of these viruses was reduced and the half-life of the L protein was decreased when Hsp 90 was inhibited (Connor *et al.*, 2007).

My results from the western blots showed that there were noticeably reduced amounts of Gw7 protein in infected A549 cells compared to Vero cells, so much so that viral protein was observed in the A549 lysates only after twice as much lysate was loaded as for Vero cells. In both cell lines, under the conditions used for infection, viral protein was barely detectable at 24 hrs p.i, while in Vero cells, the amount of viral protein increased by 48 hrs. p,i. This indicated that the restriction in growth of Gw7 occurred relatively early during the course of infection in A549 cells, and may be related to the process of transcription. The low amounts of viral protein detected in infected A549 lysates likely accounts for the inability to detect NP despite the fact that NP was visualized in infected Vero cell lysates. Alternately, this protein is not expressed at all in A549 cells, although this is unlikely, as there was some infectious Gw7 released from these cells. When viral protein synthesis was examined using

immunoprecipitation, the differences in amounts of viral protein between Vero and A549 cells were less clear. The first IP experiment displayed high amounts of viral protein at 24 and 72 hrs p.i. in Vero cells compared to A549 cells, while these results were not repeated in the second experiment. The supernatants from both experiments were assayed for infectious virus, and the results were as expected, with 3 logs less virus in the A549 cultures compared to Vero. Hence, it is difficult to explain why differences in viral protein were not more marked. One possible reason is loss of Vero cells relative to A549 cells over the course of infection. Since the samples were prepared in equal volumes, assuming equivalent number of cells, it is possible that Vero samples represented fewer cells because the high cytopathic effect (CPE) observed in these cultures. Also, the amount of viral protein was visualized after only a 1hr labeling period; longer periods of labeling might have shown a consistent difference between viral protein levels after infection of the two types of cells. Lastly, this experiment used a different human anti-mumps serum than the mouse antiserum used for the western blot experiments. The mouse serum was shown to be of high titre (Wright *et al.*, 2000), while the titre of the human serum is unknown, so it is possible that the mouse anti-serum was more efficient at detecting differences in viral protein.

The experiments comparing RNA synthesis did not display the differences in polymerase activity in the two cells types that were predicted, although in none of the experiments was there much virus-specific incorporation of label in either cell type. And in no case was visualization of viral RNA in gels achieved. This suggested that degradation of viral RNA during preparation of the samples may account, at least partially, for the low amount of virus specific counts observed. Alternately, the amount

of viral RNA synthesized under the conditions used for infection was not enough to be visualized on in a gel. Using a higher MOI and infection of a larger number of cells may have shown higher amounts of viral polymerase activity and visualization of viral RNA in the cell lines. However, I was unable to do the experiment with the higher MOI because of limited stocks of Gw7, which replicates to relatively low titre, even in Vero cells. Also, the timing, duration of labeling and amount of label used could be better optimized. In other viruses, similar experiments have been conducted with MOI= 3, and labeling with 10uCi/ml for 24 hours at infection (Storey 1987). Other approaches to observing and measuring the synthesis of viral RNA in the two cell types would have been to use Northern blots, Quantitative PCR, or Nuclease Protection Assay. Having said that, in one experiment where there was incorporation of label into viral RNA, there was a trend to higher counts in Vero than A549 cells infected with Gw7 after 24hrs. Although the difference in viral RNA synthesis in the Vero and A549 cells was small, this experiment suggests that there may be a difference in Gw7 RNA synthesis between the two cell lines.

In the Urabe model, differences in some cellular protein between Vero and A549 cells must account for the differences in viral protein synthesis and replication of Gw7 in Vero and A549 cells, whereas differences between the L proteins of Gw7 and 1004-10/2 may explain the growth differences between Gw7 and 1004 10/2 in the latter cells. Either the cellular proteins must have different sequences in human and monkey cells, or Vero cells have more or less of these cellular factors than A549 cells. For example, Vero cells may express more Hsp90, which may stabilize the L protein from Gw7, whereas in A549 cells a reduced amount of this protein may not be

sufficient to stabilize the protein from this virus while the L protein of 1004 10/2 may be less dependent on Hsp90. Connor et al (2007) has been noted that Hsp90 needs co-chaperones to stabilize the L protein and it is possible that other Hsps such as Hsp72 may act as co-chaperones to assist in Hsp90 stabilization of the L protein. To test this hypothesis, the effects of Hsp90 inhibitors on the growth of Gw7 and 1004 10/2 in Vero and A549 cells could be tested. If an L specific antibody was available, Western blots or Immunoprecipitation could investigate the stability of L proteins from the two viruses in the two cells lines, in the presence and absence of Hsp90 inhibitors. Thirdly, the amount of Hsp90 and other Hsps that act as co-chaperones could be compared in the two cells lines by Western blot.

The original observation of growth restriction of Gw7 in A549 cells suggested that lack of growth in human cells might be a marker for *in vivo* attenuation of the mumps virus. A5 and A9, the other viruses, which showed reduced growth in A549 cells, have not been assessed for virulence. At the same time, 1005, which is known to be virulent, did not replicate well in the A549 cells. So, it does not appear that growth properties in human cells correlate with *in vivo* attenuation or virulence.

Despite this, the mutations at L163 and L320 in Gw7 may still contribute to the attenuation of this virus, and likewise, the virulence of 1004 10/2 could be increased by the different residues at L1871, L512, and L1085. However, the sequence at most of the sites where 1004 and 1005 share unique sequences, including L512, L1085 have been selected in Urabe viruses passaged in chick embryo fibroblasts (CEF) or Vero cells, and these viruses and have reduced neurovirulence in the rat model compared to the starting vaccine mix of viruses (Sauder *et al.*, 2006). Thus, this study by Sauder

(2006) suggests that the presence of 1004 10/2 sequence at L512, L1085, and the non-coding change at nt10529 may not be associated with the virulence of 1004 10/2. This leaves the L1871 mutation as potentially being important for virulence.

Even though my study and previous experiments have eliminated the difference in SH, HN, and F as factors in controlling tropism, the differences in these genes can contribute the *in vivo* phenotype of attenuation of Gw7 and virulence of 1004 10/2. The SH protein has been previously shown not to be essential for viral growth in cell culture (Takeuchi *et al.*,1996), but this protein is involved in inhibiting apoptosis by blocking TNF-α, so it could still play a role in virulence *in vivo* (Wilson *et al.*, 2006). The differences in F and HN of Gw7 and 1004 10/2 can also contribute to attenuation or increase virulence as shown in studies of other mumps strains by Lemon (2007), Kovamees (1990), and Rubin (2003). Lemon (2007) showed that increased neurovirulence in the rat neurovirulence assay was due to a single mutation in the F protein. Kovamess (1990) showed that a single mutation at HN360 resulted in the reduced neurovirulence of a mutant Kilham strain of mumps in a hamster model. In another study, Rubin (2003) showed that reduced neurovirulence of the 88-1961 mumps strain after passage in CEF was due to single mutations in F, HN, and L.

Overall, the experiments in this study have narrowed control of cell tropism of Gw7 and 1004 10/2 in A549 cells to the differences in L. The differences in growth of both viruses were not due to IFN, the changes in HN and SH were shown not to contribute to tropism, and sequencing of M did not confirm the original difference observed in this gene. Future work that could be conducted to further investigate the cause of cell tropism would be to measure polymerase activity of Gw7 in A549 and

Vero cells by constructing a minigenome for Gw7, and as well measuring polymerase activity with *in vitro* polymerase assays using lysates from Gw7 infected Vero and A549 cells. In addition, polymerase activity could be investigated by observing early transcription from the Gw7 genome by Real Time PCR. Cellular protein interactions in A549 and Vero cells with components of the RNAP complex from each virus can be determined by pull down of complexes after transfection of the proteins of the complex (NP, L, and P) into cells. Non-viral proteins which consistently are associated with the complexes can be analyzed by MS. To further support the mapping of cell tropism to L, or to rule out the effects of other viral genes, reverse genetics can be used to introduce the Gw7 sequences into a 1004-10/2 genome, and rescued viruses can be assessed for growth in A549 cells. In addition, reverse genetics can be used on L from Gw7 to observed the relevance of each individual difference from L in controlling tropism in A549 cells. Further investigation of Gw7 and 1004 10/2 in the A549 human cell line and other cell lines would perhaps identify novel cellular proteins essential for paramyxovirus replication. This in turn will increase our understanding of paramyxoviruses virus/host cell interactions and assist in the development of antiviral treatments.

References

ICTVdB - The Universal Virus Database.
2006.http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/
Ref Type: Internet Communication

Mumps outbreak in the maritimes. 2008. http://www.phac-aspc.gc.ca/mumps-oreillons/prof_e.html
Ref Type: Internet Communication

Afzal, M. A., Yates, P. J., Minor, P. D., 1998. Nucleotide sequence at position 1081 of the hemagglutinin-neuramnidase gene in the Mumps Urabe vaccine strain. J.Infect.Dis 177, 265-266.

Andrewes, C. H., Bang, F. B., Burrnet, F. H., 1955. A short description of the Myxovirus group (influenza and related viruses). Virology 1, 176-184.

Balraj, V., Miller, E., 1995. Complications of Mumps. Reviews in Medical Virology 5, 219-227.

Berger, E. A., Murphy, P. M., Farber, J. M., 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu.Rev.Immunol. 17, 657-700.

Bonnet, M. C., Dutta, A., Weinberger, C., Plotkin, S. A., 2006. Mumps vaccine virus strains and aseptic meningitis. Vaccine 24, 7037-7045.

Brown, E. G., Furesz, J., Dimock, K., Yarosh, W., Contreras, G., 1991. Nucleotide sequence analysis of Urabe Mumps vaccine strain that caused meningitis in vaccine recipients. Vaccine 9, 840-842.

Brown, E. G., Dimock, K., Wright, K. E., 1996. The Urabe AM9 Mumps vaccine is a mixture of viruses differing at amino acid 335 of the hemagglutinin-neuraminidase gene with one form associated with disease. J.Inf.Dis. 174, 619-622.

Buynak E.B., Hilleman, M. R., 1966. Live Attenuated Mumps Virus Vaccine. 1. Vaccine Development. Proceedings of the Society for Experimental Biology and Medicine 123, 768-775.

Calain, P., Roux, L., 1993. The Rule of Six, a Basic Feature for Efficient Replication of Sendai Virus Defective Intefering RNA. J Virol 67, 4822-4830.

Carbone, K. M., Wolinsky, J. S., 2001. Mumps Virus. In: B. N. Fields, D. M. Knipe, P. M. Howley, R. A. Lamb, M. A. Martin, B. Roizman, S. E. Straus, D. E. Griffen (Eds.), Fields' virology. Lippincott Williams & Wilkins, Philadelphia, pp. 1381-1400.

Carlos, T. S., Fearns, R., Randall, R. E., 2005. Interferon-induced alterations in the pattern of parainfluenza virus 5 transcription and protein synthesis and the induction of virus inclusion bodies. J Virol 79, 14112-14121.

Carsillo, T., Traylor, Z., Choi, C., Niewiesk, S., Oglesbeel, M., 2006. hsp72, a Host Determinant of Measles Virus Neurovirulence. J Virol. 80, 11031-11039.a

Carsillo, T., Zhang, X., Vasconcelos, D., Niewiesk, S., Oglesbee, M., 2006. A single codon in the nucleocapsid protein C terminus contributes to in vitro and in vivo fitness of Edmonston measles virus. J Virol 80, 2904-2912.b

Cevik, B., Smallwood, S., Moyer, S. A., 2007. Two N-terminal regions of the Sendai virus L RNA polymerase protein participate in oligomerization. Virology 363, 189-197.

Champagne, S., Thomas, E., 1987. A Case of Mumps Meningitis: A Post-Immunization Complication? Can Dis Wkly Rep 13, 155-157.

Childs, K., Stock, N., Ross, C., Andrejeva, J., Hilton, L., Skinner, M., Randall, R., Goodbourn, S., 2007. mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. Virology 359, 190-200.

Clarke, D. K., Sidhu, M. S., Johnson, J. E., Udem, S. A., 2000. Rescue of Mumps Virus from cDNA. J Virol 74, 4831-4838.

Connor, J. H., McKenzie, M. O., Parks, G. D., Lyles, D. S., 2007. Antiviral activity and RNA polymerase degradation following Hsp90 inhibition in a range of negative strand viruses. Virology 362, 109-119.

Corey, E. A., Iorio, R. M., 2007. Mutations in the stalk of the measles virus hemagglutinin protein decrease fusion but do not interfere with virus-specific interaction with the homologous fusion protein. J Virol. 81, 9900-9910.

Crennell, S., Takimoto, T., Portner, A., Taylor, G., 2000. Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. Nat.Struct.Biol. 7, 1068-1074.

Curran, J., Marq, J. B., Kolakofsky, D., 1995. An N-Terminal Domain of the Sendai Paramyxovirus P Protein Acts as a Chaperone for the NP Protein during the Nascent Chain Assembly Step of Genome Replication. J Virol 69, 849-855.

De, B. P., Burdsall, A. L., Banerjee, A. K., 1993. Role of cellular actin in human parainfluenza virus type 3 genome transcription. J Biol.Chem. 268, 5703-5710.

Dillon, P. J., Parks, G. D., 2007. Role for the phosphoprotein P subunit of the paramyxovirus polymerase in limiting induction of host cell antiviral responses. J Virol 81, 11116-11127.

Dugan, A. S., Eash, S., Atwood, W. J., 2005. An N-linked glycoprotein with alpha(2,3)-linked sialic acid is a receptor for BK virus. J Virol 79, 14442-14445.

Egelman, E. H., Wu, S. S., Amrein, M., 1989. The Sendai virus nucleocapsid exists in at least four different helical states. J Virol 63, 2233-2243.

Elango, N., 1989. Complete nucleotide sequence of the matrix protein mRNA of mumps virus. Virology 168, 426-428.

Emeny, J. M., Morgan, M. J., 1979. Regulation of the interferon system: evidence that Vero cells have a genetic defect in interferon production. J Gen Virol 43, 247-252.

Enders, J. F., Kane, L. W., Cohen, S., Levens, J. H., 1945. Immunity in mumps fixing antibody following infection and experiments on immunization by means of inactivated virus and convalenscent human serum. J Exp.Med 81, 93-117.

Enders, J. F., Levens, J. H., Stokes, J., Morris, E. P., Berenberg, W., 1946. Attenuation of virulence with retention of antigenicity of mumps virus after passage in the embryonated egg. J Immunol. 54, 283-291.

Ennis, F. A., Douglas, R. D., Hopps, H. E., Meyer, H. M., Jr., Brown, E. R., Hobbins, T. E., Biehusen, F. C., 1969. Clinical studies with virulent and attenuated mumps viruses. Am.J Epidemiol. 89, 176-183.

Fearns, R., Collins, P. L., 1999. Model for polymerase access to the overlapped L gene of respiratory syncytial virus. J Virol 73, 388-397.

Fearns, R., Collins, P. L., 1999. Role of the M2-1 transcription antitermination protein of respiratory syncytial virus in sequential transcription. J Virol. 73, 5852-5864.

Finch, J. T., Gibbs, A. J., 1970. Observations on the structure of the nucleocapsids of some paramyxoviruses. J Gen Virol. 6, 141-150.

Fleischer, B., Kreth, H. W., 1982. Mumps virus replication in human lymphoid cell lines and in peripheral blood lymphocytes: preference for T cells. Infect Immun 35, 25-31.

Fleischmann, W. R., Jr., Simon, E. H., 1974. Mechanism of interferon induction by NDV: A monolayer and single cell study. J Gen Virol 25, 337-349.

Forsey, T., Mawn, J. A., Yates, P. J., Bentley, M. L., Minor, P. D., 1990. Differentiation of vaccine and wild Mumps viruses using the polymerase chain reaction and dideoxynucleotide sequencing. J Gen.Virol. 71, 987-990.

Frankova, V., Sixtova, E., 1987. Specific IgM antibodies in the saliva of mumps patients. Acta Virol 31, 357-364.

Friedman, R. M., Maheshwari, R. K., 1983. Effect of interferon on vesicular stomatitis virus proteins. Nature 302, 728.

Furesz, J., Contreras, G., 1990. Vaccine-related Mumps meningitis--Canada. Can Dis Wkly Rep 16, 253-254.

Galazka, A. M., Robertson, S. E., Kraigher, A., 1999. Mumps and Mumps vaccine: a global review. Bull World Health Organ 77, 3-14.

Gluck, R., Wegmann, A., Hoskins, J. M., 1986. Just, M, Germanier, R. Rubini, a new live attenuated Mumps vaccine virus strain for human diploid cells. Dev Biol Stand 65, 29-35.

Goh, K. T., 1999. Resurgence of mumps in Singapore caused by the Rubini mumps virus vaccine strain. Lancet 354, 1355-1356.

Gorden, I., Pavri, K., Cohen, S. M., 1956. Response of ferrets to mumps virus. J Immunol. 76, 328-333.

Gorden, J. E., Kilham, L., 1949. Ten years in the epidemiology of mumps. American Journal of the Medical Sciences 218, 338-359.

Gotoh, B., Komatsu, T., Takeuchi, K., Yokoo, J., 2002. Paramyxovirus strategies for evading the interferon response. Rev.Med Virol 12, 337-357.

Govorkova, E. A., Murti, G., Meignier, B., De Taisne, C., Webster, R. G., 1996. African Green Monkey Kidney (Vero) Cells Provide an Alternative Host Cell System for Influenza A and B Viruses. J Virol. 70, 5519-5524.

Gray, J. A., Burns, S. M., 1989. Mumps vaccine meningitis. Lancet 2, 927a.

Gray, J. A., Burns, S. M., 1989. Mumps meningitis following measles, Mumps, and rubella immunisation. Lancet. 2, 98b.

Grdzelishvili, V. Z., Smallwood, S., Tower, D., Hall, R. L., Hunt, D. M., Moyer, S. A., 2005. A single amino acid change in the L-polymerase protein of vesicular stomatitis virus completely abolishes viral mRNA cap methylation. J Virol 79, 7327-7337.

Grdzelishvili, V. Z., Smallwood, S., Tower, D., Hall, R. L., Hunt, D. M., Moyer, S. A., 2006. Identification of a new region in the vesicular stomatitis virus L polymerase protein which is essential for mRNA cap methylation. Virology 350, 394-405.

Habel, K., 1951. Vaccination of Human Beings against Mumps: Vaccine Adminstered at the Start of an Epidemic. II. am.j.hyg 54, 295-311.

Habel, K., 1945. Cultivation of Mumps virus in the developing chick embryo and its application to studies of immunity to Mumps in man. Public Health Reports 60, 201-212.

Habel, K., 1951. Vaccination of Human Beings against Mumps: Vaccine Adminstered at the Start of an Epidemic. I. Incidence and Severity of Mumps in Vaccinated and Control Groups. am.j.hyg 54, 295-311.

Henle, G., Henle, W., Wendell, K. K., Rosenberg, P., 1948. Isolation of Mumps virus from human being with induced apparent or inapparent infections. J Exp Med 88, 223-232.

Herrler, G., Compans, R. W., 1983. Posttranslational modification and intracellular transport of mumps virus glycoproteins. J Virol. 47, 354-362.

Hockin, J. C., Furesz, J., 1988. Mumps meningitis, possibly vaccine-related--Ontario. Can Dis Wkly Rep. 14, 209-211.

Homma, M., Tamagawa, S., 1973. Restoration of the Fusion Activity of L Cell-borne Sendal Virus by Trypsin . J Gen Virol 19, 423-426.

Horikami, S. M., Curran, J., Kolakofsky, D., Moyer, S. A., 1992. Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. J Virol. 66, 4901-4908.

Hourard, S., Varsanyi, T. M., Milican, F., Norrby, E., Bollen, A., 1995. Protection of hamster against experimental mumps virus (MuV) infection by antibodies raised against the MuV surface glycoproteins expressed from recombinant vaccinia virus vectors. J Gen Virol 76, 421-423.

Huntley, C. C., De, B. P., Banerjee, A. K., 1997. Phosphorylation of Sendai virus phosphoprotein by cellular protein kinase C zeta. J Biol.Chem. 272, 16578-16584.

Hwang, L. N., Englund, N., Das, T., Banerjee, A. K., 1999. Optimal Replication Activity of Vesicular Stomatitis Virus RNA Polymearse Requires Phosphorylation of a Residue(s) at Carboxy-Terminal Domain II of Its Accessory Subunit, Phosphoprotein P. Virology 73, 5613-5620.

Ibricevic, A., Pekosz, A., Walter, M. J., Newby, C., Battaile, J. T., Brown, E. G., Holtzman, M. J., Brody, S. L., 2006. Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. J Virol 80, 7469-7480.

Ilonen, J., Salmi, A., Tuokko, H., Herva, E., Penttinen, K., 1984. Immune Responses to Live Attenuated andInactivated Mumps Virus Vaccines in Seronegative and Seropositive Young Adult Males. J Med Virol 13, 331-338.

Imperiali, B., Rickert, K. W., 1995. Conformational implications of asparagine-linked glycosylation. Proc.Natl.Acad.Sci.U.S.A 92, 97-101.

Iorio, R. M., Field, G. M., Sauvron, J. M., Mirza, A. M., Deng, R., Mahon, P. J., Langedijk, J. P., 2001. Structural and functional relationship between the receptor recognition and neuraminidase activities of the Newcastle disease virus hemagglutinin-

neuraminidase protein: receptor recognition is dependent on neuraminidase activity. J Virol 75, 1918-1927.

Ito, Y., Hosaka, Y., 1983. Component(s) of Sendai virus that can induce interferon in mouse spleen cells. Infect Immun. 39, 1019-1023.

Johnson.C.D, Goodpasture, C. D., 1934. An investigation of the etiology of mumps. J.Exp.Med 59, 1-19.

Kaushik, R., Shaila, M. S., 2004. Cellular casein kinase II-mediated phosphorylation of rinderpest virus P protein is a prerequisite for its role in replication/transcription of the genome. J Gen.Virol. 85, 687-691.

Kingston, R. L., Baase, W. A., Gay, L. S., 2004. Characterization of nucleocapsid binding by the measles virus and Mumps virus phosphoproteins. J Virol 78, 8630-8640.

Kovamees, J., Rydbeck, R., Orvell, C., Norrby, E., 1990. Hemagglutinin-neuraminidase (HN) amino acid alterations in neutralization escape mutants of Kilham mumps virus. Virus Res. 17, 119-129.

Kubota, T., Yokosawa, N., Yokota, S., Fujii, N., 2001. C terminal CYS-RICH region of mumps virus structural V protein correlates with block of interferon alpha and gamma signal transduction pathway through decrease of STAT 1-alpha. Biochem.Biophys.Res.Commun. 283, 255-259.

Kubota, T., Yokosawa, N., Yokota, S., Fujii, N., Tashiro, M., Kato, A., 2005. Mumps virus V protein antagonizes interferon without the complete degradation of STAT1. J Virol 79, 4451-4459.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lamb, R. A., Kolakofsky, D., 2001. *Paramyxoviridae*: the viruses and their replication. In: B. N. Fields, D. M. Knipe, S. E. Straus, B. Roizman, M. A. Martin, R. A. Lamb, D. E. Griffen (Eds.), Fields virology. Lippincott Williams & Wilkins, Philadelphia, pp. 1305-1340.

Lawrence, M. C., Borg, N. A., Streltsov, V. A., Pilling, P. A., Epa, V. C., Varghese, J. N., Kimm-Breschkin, J. L., Colman, P. M., 2004. Structure of the haemagglutinin-neuraminidase from human parainfluenza virus type III. J Mol.Biol. 335, 1343-1357.

Lemon, K., Rima, B. K., McQuaid, S., Allen, I. V., Duprex, W. P., 2007. The F gene of rodent brain-adapted mumps virus is a major determinant of neurovirulence. J Virol 81, 8293-8302.

Levitt, L. P., Mahoney, D. H. J., Casey, H. L., Bond, J. O., 1970. Mumps in a general population. A sero-epidemiologic study. Am J Dis Child 120, 134-138.

Malik, T., Wolbert, C., Mauldin, J., Sauder, C., Carbone, K. M., Rubin, S. A., 2007. Functional consequences of attenuating mutations in the haemagglutinin neuraminidase, fusion and polymerase proteins of a wild-type mumps virus strain. J Gen Virol 88, 2533-2541.

Maximova, O., Dragunsky, E., Taffs, R., Snoy, P., Cogan, J., Marsden, S., Levenbook, I., 1996. Monkey neurovirulence test for live mumps vaccine. Biologicals 24, 223-224.

Mazumder, B., Barik, S., 1994. Requirement of casein kinase II-mediated phosphorylation for the transcriptional activity of human respiratory syncytial viral phosphoprotein P: transdominant negative phenotype of phosphorylation-defective P mutants. Virology 205, 104-111.

Merz, D. C., Wolinsky, J. S., 1981. Biochemical Features of Mumps Virus Neuraminidases and Their Relationship with Pathogenicity. Virology 114, 218-227.

Mori, C., Tooriyama, T., Imagawa, T., Yamanishi, K., 1997. Nucleotide sequence at postition 1081 of the hemagglutinin-neuraminidase gene. J.Infect.Dis 175, 1548.

Morrison, T. G., 2003. Structure and function of a paramyxovirus fusion protein. Biochim.Biophys.Acta 1614, 73-84.

Moyer, S. A., Baker, S. C., Lessard, J. L., 1986. Tubulin: a factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNAs. Proc.Natl.Acad.Sci.U.S.A 83, 5405-5409.

Moyer, S. A., Baker, S. C., Horikami, S. M., 1990. Host cell proteins required for measles virus reproduction. J Gen Virol 71 (Pt 4), 775-783.

Muhlemann, K., 2004. The molecular epidemiology of mumps virus. Infect Genet.Evol. 4, 215-219.

Murphy, S. K., Parks, G. D., 1997. Genome Nucleotide Lenghts That Are Divisible by Six Are Not Essential but Enhance Repliation of Defective Interfering RNAs of the Paramyxovirus Simian Virus 5. Virology 232, 145-157.

Murphy, S. K., Parks, G. D., 1999. RNA replication for the paramyxovirus simian virus 5 requires an internal repeated (CGNNNN) sequence motif. J Virol. 73, 805-809.

Murray, M. W., Lewis, M. J., 1989. Mumps meningitis following measles, Mumps, and rubella immunisation. Lancet. 2, 394-395.

Nakhasi, H. L., Zheng, D., Callahan, L., Dave, J. R., Liu, T. Y., 1989. Rubella virus: mechanism of attenuation in the vaccine strain (HPV77). Virus Res. 13, 231-243.

Ogino, T., Iwama, M., Ohsawa, Y., Mizumoto, K., 2003. Interaction of cellular tubulin with Sendai virus M protein regulates transcription of viral genome. Biochem.Biophys.Res.Commun. 311, 283-293.

Ogino, T., Kobayashi, M., Iwama, M., Mizumoto, K., 2005. Sendai virus RNA-dependent RNA polymerase L protein catalyzes cap methylation of virus-specific mRNA. J Biol.Chem. 280, 4429-4435.

Oglesbee, M. J., Liu, Z., Kenney, H., Brooks, C. L., 1996. The highly inducible member of the 70 kDa family of heat shock proteins increases canine distemper virus polymerase activity. J Gen Virol. 77, 2125-2135.

Olmsted, R. A., Collins, P. L., 1989. The 1A Protein of Respiratory Syncytial Virus Is an Integral Membrane Proten Present as Multiple, Structurally Distinct Species. J Virol 63, 2019-2029.

Ong, G., Gohb, K. T., Maa, S., Chew, S. K., 2005. Comparative efficacy of Rubini, Jeryl-Lynn and Urabe mumps vaccine in an Asian population Journal of Infection. J.Infect. 294-298.

Orvell, C., 1984. The Reactions of Monoclonal Antibodies with Structural Proteins of Mumps Virus. J Immunol. 132, 2622-2629.

Orvell, C., 1978. Structural polypeptides of mumps virus. J Gen Virol 41, 527-539.

Parks, G. D., 1994. Mapping of a region of the paramyxovirus L protein required for the formation of a stable complex with the viral phosphoprotein P. J Virol 68, 4862-4872.

Paterson, R. G., Lamb, R. A., 1990. RNA Editing by G-Nucleotide Insertion in Mumps Virus P-Gene mRNA Transcripts. J Virol 64, 4137-4145.

Peltola, H., Kulkarni, P. S., Kapre, S. V., Paunio, M., Jadhav, S. S., Dhere, R. M., 2007. Mumps outbreaks in Canada and the United States: time for new thinking on Mumps vaccines. Clin Infect Dis 45, 459-466.

Perry, K. R., Brown, D. W., Parry, J. V., 1993. Detection of measles, Mumps, and rubella antibodies in saliva using antibody capture radioimmunoassay. J Med Virol 40, 235-240.

Plotkin, S. A., 2004. Mumps Vaccine. In: S. A. Plotkin, W. A. Orenstein, P. A. Offit (Eds.), **Vaccines**. Saunders, New York, pp. 441-470.

Poch, O., Blumberg, B. M., Bougueleret, L., Tordo, N., 1990. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. J Gen Virol 71, 1153-1162.

Poole, E., He, B., Lamb, R. A., Randall, R. E., Goodbourn, S., 2002. The V proteins of simian virus 5 and other paramyxoviruses inhibit induction of interferon-beta. Virology 303, 33-46.

Raha, T., Pudi, R., Das, S., Shaila, M. S., 2004. Leader RNA of Rinderpest virus binds specifically with cellular La protein: a possible role in virus replication. Virus Res. 104, 101-109.

Reef, S., Dayan, G., Bellini, W., Barskey, A., Redd, S., Bi, D., Rota, P., Rota, J. Brief Report: Update: Mumps Activity---United States, January 1-October 7, 2006. Centers for Disease Control and Prevention (CDC) 55, 1152-1153. 2006.

Reyes-Leyva, J., Banos, R., Rorraz-Arguello, M., Santos-Lopez, G., Rosas, N., Alvarado, G., Herrera, I., Vallejo, V., Tapia-Ramirez, J., 2007. Amino acid change 335 E to K affects the sialic-acid-binding and neuraminidase activities of Urabe AM9 mumps virus hemagglutinin-neuraminidase glycoprotein. Microbes Infect 9, 234-240.

Rosas-Murrieta, N., Herrera-Camacho, I., Vallejo-Ruiz, V., Millan-Perez-Pena, L., Cruz, C., Tapia-Ramirez, J., Santos-Lopez, G., Reyes-Leyva, J., 2007. Differential sensitivity to interferon influences the replication and transcription of Urabe AM9 mumps virus variants in nerve cells. Microbes.Infect 9, 864-872.

Rubin, S., Mauldin, J., Chumakov, K., Vanderzanden, J., Iskow, R., Carbone, K., 2006. Serological and phylogenetic evidence of monotypic immune responses to different mumps virus strains. Vaccine 24, 2662-2668.

Rubin, S. A., Snoy, P. J., Wright, K. E., Brown, E. G., Reeve, P., Beeler, J. A., Carbone, K. M., 1999. The mumps virus neurovirulence safety test in Rhesus monkeys: a comparison of mumps virus strains. J Infect Dis. 180, 521-525.

Rubin, S. A., Pletnikov, M., Taffs, R., Snoy, P. J., Kobasa, D., Brown, E. G., Wright, K. E., Carbone, K. M., 2000. Evaluation of a neonatal rat model for prediction of mumps virus neurovirulence in humans. J Virol 74, 5382-5384.

Rubin, S. A., Amexis, G., Pletnikov, M., Li, Z., Vanderzanden, J., Mauldin, J., Sauder, C., Malik, T., Chumakov, K., Carbone, K. M., 2003. Changes in mumps virus gene sequence associated with variability in neurovirulent phenotype. J Virol 77, 11616-11624.

Russell, R., Paterson, R. G., Lamb, R. A., 1994. Studies with cross-linking reagents on the oligomeric form of the paramyxovirus fusion protein. Virology 199, 160-168.

Sanderson, C. M., Wu, H. H., Nayak, D. P., 1994. Sendaivirus M Protein binds independently to either the F or the HN glycoprotein in vivo. J Virol. 69-76.

Santos-Lopez, G., Cruz, C., Pazos, N., Vallejo, V., Reyes-Leyva, J., Tapia-Ramirez, J., 2006. Two clones obtained from Urabe AM9 mumps virus vaccine differ in their replicative efficiency in neuroblastoma cells. Microbes Infect 8, 332-339.

Sauder, C. J., Vandenburgh, K. M., Iskow, R. C., Malik, T., Carbone, K. M., Rubin, S. A., 2006. Changes in mumps virus neurovirulence phenotype associated with quasispecies heterogeneity. Virology 350, 48-57.

Scheid, A., Choppin PW, 1974. Identification of biological activities of Paramyxovirus glycoprotein. Activation of cell fusion, hemolysis, and infectivity of proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology 57, 475-490.

Schmitt, A. P., He, B., Lamb, R. A., 1999. Involvement of the cytoplasmic domain of the hemagglutinin-neuraminidase protein in assembly of the paramyxovirus simian virus 5. J Virol 73, 8703-8712.

Schultze, B., Cavanagh, D., Herrler, G., 1992. Neuraminidase treatment of avian infectious bronchitis coronavirus reveals a hemagglutinating activity that is dependent on sialic acid-containing receptors on erythrocytes. Virology 189, 792-794.

Shah, D., Wright, K. E., Rubin, S., Videl, S. Identification of genetic mutations associated with attenuation and changes in tropisms of Urabe mumps virus. J Med Virol . 2007.
Ref Type: In Press

Shiroki, K., Ishii, T., Aoki, T., Ota, Y., Yang, W. X., Komatsu, T., Ami, Y., Arita, M., Abe, S., Hashizume, S., Nomoto, A., 1997. Host range phenotype induced by mutations in the internal ribosomal entry site of poliovirus RNA. J Virol 71, 1-8.

Sidhu, M. S., Menonna, J. P., Cook, S. D., Dowling, P. C., Udem, S. A., 1993. Canine distemper virus L gene: sequence and comparison with related viruses. Virology 193, 50-65.

Smorodintsev, A., Luzyanina, T. Y., Milcutskaya, B. A., 1963. Immunogenic and Reactogenic Properties of Live Tissue Culture Anti-Mumps Vaccine. 7 562, 562-563.

Storey, D. G. Structural Characterization of Human Parainfluenza Virus 3 and Cloning of Viral Specific Genes. 1987.
Ref Type: Thesis/Dissertation

Strazielle, N., Ghersi-Egea, J. F., 2000. Choroid plexus in the central nervous system: biology and physiopathology. J Neuropathol Exp Neurol 59, 561-574.

T.E.C.Jr., 1970. The first detailed description of Mumps by Robert Hamilton, M.D. Pediatrics. 45, 253.

Tahara, M., Takeda, M., Yanagi, Y., 2005. Contributions of matrix and large protein genes of the measles virus edmonston strain to growth in cultured cells as revealed by recombinant viruses. J Virol 79, 15218-15225.

Takeuchi, K., Tanabayashi, K., Hishiyama, M., Yamada, Y. K., Yamada, A., Sugiura, A., 1990. Detection and characterization of mumps virus V protein. Virology 178, 247-253.

Takeuchi, K., Tanabayashi, K., Hishiyama, M., Yamada, A., 1996. The mumps virus SH protein is a membrane protein and not essential for virus growth. Virology 225, 156-162.

Takimoto, T., Taylor, G. L., Crennell, S. J., Scroggs, R. A., Portner, A., 2000. Crystallization of Newcastle disease virus hemagglutinin-neuraminidase glycoprotein. Virology 270, 208-214.

Takimoto, T., Taylor, G. L., Connaris, H. C., Crennell, S. J., Portner, A., 2002. Role of the hemagglutinin-neuraminidase protein in the mechanism of paramyxovirus-cell membrane fusion. J Virol 76, 13028-13033.

Tanabayashi, K., Takeuchi, K., Okazaki, K., Shiyama, M. I. Y. A., 1992. Expression of Mumps Virus Glycoproteins in Mammalian Cells from Cloned cDNAs: Both F and HN Proteins Are Required for Cell Fusion. Virology 187, 801-804.

Thompson, S. D., Laver, W. G., Murti, K. G., Portneri, A., 1988. Isolation of a Biologically Active Soluble Form of the Hemagglutinin-Neuraminidase Protein of Sendai Virus. J Virol. 62, 4653-4660.

Tischer, A., Gerike, E., 2000. Immune response after primary and re-vaccination with different combined vaccines against measles, mumps, rubella. Vaccine 18, 1382-1392.

Turner, P. C., Forsey, T., Minor, P. D., 1991. Comparison of the nucleotide sequence of the SH gene and flanking regions of Mumps vaccine virus (Urabe strain) grown on different substrates and isolated from vaccines. J Gen.Virol. 72, 435-437.

Tyler, K. L., Fields, B. N., 2001. Pathogenesis of viral infection. In: B. N. Fields, D. M. Knipe, D. E. Griffen, P. M. Howley, R. A. Lamb, M. A. Martin, B. Roizman, S. E. Straus (Eds.), Fields Virology. Lippincott, Williams & Wilkins Co, Philadelphia, pp. 173-218.

Ukkonen, P., Granstrom, M., Penttinen, K., 1981. Mumps-Specific Immunoglobulin M and G Antibodies in Natural Mumps Infection as Measured by Enzyme-Linked lmmunosorbent Assay. J Med Virol 8, 131-142.

Ulane, C. M., Rodriguez, J. J., Parisien, J. P., Horvath, C. M., 2003. STAT3 ubiquitylation and degradation by mumps virus suppress cytokine and oncogene signaling. J Virol 77, 6385-6393.

Ulane, C. M., Kentsis, A., Cruz, C. D., Parisien, J. P., Schneider, K. L., Horvath, C. M., 2005. Composition and assembly of STAT-targeting ubiquitin ligase complexes: paramyxovirus V protein carboxyl terminus is an oligomerization domain. J Virol. 79, 10180-10189.

Vasconcelos, D. Y., Cai, X. H., Oglesbee, M. J., 1998. Constitutive overexpression of the major inducible 70 kDa heat shock protein mediates large plaque formation by measles virus. J Gen Virol 79 (Pt 9), 2239-2247.

Villar, E., Barroso, I. M., 2006. Role of Sialic Acid-containing molecules in paramyxovirus entry into the host cell: A minreview. Glycoconj 23, 5-17.

Viola, L., Chiaretti, A., Castorina, M., Tortorolo, L., Piastra, M., Villani, A., Valentini , P., Polidori , G., 1998. Acute hydrocephalus as a consequence of Mumps meningoencephalitis. Pediatr Emerg Care. 14, 212-214.

Wang, Z., Mirza, A. M., Li, J., Mahon, P. J., Iorio, R. M., 2004. An oligosaccharide at the C-terminus of the F-specific domain in the stalk of the human parainfluenza virus 3 hemagglutinin-neuraminidase modulates fusion. Virus Res. 99, 177-185.

Wanger, E. K., Hewlett, M. J., 1999. Basic Virology. Blackwell Science, Malden, Massachusetts.

Wansley, E. K., Parks, G. D., 2002. Naturally occurring substitutions in the P/V gene convert the noncytopathic paramyxovirus simian virus 5 into a virus that induces alpha/beta interferon synthesis and cell death. J Virol 76, 10109-10121.

Wansley, E. K., Dillon, P. J., Gainey, M. D., Tam, J., Cramer, S. D., Parks, G. D., 2005. Growth sensitivity of a recombinant simian virus 5 P/V mutant to type I interferon differs between tumor cell lines and normal primary cells. Virology 335, 131-144.

Waxham, M. N., Wolinsky, J. S., 1986. A Fusing Mumps Virus Variant Selected from a Nonfusing Parent with the Neuraminidase Inhibitor 2-Deoxy-2,3-dehydro-IV-acetylneuraminic Acid. Virology 151, 286-295.

Waxham, M. N., Aroncjwski, J., 1988. Identification of Amino Acids involved in the Sialidase Activity of the Mumps Virus Hemagglutinin-Neuraminadase Protein. Virology 187, 226-232.

Weibel, R. E., Buynak, E. B., Stokes, J. J., Hilleman, M. R., 1970. Persistence of immunity four years following Jeryl Lynn strain live Mumps virus vaccine. Pediatrics. 45, 821-826.

Wilson, R. L., Fuentes, S. M., Wang, P., Taddeo, E. C., Klatt, A., Henderson, A. J., He, B., 2006. Function of small hydrophobic proteins of paramyxovirus. J Virol 80, 1700-1709.

Wright, K. E., Dimock, K., Brown, E. G., 2000. Biological Characteristics of Genetic Variants of Urabe AM9 Mumps Vaccine Virus. Virus Research 67, 49-57.

Yamada, A., Takeuchi K, Hishiyama M, 1988. Intracellular processing of mumps virus glycoproteins. Virology 165, 268-273.

Yamada, A., Takeuchi, K., Tanabayashi, K., Hishiyama, M., Takahashi, Y., Sugiura, A., 2008. Differentiation of the Mumps vaccine strains from the wild viruses by the nucleotide sequences of the P gene. Vaccine 8, 553-557.

Yamanishi, K., Takahashi, M., Kurimura, T., Ueda, S., Minekawa, Y., 1970. Studies on live Mumps virus vaccine. 3. Evaluation of newly developed live Mumps virus vaccine. . 1970 Sep;13(3):157-61. Biken J 13, 157-161.

Yamanishi, K., Takahashi, M., Ueda, S., Minekawa, Y., Ogino, T., 1973. Studies on live Mumps virus vaccine. V. Development of a new Mumps vaccine "AM 9" by plaque cloning. Biken J. 16, 161-166.

Yates, P. J., Afzal, M. A., Minor, P. D., 1996. Antigenic and genetic variation of the HN protein of mumps virus strains. J Gen Virol 77 (Pt 10), 2491-2497.

Yawata, M., 1994. Japan's troubles with measles Mumps-rubella-vaccine (commentary). Lancet 343, 105-106.

Yoshida, T., Nagai, Y., Maeno, K., Iinuma, M., Hamaguchi, M., Matsumoto, T., Nagayoshi, S., Hoshino, M., 1979. Studies on the role M protein in virus assembly using a ts mutant of HVJ (Sendai virus). Virology 92, 139-154.

Yoshida, T., Nagai, Y., Maeno, K., Iinuma, M., Hamaguchi, M., Matsumoto, T., Nagayoshi, S., Hoshino, M., 1979. Studies on the role M protein in virus assembly using a ts mutant of HVJ (Sendai virus). Virology 92, 139-154.

Yuan, P., Thompson, T. B., Wurzburg, B. A., Paterson, R. G., Lamb, R. A., Jardetzky, T. S., 2005. Structural studies of the parainfluenza virus 5 hemagglutinin-neuraminidase tetramer in complex with its receptor, sialyllactose. Structure. 13, 803-815.

Zaitsev, V., von, I. M., Groves, D., Kiefel, M., Takimoto, T., Portner, A., Taylor, G., 2004. Second sialic acid binding site in Newcastle disease virus hemagglutinin-neuraminidase: implications for fusion. J Virol. 78, 3733-3741.

Zhirnov, O. P., Klenk, H. D., 2007. Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling. Apoptosis. 12, 1419-1432.