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Functional Effects of Mutations in Mumps Virus Polymerase Protein

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FUNCTIONAL EFFECTS OF MUTATIONS IN MUMPS VIRUS POLYMERASE PROTEIN

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ABSTRACT

The Urabe AM9 mumps vaccine was removed from use in many countries due to an unacceptable incidence of post-vaccination disease. The vaccine was found to be a mixture of closely related viruses, presumably with differing degrees of attenuation. One virus isolated from the vaccine has been shown to be fully attenuated in a rodent model. This virus, Gw7, is characterized by growth in Vero cells (titers $2x10^6$ pfu/ml) but extremely limited growth in human cell lines ($<10^4$ pfu/ml), while a virulent Urabe virus, 1004, from a patient with post-vaccination meningitis, grows well ($>2x10^7$ pfu/ml) in all the cell lines tested to date. Initial sequencing of the genome of the two viruses has identified genetic differences in 6 of the 7 genes, including the two proteins of the replication complex, the polymerase, L (Large protein), and in the P protein (phosphoprotein). The hypothesis was that the amino acid differences observed in L and P play a role in the differences in attenuation *in vivo* and growth differences in tissue culture cells.

The first objective was to confirm the sequence differences in the L and P genes of the viruses and the sequencing results confirmed all the reported differences in the L gene between 1004 and Gw7 plus identified another sequence difference in the L gene which had not been reported in the original sequencing; it also showed that there was no sequence difference in the P gene of the two viruses.

The next objective was to compare the polymerase activity of the two viruses. Two methods have been employed to measure the polymerase activity of the viruses; *In vivo* incorporation of ^3H UTP in the presence of Actinomycin D, and transcription of

luciferase reporter gene from the Urabe mini-genome construct. For the first assay, the polymerase activity was examined under two different conditions; and in one case, a higher polymerase activity was observed for 1004, but this was not confirmed in the second set of experiments. To compare the polymerase activities of the two viruses with the help of a mini-genome system, the Urabe mini-genome was constructed and the polymerase activity was determined in two ways; using the polymerase complex (L, P and NP proteins) of each virus or the whole viral particles to drive the luciferase expression from the mini-genome. In either case, a significant difference in the polymerase activity of the two viruses was not detected. Thus, it can be concluded that no inherent difference in the polymerase activity between 1004 and Gw7 was observed by the performed experiments in this study.

This thesis is dedicated to all the Baha'i youths in Iran who are deprived of higher education solely because of their beliefs.

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Finally many thank to my family especially my mom and my husband Naeim for their love and support. Without their encouragement I could not be where I am today.

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LIST OF ABBREVIATIONS

CDV	Canine Distemper Virus
EtBr	Ethidium Bromide
GHLS	Group Histologic Lesion Score
HPIV	Human Parainfluenza Virus
LUC	Luciferase
MNVT	Monkey Neurovirulence Test
NDV	Newcastle Disease Virus
ORF	Open Reading Frame
PrE	Promoter Element
RNP	Ribonucleoprotein
RNVT	Rat Neurovirulence Test
SV5	Simian Virus 5
UTR	Untranslated Rigion
VSV	Vesicular Stomatis Virus

Chapter One: Introduction

Mumps disease

Mumps is a viral disease that was first described around the 5th century BC by Hippocrates. The name "mumps" was probably derived from mumbling speech of the patients who suffered from the disease (Bellini and Sever, 2000). In 1934, Johnson and Goodpasture, identified the etiologic agent as a virus by passing the saliva from patients with mumps through a series of filters and then inoculating it into *M. rhesus* and reproducing the disease (Johnson and Goodpasture, 1934).

Mumps virus can infect all tissues and organs systems but mumps disease is mainly characterized by pain and swelling of one or both parotid glands, although asymptomatic mumps occurs in one-third of infections. The involvement of the central nervous system (CNS) has been reported in 11% of clinical mumps cases in unvaccinated populations and mumps was reported to be the most common cause of aseptic meningitis in the United States until 1975 (Carbone and Rubin, 2007). Post pubertal infection with mumps virus may cause orchitis in about 20% to 50% of male patients and pancreatitis can occur in 10% of the cases. Other complications of mumps virus include arthritis, myocarditis, thyroiditis and mastitis (Arguedas, et al, 2004).

Mumps is a worldwide infection. In unvaccinated populations, the highest incidence is in children between the ages of 5 to 9 years (Morgan-Capner, 2004). Mumps is a seasonal infection which mostly occurs in late winter and spring in the northern hemisphere and has a higher prevalence in urban areas; although vaccination has changed the epidemiological pattern significantly. It is also noteworthy to mention that mumps

outbreaks are still reported even among vaccinated populations in developed countries (Carbone and Rubin, 2007).

Mumps virus

Mumps is an enveloped virus which belongs to the *Rubulavirus* genus of the *Paramyxoviridae* family. Parainfluenza virus 5 (previously called simian virus 5 [SV5]) and human parainfluenza virus type 2 (HPIV2) are among other viruses categorized in this genus. Like other members of the *Paramyxoviridae,* mumps has a pleomorphic virion. The virus size ranges from 100 to 600 nm (Fauquet, et al. 2005). Inside the host cell- derived bilayer lipid envelope lies a helical ribonucleocapsid core containing the viral genome which is a non-segmented, single stranded RNA with negative polarity and is comprised of 15,384 nucleotides containing 7 linked transcription units encoding 9 proteins (Figure1.1). The viral glycoproteins are inserted through the envelope and protrude around 12-15 nm from the surface. Between the envelope and the helical nucleocapsid is the viral matrix (M) protein that has an important role in budding and maintaining the structure of the virion (Carbone and Rubin, 2007).

Genome and encoded proteins

As already mentioned, the genome of the mumps virus contains 7 genes encoding 9 proteins including the Nucleoprotein (N, or NP), V/I/phosphoprotein (P), matrix (M) protein, fusion (F) protein, small hydrophobic (SH) protein, hemagglutinin-neuraminidase (HN) protein, and the large (L) protein plus the 3' leader (55 nt) sequence with adjacent 90-nt NP gene untranslated region (UTR) and the 5' trailer (24 nt) sequence

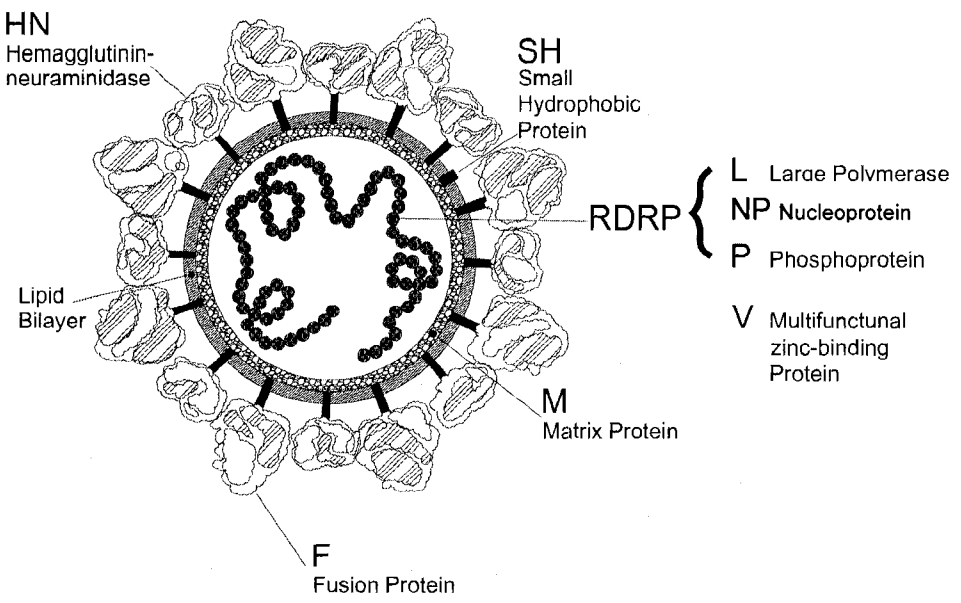

Figure 1.1 Schematic diagram of mumps virus (not drawn to scale). The outer layer surrounding the virion is the host-derived lipid bilayer and underlying the lipid bilayer is the matrix protein. The hemagglutinin-neuraminidase (HN) attachment glycoprotein and the (F) fusion glycoprotein are inserted through the lipid bilayer. The small hydrophobic protein (SH), is shown as a small integral membrane protein. The HN protein is believed to have a stalk region and a globular head, and the F protein consists of two disulfide-linked chains F1and F2. The HN protein is a tetramer, and the F protein a trimer. Inside the virus is located the helical ribonucleoprotein (RNP) which consists of the negative-strand virion RNA, which is encapsidated with the nucleocapsid protein (NP) and is associated with the L and P proteins. Together this complex is called the RNA-dependent RNA polymerase (RDRP) which performs all the polymerase activity of the virus. Adapted from (Lamb and Parks, 2007).

adjacent to the 137-nt L gene untranslated region (Clarke et al., 2000). These regions are essential for controlling transcription and replication. The order of the genes is: 3'NP-P-M-F-SH-HN-L-5'(Figure 1.2).

The NP gene encodes for one protein, the Nucleoprotein (NP), or Nucleocapsid (N) which is comprised of 549 amino acids and encapsidates both genomic (-) sense and antigenomic (+) sense RNA to protect them from cellular ribonucleases (RNases). Studies with related viruses in the *Paramyxoviridae* family have shown that each NP protein is associated with 6 nucleotides to form each turn of the helical nucleocapsid (Kolakofsky et al. 2005). The encapsidated RNA, rather than the naked RNA, is the template for all RNA synthesis. In infected cells, NP protein exists in two forms, one is associated with genomic or anti-genomic RNA in a nucleocapsid structure and a second unassembled soluble form called NP^0 , which has been shown to be associated with P protein in many paramyxoviruses (Figure 1.4). The NP protein, together with P and L proteins, makes the viral RNA dependent RNA polymerase (RDRP) complex (Lamb and Parks, 2007).

The P gene is the only gene in the mumps genome that produces more than one polypeptide. The faithful transcription of the P gene by the viral polymerase generates the V protein, while the Phosphoprotein (P) is made from mRNA which has two non-templated G residues added through an RNA editing process. Insertion of four G residues in the same region generates the I protein, which is a nonstructural protein with an unknown role in the life cycle of the mumps virus. It is 171aa long (Carbone and Rubin, 2007). Therefore, all three proteins share a common N-terminus but have different C-terminal regions (Figure 1.3). The P protein is 391aa long and is heavily phosphorylated

at serine and threonine residues (Lamb and Parks, 2007). It is a cofactor for RDRP and plays an important role in both mRNA synthesis and genome replication which will be discussed later in this chapter (Carbone and Rubin, 2007; Lamb and Parks, 2007). Deletion studies of Sendai virus have shown that residues 1-77 in the N-terminal half of the P protein are essential for P-NP^0 binding, which delivers NP^0 to nascent RNA and leads to encapsidation during RNA replication(Curran et al., 1995;Curran et al., 1994).

The V protein is made up of 224aa and has a cysteine rich C –terminal domain which binds two zinc molecules (Lamb and Parks, 2007). The V protein is known to inhibit interferon (IFN) production and IFN induced responses, interacting with signal transducer and activator of transcription 1 (STAT1), which is important for cellular responses to IFN. This interaction leads to STAT1 ubiquitination and degradation through the proteosome pathway (Kubota et al., 2002;Yokosawa et al., 2002). The V protein has also been shown to block activation of the IFN-β promoter by binding to the IFN-inducible RNA helicase, mda-5 (Andrejeva et al., 2004). It has also been shown that V protein inhibits viral RNA synthesis (Lin et al., 2005) and since it shares the N-terminal with P protein, it has been proposed that it competes with P in forming a complex with NP and thus reducing RNA synthesis (Horikami et al., 1996).

The M gene encodes for the Matrix (M) protein which is comprised of 375aa. It functions in viral assembly and budding. Inside the virion, it associates with the cytoplasmic domains of the viral glycoproteins on one side, and the helical nucleocapsid on the other. In infected cells, M guides the ribonucleoprotein (RNP) to the sites where viral glycoproteins are embedded in cell membrane and mediates budding (Carbone and Rubin, 2007; Lamb and Parks, 2007).

Figure 1.2 Schematic diagram of the mumps virus genome showing genes, intergenic and 3' leader and 5' trailer regions. The genes are shown as boxes that are drawn to approximate scale

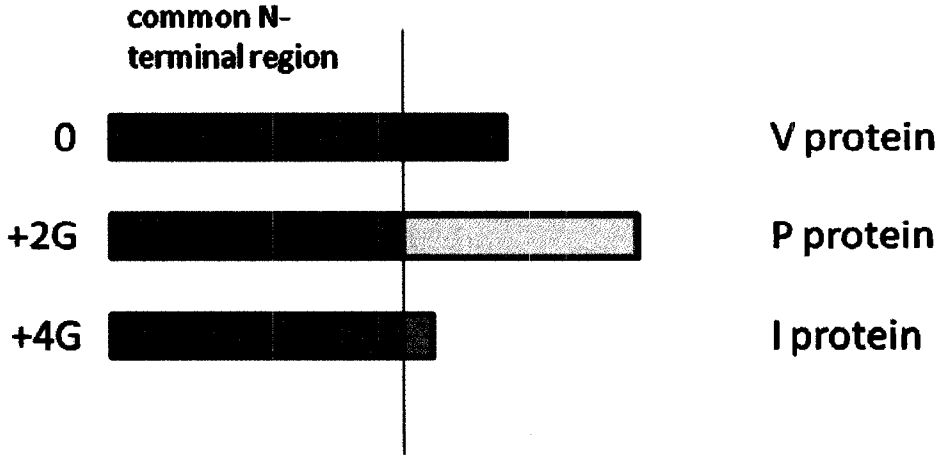

FIGURE 1.3 Schematic diagram of different proteins generated from the P gene in mumps virus. Faithful transcription of the P gene produces the V protein, while the Phosphoprotein (P), is made by adding two non-templated G residues through RNA editing process. Also, the I protein is generated by insertion of four G residues in that same region.

Mumps virus, like other Paramyxoviruses, has two essential envelope glycoproteins; the Fusion (F) and Hemagglutinin-Neuraminidase (HN). Mumps also possesses a third integral membrane protein called the Small-hydrophobic (SH) which has been shown to be non-essential for the life cycle of the virus in tissue culture (Takeuchi et al., 1996). The F protein contains 538aa and mediates membrane fusion at neutral pH which results in the delivery of the viral RNP to the cytoplasm. It is synthesized as an inactive form, F_0, which undergoes N-glycosylation in the endoplasmic reticulum and then is transported to the Golgi network where it is cleaved by Furin between amino acids 102 and 103 to produce two disulfide-linked heterodimers, F_1 and F_2. This cleavage is essential for the proper function of the F protein (Carbone and Rubin, 2007).

The other integral glycoprotein, HN, with 582aa, exists as disulfide-bonded homotetramers and is held in the viral envelope via a hydrophobic domain near the N-terminus. The ectodomain of the protein consists of a stalk that supports a globular head which binds to sialic acid and mediates cell attachment and virus entry. The HN protein also has neuraminidase activity in order to prevent self-aggregation of viral particles during budding from the plasma membrane. The HN protein also is essential for promotion of fusion mediated by the F protein (Carbone and Rubin, 2007; Lamb and Parks, 2007).

The SH protein of mumps virus is only composed of 57aa and in infected cells, it has been shown that the SH is an integral protein with its C-terminal exposed to the cytoplasm (Wilson et al., 2006). It has not been determined whether this protein is part of virions or is only expressed in infected cells. Recent studies have demonstrated that the

SH protein has a role in inhibition of apoptosis in infected cells through blocking the TNF-α pathway (Wilson et al., 2006).

The Large protein (L), encoded by the L gene, is the catalytic subunit of the RDRP and therefore responsible for all of the viral RNA synthesis, including replication and transcription. It also functions in mRNA 5'cap methylation and 3' polyadenylation (Grdzelishvili et al., 2005;Hercyk et al., 1988). The L protein is 2,261aa long and studies on other non-segmented negative sense RNA viruses, including viruses from the *Paramyxoviridae* family, have identified six highly conserved domains in L proteins based on sequence homologies (Figure 1.6). Originally, it was suggested that each of these domains was responsible for at least one of the multiple functions of the L protein (Lamb and Parks, 2007). The function of these domains and other conserved motifs of the L protein will be elaborated in a later section.

The replication cycle

As it has been already mentioned, sialic acid, which is the acyl derivative of neuraminic acid, serves as cell surface receptor for the HN protein of mumps virus. It has been demonstrated that the HN protein of the mumps virus binds to sialic acid types sialyl α2,3 lactose and sialyl α2,6 lactose (Reyes-Leyva et al., 2007). Following the attachment of the HN protein to sialic acid through its globular head, the F protein undergoes a series of conformational changes which expose the conserved hydrophobic domain, causing viral and host cell membrane fusion (Lamb and Parks, 2007; Carbone and Rubin, 2007). Two models have been suggested to rationalize the interaction between

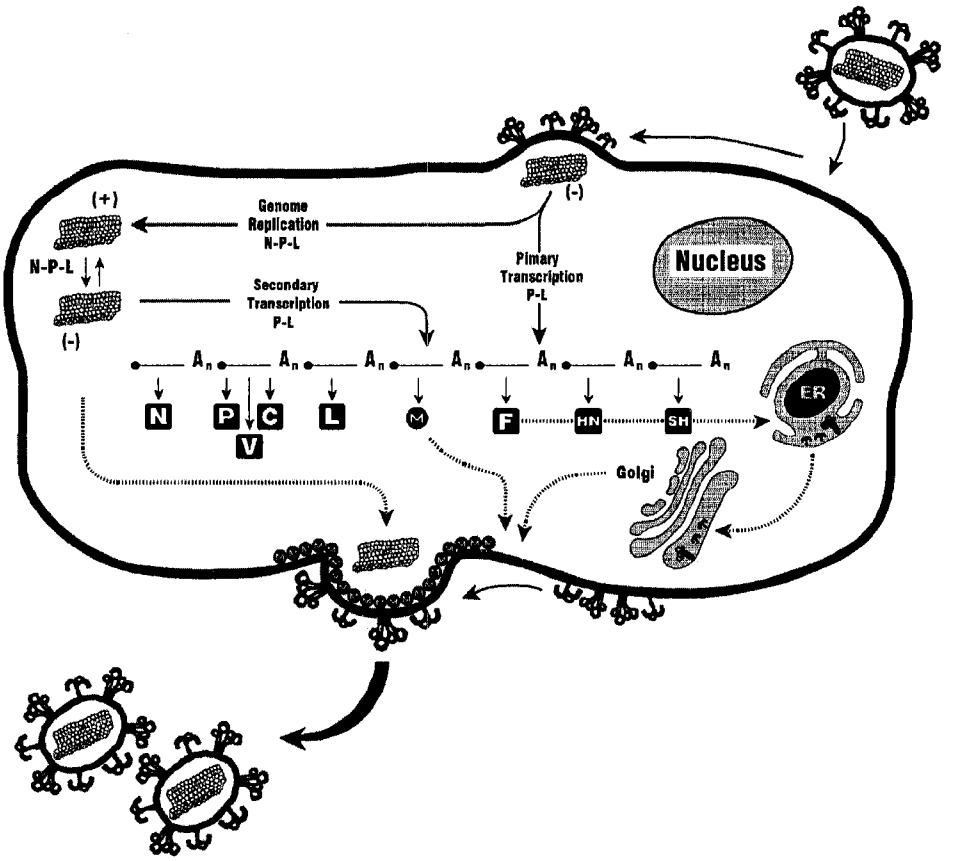

FIGURE 1.4 Schematic diagram of the life cycle of mumps virus. The top of the figure shows an incoming virion that fuses with the plasma membrane to release the (-) sense nucleocapsid in the cytoplasm. Viral mRNAs are indicated by lines with the 5' mRNA cap denoted by a filled circle and 3' poly A tail by A_n. Solid lines denote primary and secondary transcription carried out by a P-L complex and genome replication carried out by an N-P-L complex. Dotted lines denote intracellular transport of nucleocapsid and M protein to the plasma membrane and the viral glycoproteins F, HN, and SH from the ER to Golgi to plasma membrane. The large arrow denotes release of progeny virions from the plasma membrane by a budding process.

HN and F proteins; in the first model, upon binding to sialic acid, HN undergoes a conformational change which allows it to interact with F, which in turn triggers F to its fusion-active conformation (Lamb, 1993). The second model proposes that in the absence of sialic acid, HN can interact with F and then upon binding to sialic acid, F is released and becomes activated for membrane fusion (Lamb et al., 2006;Yuan et al., 2005).

The whole life cycle of mumps virus occurs in the cytoplasm (Figure 1.5). Upon binding and fusion to the host cell membrane, the virus is uncoated and the RNP is released into the cytoplasm where it becomes the template for the accompanied viral RDRP, which starts transcription at the 3' end of the genomic RNP to generate the (+) leader RNA and the successive capped (meG-Cap) and polyadenylated (PolyA) mRNAs. Primary transcription produces all the viral proteins. Because RDRP occasionally fails to reinitiate the transcription of downstream mRNAs at each gene junction, there is a gradient of decreasing transcription from NP to L as it gets further from the 3' end of the genomic RNA (Lamb and Parks, 2007). Viral mRNAs become translated and when there is enough NP^0 protein available, new viral RNA becomes encapsidated as it is transcribed. Under these conditions, instead of terminating and reinitiating transcription at each gene junction, the RDRP uninterruptedly produces an exact complementary (+) sense antigenomic RNA, which is fully encapsidated and serves as the template for genomic RNA synthesis. The promoter that directs transcription and replication is located at the 3' end of the genomic RNA. The 3' end of the antigenomic RNA also contains a promoter for replication which is believed to be stronger than the genomic promoter for RNA replication. In paramyxoviruses, the promoter consists of two discontinuous and

conserved elements called promoter element I (PrE-I) and PrE-II (Walpita & Peters, 2007;Cordey & Roux, 2007;Tapparel et al., 1998).

Secondary transcription occurs when there are abundant progeny genomes available and leads to production of much higher levels of viral mRNA (Clarke et al., 2000;Afzal et al., 1990). Formation of viral ribonucleoprotein (RNP) occurs through a self-assembly process in which association of NP subunits with genomic or antigenomic RNA leads to encapsidation. It has been supposed that there are specific sequences in (+) leader (5' end of the antigenome) and trailer (5' end of the genome) regions responsible for initiating the encapsidation process (Lamb and Parks, 2007). Later on, viral glycoproteins are transported to the host cell membrane, M proteins are positioned beneath the cell membrane where they can associate with both the RNP and envelope glycoproteins, then a bud is induced and virus particles release though neuraminidase activity of HN protein. The whole process is called budding (Lamb and Parks, 2007) (Figure 1.5).

Pathogenesis

Humans are the only natural reservoir for the mumps virus, although experimental infection in several species such as monkey, mouse, rat, hamster and developing chick embryo has been induced (Carbone and Rubin, 2007). The natural infection occurs by droplet spread and the incubation period for the disease is usually 16-18 days. Mumps virus first replicates within the epithelial cells of the upper respiratory tract and then spreads to the local lymph nodes, from where the primary viremia occurs which can virtually infect every tissue and organ but mainly targets parotid glands, meninges,

pancreas and gonads (Morgan-Capner, 2004). During the primary viremia, the virus infects the ductal epithelium of parotid glands causing swelling, inflammation, and tissue damage (Carbone and Rubin, 2007). The involvement of the CNS occurs when infected mononuclear cells cross the endothelium of the choroid plexus or plasma virions cross the endothelium of the choroid plexus directly and infect the choroidal epithelium, which can result in meningitis. Mumps virus may enter the subarachnoid space where it can occasionally infect neurons (Carbone and Rubin, 2007). The gonadal involvement occurs in many infections but is most noted in postpubertal men and the virus has been isolated from affected testis. It has been suggested that the seminiferous tubules are the primary site of viral replication (Carbone and Rubin, 2007).

Only one serotype of mumps virus exists and lifetime immunity is expected after natural infection. However, reinfection has been reported in 1-2% of the cases (Knowles and Jin, 2005; Lienikki, 2004). Both humoral and cell-mediated immune responses are involved in recovery from primary infection and long-term protection from mumps disease. Studies have shown that antibodies against both the HN and F proteins can neutralize the virus *in vivo* while *in vitro,* only antibodies to HN can neutralize virus (Houard et al., 1995;Orvell, 1984). Studies in animal models have shown that clearance of the virus correlates with the rise in neutralizing antibody titer (Carbone and Rubin, 2007; (Wolinsky et al., 1985). Cytotoxic T-cell responses and mononuclear cell inflammation have been also reported to be induced during infection with mumps virus and might be important in clearance of the virus (Kreth et al., 1982;Chiba et al., 1982).

Mumps vaccines

Two types of vaccines against mumps disease have been developed; formalin-inactivated or killed vaccines and live, attenuated vaccines. A killed vaccine was used in the United States from 1950 to 1978. The problems with this type of vaccine were short-term (less than 1 year) immunity and low protective efficiency (Carbone and Rubin, 2007). Nowadays, all available mumps vaccines are composed of live, attenuated viruses and are usually administered together with measles and rubella vaccines (MMR). So far, several live, attenuated vaccines have been developed, and all of them have been made by serial passage of wild-type isolates (Table 1.1) (Bonnet et al., 2006). It was believed that immunization with a single dose of live, attenuated virus provided complete protection from natural mumps, and neutralizing antibody has been shown to persist for more than 19 years (Pipkin et al., 1999;Brunell et al., 1969). High rates of mumps outbreaks among vaccinated individuals, including the 2006 mumps outbreak in the United States, have increased concerns regarding mumps vaccine failure (Date et al., 2008). Recent studies have demonstrated that the effectiveness of vaccination against mumps decreases in epidemic conditions. For example the widely used Jeryl Lynn vaccine demonstrated an efficacy of 95%, but in epidemic conditions, the effectiveness has been as low as 62% (Peltola et al., 2007a). So in 1990 the use of a second dose of mumps vaccine was begun among U.S. schoolchildren (Dayan et al., 2008).

At present, the Jeryl Lynn strain is the most widely used vaccine against mumps (Amexis et al., 2002). Other widely used vaccines include the Urabe AM9, Leningrad-Zagreb, and Rubini strains. Nonetheless, the use of these vaccines has been limited or discontinued due to several reports of post-vaccination adverse effects such as aseptic

TABLE 1.1 Mumps Virus Vaccines in Recent Use		
Vaccine Strain	**Manufacturer**	**Area of Use**
Hoshino	Kitasato Institute	Japan, Korea
Jeryl Lynn (MumpsVax)	Merck and Co.	Worldwide
Leningrad-3	Moscow State Facility for Bacterial Preparations	Former Soviet Union
Leningrad-Zagreb	Institute of Immunology of Zagreb	Croatia, India, and Slovenia
Miyahara	Chem-Sero Therapeutic Research Institute	Japan
NK M-46	Chiba	Japan
Pavivac	Sevapharma	Czech Republic
RIT-4385	GlaxoSmithKline Biologicals	Europe
Rubini (discontinued)	Swiss Serum Institute	Switzerland
S-12	Razi State Serum and Vaccine Institute	Iran
Sofia-6	Center for Infectious and Parasitic Diseases	Bulgaria
Torii	Takeda	Japan
Urabe	GlaxoSmithKline Biologicals (discontinued)	Worldwide
	Sanofi-Pasteur	Worldwide
	Biken	Japan
	Chiron Therapeutics and Vaccines	Germany, Italy, Asia, Latin America

meningitis for the first two, and concerns regarding the efficiency of the third (Arruda & Kondageski, 2001;Dourado et al., 2000;Goh, 1999;Afzal & Minor, 1999).

Urabe AM9

The Urabe AM9 vaccine was first developed and licensed in Japan before it was introduced in many countries, including Canada and United Kingdom, in the early 1980's (Peltola et al., 2007b;Bonnet et al., 2006a). The vaccine was made by 6 passages of a wild isolate in chicken embryonic cavity, 2 plaque purifications in quail fibroblast cell culture and a subsequent 4 passages in chicken embryonic cavity. At the end, after testing in children, the clone (AM9) that induced the highest neutralizing antibody with no side effects was introduced to the market as Urabe AM9 (Brown & Wright, 1998;Yamanishi et al., 1973). The vaccine was licensed in Canada, Japan and Europe.

Shortly after introduction, several cases of post-vaccination aseptic meningitis were reported. In each case, Urabe mumps virus was isolated from patients' cerebrospinal fluid. The overall rate of vaccine-associated aseptic meningitis was approximately 1 in 10,000 vaccinees (Dourado et al., 2000;Hockin & Furesz, 1988). This unacceptable side effect, together with other symptoms such as parotitis and orchitis, led to withdrawal of the vaccine from use in the early 1990's (Amexis et al., 2001a;Colville et al., 1994). It is noteworthy to mention that no cases of aseptic meningitis have been associated with Jeryl Lynn vaccine so far (Miller et al., 1993). Further studies revealed that the vaccine was a mixture of closely related viruses which could be distinguished based on sequence differences in the HN gene; with wild type (A) and variant (G) at position nt 1081 which results in a Lys→ Glu change at aa335 of the HN protein. Those

viruses isolated from post-vaccination meningitis or parotitis patients were predominantly A (98%-100%) at 1081 nt while the proportion of the A viruses within the original Urabe AM9 is 25%-54%. It is notable that the variant G form has not been associated with any symptom and therefore it has been suggested that these viruses are more attenuated and safer than the original Urabe AM9 vaccine (Wright et al., 2000;Brown & Wright, 1998;Afzal et al., 1998;Brown et al., 1996;Brown et al., 1991).

Unfortunately, apart from failure to produce clinical symptoms in vaccinees, there is no marker for determining the attenuation status of mumps isolates (Carbone and Rubin, 2007). Since wild type mumps viruses are often neurotropic and can cause aseptic meningitis, the monkey neurovirulence safety test (MNVT) was evaluated for its ability to predict the neurovirulence potential of the vaccines against mumps in humans. For this purpose, several mumps viruses, including some Urabe isolates were tested in Rhesus monkeys and the group histologic lesion score (GHLS) representing each group of monkeys, was calculated. One of the Urabe strains tested in this assay was Gw7, which is a 1081G virus plaque purified directly from the vaccine (Wright et al. 2000), and the other was an Urabe post-vaccination meningitis isolate, 87-1004, which is a 1081A variant. Although in the test 87-1004 showed a higher GHLS (1.20) in comparison to Gw7 (1.13) (Rubin et al., 1999), the MNVT proved not to be the best model to study the neurovirulence of mumps virus, since a completely attenuated virus, Jeryl Lynn, scored higher (1.46) in this test than 87-1004 (1.20)! Consequently another test has been developed. Mumps neurovirulence in humans appears to be correlated with the severity of hydrocephalus caused in rats compared to lesion scores in the monkeys, therefore a rat neurovirulence test (RNVT) was developed to better address the attenuation of mumps

vaccine strains (Rubin et al., 2005;Rubin et al., 2003;Rubin et al., 2000). The RNVT was performed on Gw7 (possessing the potentially attenuated genotype) and 1004-10/2, which was isolated from 87-1004 by two plaque purifications in Vero cells. The results showed that 1004-10/2 induced a higher hydrocephalus score (4.7) than Gw7 (1.37) and importantly Gw7 scored almost identical to Jeryl Lynn (1.8) (unpublished Shah et al., 2008). These results support the suggestion that Gw7 is an attenuated Urabe virus.

Other biological differences between Gw7 and 1004-10/2

Previous studies have indicated that Gw7 induces higher fusion in Vero cells compared to 87-1004. Also, the morphology of the plaques produced by each virus is different from the other. Plaques produced by Gw7 in Vero cells are clear and smaller (1.1mm in diameter) while plaques produced by 1004 are opaque and slightly larger (1.2mm in diameter) (Wright et al., 2000). Interestingly, these two viruses also show different growth abilities in different cell lines. Gw7, like other G viruses, grows to 1 log lower titer in Vero cells, which is a monkey kidney cell line, than 1004-10/2 (Table 1.2). The difference in growth was more drastic when these viruses were tested in human cell lines; particularly there was an interest to examine the growth in the human lung epithelial line, A549, which represents cells at the natural site of replication. It has been discovered that Gw7 grows to 3 logs lower in A549 cells than in Vero, and 4 logs lower in A549 than 1004-10/2 during time points from 24 hours to 96 hours post infection (p.i) (Wright et al., 2000). In order to determine whether this result was specific to lung epithelial cells or to human cells of any origin, the growth was examined in another human cell line, Hela T4 and the same growth pattern as in A549 was observed

(unpublished Shah et al., 2008). Therefore it is proposed that the *in vivo* attenuation of Gw7 and the reduced growth in human cultured cells may be connected.

TABLE 1.2 Growth differences in different cultured cells			
Virus	**A549**	**HELA-T4**	**Vero**
Gw7	1.15×10^3	5.8×10^3	2×10^6
1004-10/2	6.6×10^7	1.2×10^7	2×10^7

Sequence comparison of Gw7 and 1004-10/2

As demonstrated in Table 1.3 and 1.4, initial sequencing of the genomes of the two viruses found 11 coding nucleotide differences in 5 genes and 4 non-coding differences (Unpublished Shah, et al 2008). The sequences were also compared to a consensus sequence for Urabe virus, and the nucleotides which are bolded are those differing from the Urabe AM9 consensus sequence. The Urabe consensus sequence was derived from 8 published sequences of Urabe viruses and direct sequencing of quasispecies from different vaccine lots, including SKB, Chiron and Biken, together with AM9 parental, AM9-A and AM9-B (Sauder et al., 2006;Amexis et al., 2001). The sequence for wild type Urabe is not available.

These sequence differences must control both differences in virulence *in vivo* and cell tropism. Previous work in the lab has shown that there is no significant reduction in

Gene/AA	**Gw7**	**1004-10/2**	Consensus/ 87-1004	87-1005	AM9
1387 / NP	**a**	g	g	g	g
1880 / NP-P	c	t	c	t	c
4817 / F91	c - Ala	**t - Val**	c - Ala	c - Ala	c- Ala
4903 / F120	**g - Glu**	c - Gln	c - Gln	c - Gln	**c/g Gln/Glu**
6385 / SH40	c - His	a - Asn	c - His	a - Asn	c - His
7573	t	c	t	c	t
7616 / HN335	**g - Glu**	a - Lys	a - Lys	a -Lys	**a/g – Lys/Glu**
8005 / HN464	c - Asn	a - Lys	C - Asn	a - Lys	c/a – Asn/Lys
8191 / HN526	a - Lys	**c - Asn**	a - Lys	a - Lys	a - Lys
8925 / L163	**a - Asn**	c - Thr	c - Thr	c - Thr	c - Thr
9397 / L320	**g - Met**	a - Ile	a - Ile	a - Ile	a - Ile
10529/L	c	t	c	t	c/t
11692 / L1085	g - Leu	t - Phe	g - Leu	t - Phe	g/t – Leu/Phe
14049 / L1871	c - Ala	t - Val	c - Ala	t - Val	c- Ala
15328	**a**	g	g/a	g	g

Table 1.3 Sequence differences between 1004-10/2 and Gw7. The bolded sequences are the ones different from the consensus

binding of Gw7 to A549 compared to Vero cells and no reduction in binding of Gw7 to either cell type compared to 1004-10/2 (Unpublished Shah, et al 2008). Also it has been shown that Gw7 has a higher fusion activity relative to 1004-10/2. Hence the observed differences in growth and cell tropism between these two viruses are not a result of reduced binding or cell entry of Gw7 to A549 cells. Work completed by another student eliminates SH and M as important for growth differences in A549 and implicates the differences in polymerase activity as controlling the different growth phenotypes of the two viruses.

Mutations in the L polymerase protein have been found to be associated with *in vivo* attenuation in many RNA viruses including the Paramyxoviruses (Skiadopoulos et al., 1998;Murphy et al., 1997). For example, mutations in the L protein of Human Parainfluenza virus type 1 and 3 (HIPV1,3) and Respiratory Syncytial Virus (RSV) were discovered to cause temperature-sensitivity (ts) *in vitro* and attenuation *in vivo* (Bartlett et al., 2007;McAuliffe et al., 2004;Collins & Murphy, 2002;Skiadopoulos et al., 1999). Amino acid changes in the L protein of measles virus have also been shown to be responsible for attenuation *in vivo* (Takeda et al., 1998). Furthermore, it has been demonstrated that mutations in the polymerase protein of mumps virus selected through passage in different cell lines affected virulence of the virus in the RNVT (Rubin et al., 2003). Additionally, mutations in the polymerase of measles virus have been associated with changes in tropism in tissue culture lines (Bankamp et al., 2008) which contributes to the assumption that polymerase activity may be important in the different phenotypes of 1004-10/2 and Gw7.

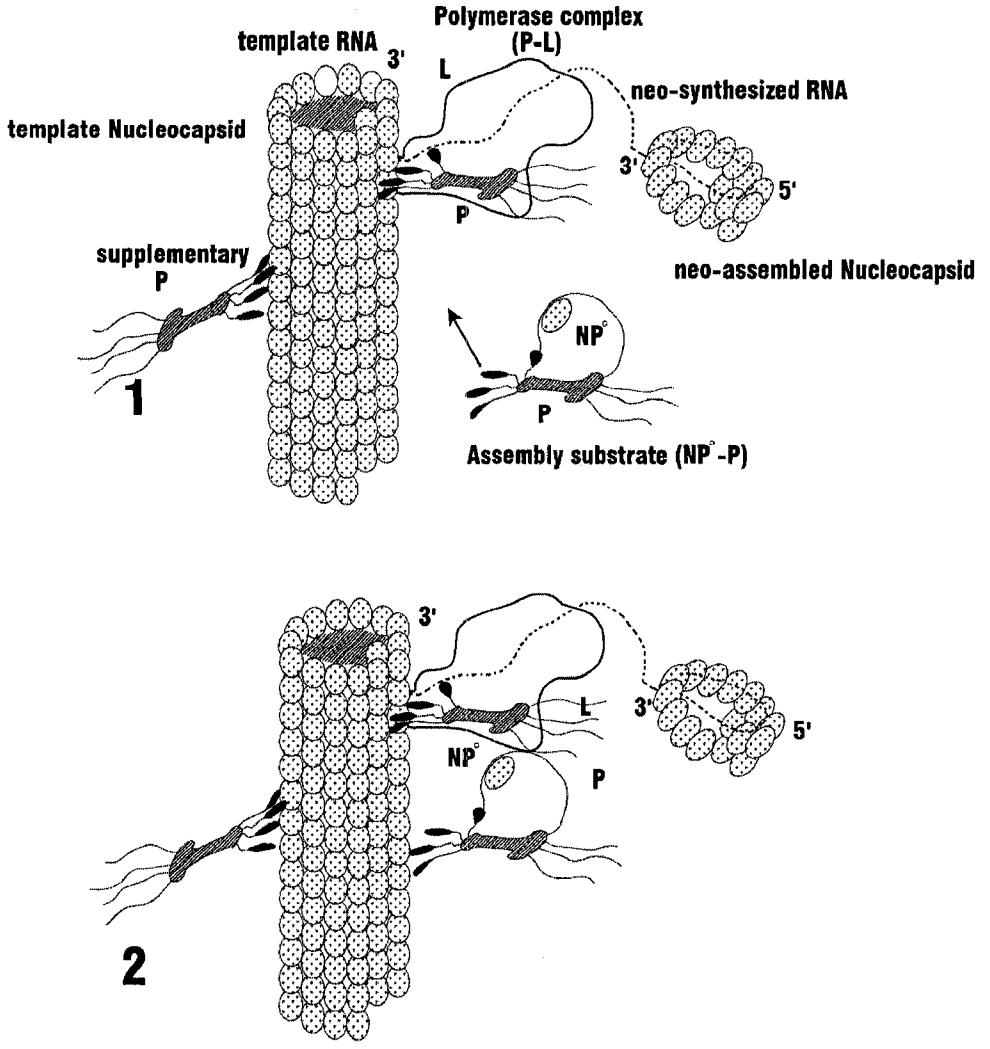

Figure 1.5 Model of the polymerase complex actively replicating genomic RNA. The P protein forms a tetramer which delivers NP° has been represented distinct from that within the L–P complex. The numbering of the different panels shows the chronology of events. (Top) L is bound to a P tetramer. A supplementary P molecule, not bound to L, is also shown (right). The newly-synthesized RNA is shown as already partially encapsidated. (Bottom) The encapsidation complex, NP°–P, binds to the nucleocapsid template through three of its four arms. Adapted from (Bourhis et al., 2006).

The polymerase complex

Studies on other members of *Paramyxoviridae* have revealed that the functional RNA dependent RNA polymerase (RDRP) is comprised of P and L proteins, although some references include NP protein as well (Lamb and Parks, 2007). RDRP recognizes the encapsidated viral RNA (RNP) as the template and is able to perform all necessary RNA synthesis for the virus life cycle. It has been demonstrated that the L protein is the catalytic subunit of RDRP while P binding stabilizes the L protein. Also, since there is no recognized NP binding site on the L protein, it seems that P serves as the bridge which provides an NP-RNA template link to the L protein (Figure 1.4) (Cevik et al., 2004;Horikami et al., 1997;Curran et al., 1994;Horikami et al., 1992). The N-terminal region of the NP protein is important for formation of the NP^0-P complex, and these domains are different from those involved in binding of P to NP in the assembled nucleocapsid (Homann et al., 1991). The P protein forms a tetramer and its interaction with the L protein has been mapped to aa 412–479 of the P protein in SeV (Figure 1.4) (Cevik et al., 2007;Tarbouriech et al., 2000;Curran et al., 1994).

As mentioned earlier, based on sequence homology, 6 domains have been considered for L protein and the function of some of these domains is understood. For example, due to the high positive charge of domain II, it was proposed that it contained RNA binding motifs (Smallwood et al., 1999). Domain I has recently been shown to have sites for binding to P protein and L (L-L oligomerization) (Smallwood & Moyer, 2004;Cevik et al., 2004;Cevik et al., 2003). Site directed mutagenesis of nucleotides in domain III has shown that the highly conserved GDNQ motif serves as the active site for

phosphodiester bond formation in nucleotide polymerization (Chattopadhyay et al., 2004). Mutations of certain residues in conserved regions of domain IV and V reduced but did not abolish RNA synthesis, with more effect on RNA replication than transcription (Cortese et al., 2000;Feller et al., 2000).

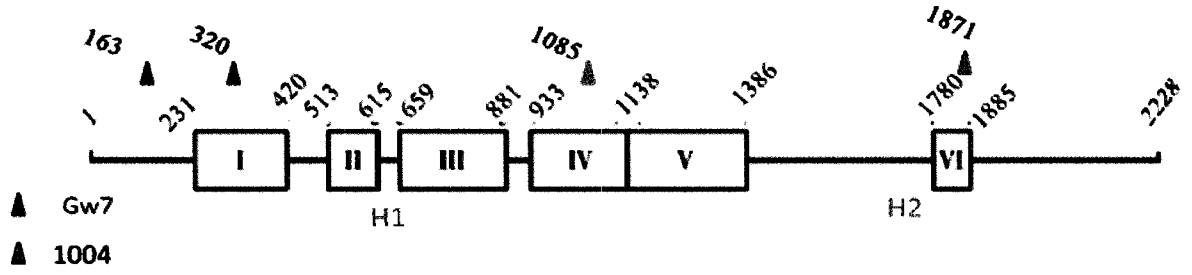

Figure 1.6 Schematic diagram of the Large protein with its 6 domains. The blue triangles represent the differences from consensus in the L protein of Gw7 and the red triangles represent the differences in the L protein of 1004-10/2.

Domain VI has been identified to have mRNA cap methylation motifs (Grdzelishvili et al., 2006;Grdzelishvili et al., 2005). Sequence alignment has also shown two non-conserved hinge regions (H1 and H2; Figure 1.6), and insertion of Green Fluorescent Protein (GFP) in these regions of measles and rinderpest viruses produces active recombinant viruses that can be used to identify sites of L localization during infection (Brown et al., 2005b;Duprex & Rima, 2002;Duprex et al., 2002).Although there is not much known about L protein structure, it has been shown that L protein forms an oligomer. The L-L binding site is mapped to aa1-174 of the Sendai virus L protein (Cevik et al., 2007;Smallwood et al., 2002) and is distinct from the P-L binding site which is within aa 21-350 of L (Holmes & Moyer, 2002) (Figure 1.4).

The L protein is also known to interact with host cell proteins. For example, the interaction of L protein with tubulin in measles virus and Sendai virus is believed to promote L activity and the same studies have also suggested that tubulin may be a subunit of the viral polymerase complex (Moyer et al., 1990;Moyer et al., 1986). Other cellular proteins that have been shown to interact with the L protein are Heat Shock Protein 90 (Hsp90), Hsp72 and Striatin (Connor et al., 2007;Carsillo et al., 2006;Sleeman & Baron, 2005). It has been demonstrated that inhibiting Hsp90 activity or silencing its expression reduces the replication of VSV and paramyxoviruses simian virus 5 (SV5) and HPIV-2 by shortening the half-life of L protein (Connor et al., 2007). Also, when transgenic mice created to constitutively over-express hsp72 in neurons were inoculated with measles virus, total viral RNA burden in the brain increased twice as much as in non-transgenic mice caused by an increase in viral transcription due to the presence of hsp72 (Carsillo et al., 2006).

There are four coding differences in the L gene of 1004-10/2 and Gw7 and Figure 1. 6 shows where they are located. For two sites, 1004-10/2 has the consensus amino acids and for the remaining sites Gw7 has the consensus amino acids. Gw7 L^{163} is located upstream of domain I and falls within the region (aa 20-178) shown to be important for L oligomerization in Sendai virus (SV) (Cevik et al., 2007;Cevik et al., 2003b). Gw7 L^{320}, is inside domain I but it does not fall within the highly conserved stretches in domain I (aa 280–300), and (aa 348–379) which have been shown to be crucial for transcription and replication in Sendai and measles virus (Chandrika et al., 1995). This site is, however, located within a broad region (aa21-350) shown in Sendai virus to be important for P binding (Holmes & Moyer, 2002).

The L^{1085} change in 1004-10/2 is located in a highly conserved stretch of amino acids in domain IV and in SV, changing the amino acids located adjacent to this conserved region by site-directed mutagenesis caused a decrease in *in vitro* replication but had no effect on transcription (Feller et al., 2000). The L^{1871} change is inside domain VI and is located not very far from a stretch of conserved residues (aa 1643–1843) that has been identified to have methyl transferase (MTase) activity in vesicular stomatitis virus (VSV) (Grdzelishvili et al., 2006;Grdzelishvili et al., 2005).

Lastly, the non-coding difference at 15328 nt is located in a 96 nt sequence stretch in the 5' untranslated region (UTR) of the genomic RNA where the anti-genomic promoter (AGP) essential for replication is situated (Cordey & Roux, 2007). However, it is unlikely that this position falls into any of the conserved promoter elements defined for Rubulaviruses, since it is located 56nt from the 3' end of the anti-genomic RNA; the first promoter element (PrE-I) of the AGP for Rubulaviruses is located between nucleotides 1

and 19 and the second promoter element (PrE-II) is located between nucleotides 73 and 90 (Keller & Parks, 2003;Murphy & Parks, 1999;Murphy et al., 1998). However, it is worthy of mention that 15328 nt does fall into a nonessential enhancer of replication (bases 51-66) located between anti-genomic PrE-I and PrE-II identified for PIV5 (Keller & Parks, 2003;Keller et al., 2001).

In the original sequencing, a K→R change at the position of aa 274 of the P gene of Gw7 was reported which does not fall into any of the conserved motifs of the P protein with known function. In fact, deletion of residues 78 to 324 of P protein of Sendai virus did not inhibit mini-genome replication in transfected cells (Curran et al., 1995).

Based on the data obtained from studying viral attachment and entry, polymerase activity has been suggested to control growth differences between 1004-10/2 and Gw7 in Vero and A549 cells. The polymerase protein performs all the enzymatic activities necessary for viral transcription and replication and as explained earlier, there are 4 coding differences in the polymerase proteins of the two viruses which can potentially affect the polymerase activity. It is also assumed that differences between the polymerases of the 2 viruses contribute to differences in virulence *in vivo*.

Hypothesis

The genetic differences between 1004-10/2 and Gw7 in the large protein result in differences in the activity of the polymerase complex, which in turn, affect attenuation *in vivo* and growth differences in tissue culture cells.

Objectives

1) To confirm sequence differences in P and L genes of Gw7 and 1004-10/2

2) To measure and compare activity of the polymerases from Gw7 and 1004-10/2

   a) *In vivo* incorporation of ^{3}H UTP in the presence of Actinomycin D

   b) To establish Urabe mini-genome system

      i) To clone NP (same sequence between viruses) and P

      ii) To clone the L genes from each virus

      iii) To construct a mini-genome with Luciferase as the reporter gene

   c) To compare polymerase activity using the Urabe mini-genome

Chapter Two: Materials and Methods

Cell culture and virology

Cell lines and culture

The African green monkey kidney fibroblastic cell line, Vero, and the human alveolar basal epithelial line, A549, were originally obtained from ATCC. The human epithelial-like cell line, Hela T4, was obtained from the National Institutes of Health (NIH AIDS Research and Reference Resource Program). All cell lines were maintained in Dulbecco's modified Eagle's essential medium (DMEM) (Wisent INT) supplemented with 7% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin sulfate and 2 mM L-glutamine at 37°C in an atmosphere of 5% CO_2 in a CO_2 water jacked incubator (Forma Scientific). All cell lines were passaged once or twice a week at a ratio of 1:2 to 1:10 depending on growth. Cells were washed once with pre-warmed 1x phosphate buffered saline (PBS), then 25% Trypsin (Wisent) in PBS was added to detach the cell monolayer. Fresh medium was added to inactivate Trypsin and dilute the cells to the proper ratio, and the cells were transferred to new culture flasks (Sarstedt).

Viruses

Gw7 had previously been isolated from Urabe/PM vaccine (Institute Pasteur Merieux, France) by three plaque purifications in Vero cells (Wright et al., 2000). The stock of Gw7 used in all the experiments had undergone no more than 5 passages in Vero cells. 1004-10/2 was previously isolated by two plaque purifications in Vero cells from 87-1004, Urabe virus associated with post vaccination disease, which was received from the Bureau of Biologics, Health Canada. The stock of 1004-10/2 used in all experiments

had been passaged three times in Vero cells after purification. The Vaccinia virus expressing the T7 polymerase, VTF73, was a kind gift from Bernard Moss (Fuerst & Moss, 1989). High titered stocks were available in the lab.

RNA preparation and analysis

RNA isolation

To isolate viral RNA, cell monolayers were infected at a multiplicity of infection (MOI) of 0.1 with one or another Urabe virus and incubated at 37°C for 3 days, then the total RNA was isolated as follows: Trizol reagent (Invitrogen) was used to harvest the cells (4ml for approximately $3x10^6$ cells), then 200µl of phenol: chloroform: isoamyl alcohol (PCI) (Invitrogen) per one milliliter of Trizol, were added to the sample to deproteinize it and the sample was centrifuged for 15 minutes at 12000 x g at 4°C (Baxter Canlab). The aqueous phase was harvested and RNA was precipitated using 750µl isopropanol per 1ml of Trizol and centrifugation for 10 min. at 12000 x g at 4°C. The RNA pellet was then washed with 75% ethanol; air dried and resuspended in 20µl (per 1ml of Trizol) of diethyl pyrocarbonate (DEPC) treated double distilled water.

Reverse transcription

Five microliters of RNA were used as the template for a 50µl reverse transcription (RT) reaction. The recipe for RT was: 100ng of the specific primer (Table 2.1) were added to the RNA solution, heating to 70°C for 10 minutes, quick chill on ice, then 10µl of 5x RT buffer, 5 µl of 0.1 mM DTT, and 1µl of 25mM dNTP (all obtained from Invitrogen) were added and the mixture was incubated at 42°C for 2 min, then 200 U of SuperScript™ II RT (Invitrogen) were added to the mixture as per the manufacturer's

instructions. The RT reaction was performed in a Techgene thermal cycler using the following program; (10 minutes at 25°C, 1 hour at 42°C, 30 minutes at 50°C).

Denaturing agarose-Formaldehyde RNA gel electrophoresis

In order to make a stock of DEPC treated water, 0.1 ml of DEPC was added per 100ml of H_2O and the solution was incubated at 37°C overnight, prior to autoclaving to remove any trace of DEPC. A Bio-Rad Mini Sub™ DNA cell apparatus was either soaked in or wiped with 1% SDS and 10% bleach and then was rinsed with DEPC treated water to remove any ribonucleases (RNase). The gel was prepared by adding 8.9 ml of 37% Formaldehyde and 10 ml of 5x gel running buffer (MOPS 0.1M, NaAc 40 mM, EDTA 5 mM pH 8, protected from light) to 31.1 ml of 2% agarose in DEPC treated H_2O at 60°C. For sample preparation, 2 µl of 5x gel running buffer, 3.5 µl 37% Formaldehyde, 10 µl Formamide, 1 µl Ethidium Bromide (1µg/µl diluted stock), 2 µl Sample loading buffer (50% glycerol, 1 mM EDTA pH 8, 0.03% Bromophenol blue) were added to 5 µl of RNA and the samples were heated for 15 minutes at 65°C then chilled on ice. Then the samples were loaded together with 5 µl of an RNA marker ladder (Invitrogen) and the gel electrophoresis was carried out at 5-7 V/cm. The picture of the gel was taken using AlphaEase™ imager with ultraviolet light box (Alpha Innotech Corporation). The gel was then incubated in fixative solution (30% methanol, 10% acetic acid) for 30 minutes and soaked in NAMP100VAmplify (Amersham) for 30 minutes to enhance detection of radioactivity, vacuum dried and exposed to X-ray film (Kodak).

Polymerase activity analysis

Vero cells were grown in 6-well plates (0.5-1.0 $x10^6$ cells per well) and infected with either Gw7 or 1004-10/2 at different MOI depending on the experiment, and were

labeled with 50μCi of ^{3}H UTP (Amersham) at different time points, then incubated at 37°C for 8 hours. Cells were treated with 5μg/ml of Actinomycin D (Sigma) (Storey, 1987) for 1hour prior to labeling and Actinomycin D was present in the media during the labeling period. The total RNA was then isolated as described above. Five μl of RNA solution were added to small pieces of filter paper (Waterman) which were placed in 7ml Mini Vials® (Fluke Biomedicals). Five ml of scintillation liquid (Fisher Scientific) were added to the samples, which were counted in a 1214 Rackbeta scintilation counter (LKB Instruments).

Gel densitometry

Quantification of the ribosomal RNA bands in agarose gels was performed with the help of UN-SCAN-IT gel Version 6.1 software (Silk Scientific) that digitizes electrophoresis gels.

DNA preparation and analysis

Polymerase chain reaction (PCR)

PCR amplification was done using 50ng-200ng of cDNA, linear DNA or plasmid DNA as a template together with 100ng of specific primers (Invitrogen or Sigma; Table 2.1), for a 50 μl reaction. Taq polymerase and other PCR reagents were obtained from MBI or Invitrogen (Platinum High Fidelity Taq polymerase) and were used according to the manufacturer's protocol. PCR was performed in a Techgene thermal cycler. The programs for all the PCRs performed in this research are shown in Table 2.2.

Agarose gel electrophoresis

DNA electrophoresis was carried out in 0.8-2% agarose gels in 1x Tris-acetate buffer (TAE; 40mM Tris-acetate, 1mM EDTA, pH 8). One µl of 6x gel loading buffer (GLB; 30% glycerol, 0.03% Bromophenol blue, 0.03% xylene cyanol) was added to 5 µl of DNA solution and the mixture was loaded on the gel. Electrophoresis was carried out in a Horizon® 58 apparatus (Life Technologies) at 5-7 V/cm. Then the DNA was stained with 1µg/ml Ethidium Bromide solution. Photos were taken using AlphaEase™ imager with an ultraviolet light box (Alpha Innotech Corporation).

DNA purification from agarose gel

DNA fragments used for cloning or making genetic constructs were purified from 0.8-2% agarose gel in TAE. In all cases, 5 µl of the DNA sample were run in a separate well and only this portion of the sample was sacrificed and exposed to 312nm UV light on a mid-range UV transilluminator (Fotodyne INC) and the correct position of the DNA band was marked, then the rest of the gel was aligned to the part which was exposed and the relevant section was excised accordingly. DNA was extracted from the unexposed part of the gel, using the GenElute™ Gel Extraction Kit (Sigma-Aldrich). By applying this method, the damage usually caused by UV light was avoided.

Plasmid DNA preparation

For small scale plasmid isolation or Mini-preps, 5-7 ml of Luria-Bertani medium (LB) containing Ampicilin (100µg/ml) were inoculated with a portion of a bacterial colony or frozen bacterial stock and the culture was incubated on an orbital shaker

Name	Sequence
NP+146	5'-ATGTCGTCTGTGCTCAAAGCA-3'
NP-1795	5'-TTACTCATCCCAGTCGCCCACT-3'
P+1970	5'-GGGCAAGCCATGGACCAA-3'
P-3152	5'-TCATATGGCGCTCTTATG-3'
LFR1+ (8436+)	5'-GAATGGCGGGCCTAAATGAGAT-3'
LFR1-(11692-)	5'-CAGGACCCAGATATCTAGACAG-3'
LFR2+ (11311+)	5'-CATAGGAGACCCATTAGTGTCT-3'
LFR2-(15246-)	5'-TGCCAGAGGAGTCTTTAGTCGA-3'
Leader+(Le+)	5'-CCACCAAGGGGAAAATGAAGATGGA- 3'
Leader-(Le-)	5'-CATAACGTTTCCAGGTAGTGTCAAA-3'
Trailer+(Tr+)	5'-TCTGCGGCCGCAATCGACTAAAGACTCCTC-3'
Trailer-(Tr-)	5'-GGATCCATTATGCTGAGTGATATCCCACCAAGGGGA GAAAGTAA-3'

Table 2.1 Primers used for RT-PCR

Program	# of cycles	Denaturing	Annealing	extension	Final ext.
Large	30	94°C 30 sec	55°C 1min	72°C 45 sec	72°C 5 min
Phospho	30	94°C 30 sec	51°C 1min	72°C 45 sec	72°C 5 min
NP	30	94°C 30 sec	60°C 1 min	72°C 45 sec	72°C 5 min
Le/Tr/Luc	30	94°C 30 sec	51°C 30 sec	72°C 45 sec	72°C 5 min
Mutagenesis	20	94°C 30 sec	45°C 1 min	72°C 45 sec	68°C 15 min
Long	30	94°C 30 sec	44-48°C 30 sec	72°C 45 sec	72°C 10 min

Table 2.2 PCR programs

(Forma Scientific) at 37 °C overnight. The plasmid DNA was purified using the Sigma GenElute™ Plasmid Miniprep Kit. Cracking is a fast way of screening bacterial colonies in which a portion of the colony was dissolved in 4 µl of TE buffer (25mM Tris-HCl, pH8.0, 10mM EDTA), then 7 µl of lysis buffer (400mM NaOH, 2% SDS),and 2µl of GLB was added and then the mixture was loaded on 0.8% agarose gel in TAE. The gel was incubated at room temperature for 10 minutes and then 1x TAE buffer was added and the electrophoresis was carried out as described previously.

For large scale plasmid isolation or Midi-preps, 100ml of LB medium containing Ampicilin (100µg/ml) were inoculated with a bacterial colony and the culture was incubated on an orbital shaker at 37 °C overnight. The plasmid DNA was extracted using The Wizard® Plus Midipreps DNA Purification System (Promega).

DNA sequencing

DNA samples were sequenced at the Ontario Genomics Innovation Centre (OGIC), using an automated fluorescent DNA analyzer (Applied Biosystems 3730 DNA Analyzer).

Site directed mutagenesis

Site directed mutagenesis was performed using the enzyme PFU Turbo (Stratagene) and the primers listed on Table 2.3 and 2.4 All the primers used for mutagenesis were from Sigma-Aldrich and were cartridge purified. Mutagenesis of 100ng plasmid DNA in a 50µl reaction was as follows; 5µl of 10x PFU reaction buffer, 125 ng of each pair of specific primers, 1µl of 10mM dNTPs and 2.5U of PFU Turbo in a Techgene thermal cycler (Table 2.2). In order to remove the methylated parental plasmid, the DNA was digested with 6U of DpnI (New England Biolabs) for 2-3

Name	Sequence
mt1004nt406+	5'-GTATTACACA**T**CAGCAATCAG-3'
mt1004nt406-	5'- CTGATTGCTGA**T**GTGTAATAC-3'
mt1004nt521+	5'- TTAACAACGA**G**TGGAGTTGGA-3''
mt1004nt521-	5'- TCCAACTCCA**C**TCGTTGTTAA-3'
mt1004nt4001+	5'- GAGATCGATG**A**TCAGGTAACA-3'
mt1004nt4001-	5'- TGTTACCTGA**T**CATCGATCTC-3'
mt1004nt4541+	5'-TACTTAAGAA**T**TCAAGGCATC-3'
mt1004nt4541-	5'- GATGCCTTGA**A**TTCTTAAGTA-3'
mt1004nt4757+	5'- ACTAAGCAGG**T**TTTGACCAAC-3'
mt1004nt4757-	5'- GTTGGTCAAA**A**CCTGCTTAGT-3'
mt1004nt5726+	5'- GTAGGGGCTG**A**TACATGTGCA-3'
mt1004nt5726-	5'- TGCACATGTA**T**CAGCCCCTAC-3'

Table 2.3 Primers used for mutagenesis of 1004-10/2 L gene. The bolded bases are the target nucleotides.

Name	Sequence
2nd Gw7+	5'-AGATGCGGCATGATA**A**TTCAGAGCTTAAGT-3'
2nd Gw7-	5'-ACTTAAGCTCTGAAT**T**ATCATGCCGCATCT-3'
3rd Gw7+	5'-ATTTCTTCATATAC**A**GTAAGAGGGTGTTT-3'
3rd Gw7-	5'-AAACACCCTCTTAC**T**GTATATGAAGAAAT-3'
4th Gw7+	5'-TTCAAGGTTACTTG**G**ATTCCACTAGAACT-3'
4th Gw7-	5'-TAGTTCTAGTGGAAT**C**CAAGTAACCTTGA-3'
5th Gw7+	5'-TTCAAGCAGGTATA**G**CAGCAGGAAGTGGT-3'
5th Gw7-	5'-CACCACTTCCTGCTG**C**TATACCTGCTTGAA 3'
6th Gw7+	5'-AGATGGTAAATAGC**A**TCACCGGGCTTGAAG-3'
6th Gw7-	5'-CTTCAAGCCCGGTGA**T**GCTATTTACCATC-3'
V to P+	5'-GAATTTAAGAGGGGG**GG**GCCGGGAGCGGCTGC-3'
V to P-	5'-GCAGCCGCTCCCGGC**CC**CCCCCTCTTAAATT-3'

Table 2.4 Primers used to mutagenize the V coding sequence to P and the L gene of Gw7. The bolded sequences are the target bases.

hours at 37 °C. The mixture was then extracted with phenol: chloroform: isoamyl alcohol (PCI) and the DNA was precipitated in 2 volumes of Sodium Acetate in Ethanol at -20°C. The DNA was then washed with 70% ethanol and resuspended in 10µl of ddH_2O before transformation of bacteria.

Molecular cloning

Digestion

Restriction endonucleases were obtained from New England Biolabs, Pharmacia or Invitrogen. The digestion reactions were carried out in a 37 °C waterbath (Fisher Scientific) for 1-3 hours and then the enzyme was inactivated according to the manufacturers' instructions.

Ligation

DNA fragments were ligated to each other or to a plasmid vector using 1U of T4 DNA ligase (for a 20 µl reaction) and the related buffers from Invitrogen. For ligation of linear DNA fragments, they were mixed in a ratio of 1:1, but for ligating a DNA fragment to a plasmid, the ratio was 3:1. In some cases the PCR products were T/A cloned into pDrive (Qiagen) using a PCR cloning kit from Qiagen. In either case, the reaction mixture was incubated overnight at 4°C. Another vector that has been used in this research is pCITE-2a(+) (Novagen) which is designed for enhanced translation of cloned sequences in mammalian cells because it contains a Cap-Independent Translation Enhancer (CITE) which has the IRES (internal ribosome entry sequence). In addition the CITE sequence is located immediately downstream from a T7 promoter and is followed by the multiple cloning sites. Also, pUC19 vector (Invitrogen) has been used in

subcloning of the mini-genome construct which is a small, high copy number, *E.coli* plasmid and does not contain the T7 promoter, which allows the transcription to take place from the T7 promoter designed inside the construct.

Transformation

For cloning purposes, Qiagen EZ chemically competent cells were transformed according to the manufacturer's instructions, while for subcloning purposes, Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) were transformed as follows; 1-5 µl of plasmid DNA were added to 50 µl of DH5α cells, which were thawed on ice, the mixture was incubated on ice for 30 minutes, heated at 42 °C for 30 seconds and then incubated on ice for 2 minutes. Five hundred µl of SOC medium (Bacto-tryptone 20 g/l, Bacto-yeast extract 5 g/l, NaCl 5g/l, 1M KCl 2.5 ml), were added to the mixture and it was incubated on an orbital shaker (Forma Scientific) at 37°C for about an hour. After that, bacteria were plated in various volumes on LB plates containing 0.1% Ampicillin, IPTG (50 µM) and X-gal (80 µg/ml) for blue/white screening; incubated at 37°C overnight. White colonies were selected for further screening, which was based on size differences.

Gene expression and protein analysis

Transient transfection

Vero cells were grown to 70-90% confluency in a 12-well plate (≤500,000 cells per well). Lipofectamine™ 2000 (Invitrogen) was used as the transfection reagent and transfection was performed according to the manufacturer's protocol. In general, 500ng of each plasmid (NP, P, L, minigenome, β-galactosidase) were transfected in each well

and since all these genes are under the control of T7 promoter, transfected cells were infected with recombinant T7 vaccinia virus, vTF73, as the source for T7 polymerase, at MOI 10. In order to reduce the cytopathic effects caused by the Vaccinia virus, Arabinosylcytosine hydrochloride (AraC; Sigma-Aldrich) at a final concentration of 36μg/ml in 1ml of media containing 7% FBS and was added to the cells after being incubated with the transfection mixture for 4-5 hours. Transfected cells were incubated at 37°C for 24 hours.

Preparation of cell lysate

The medium was gently removed from the transfected cells and the cells were washed once with 1x PBS, then were lysed in 100-150 μl of 1x Luciferase cell lysis buffer (Promega). The lysates were transferred to 1.5 ml micro-centrifuge tubes, vortexed for 15 seconds, and then centrifuged at 11000 rpm for 5 minutes. The supernatants were transferred to new tubes and kept on ice or stored at -80°C until the next analysis.

SDS-PAGE

Protein expression in transfected cells was assessed by polyacrylamide gel electrophoresis (PAGE). For this purpose, 20-50 μl of the lysate were mixed with an equal volume of 2x denaturing sample buffer (0.125M Tris, pH 6.8; 4% SDS, 20% glycerol, 0.02% Bromophenol Blue, 2% β-mercaptoethanol) and the mixture was boiled for 10 minutes. Samples were loaded on an 8-10% polyacrylamide gel containing SDS and electrophoresis was carried out at 25mA in the Bio-Rad Mini Protean apparatus. The gel was either prepared for wet transfer to membrane for Western blot, or was incubated in fixative (30% methanol, 10% acetic acid) for 30 minutes and then in Coomassie blue solution (0.1 coomassie blue, 20% methanol, 10% acetic acid) for 1-2 hours. The gel was

then destained by gentle agitation in destaining solution (50% methanol, 10% acetic acid).

Western blot analysis

Proteins were transferred to polyvinylidene fluoride membranes (PVDF; Millipore) overnight at 4°C at 22 V in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, 0.1%SDS) in the Bio-Rad Mini Trans-Blot cell. The membrane was then removed, soaked in methanol for 30 seconds and ddH_2O for 2 minutes, then it was incubated in blocking buffer (5% non-fat milk, or 3% BSA in Tris buffered saline, TBS,: 0.01M TRIS pH7.5, 0.08M NaCl) for 1 hour at room temperature or overnight at 4°C. The membrane was then incubated overnight at 4°C in mouse anti-Urabe polyclonal antibody (Wright et al. 2000) or human sera known to be positive for mumps antibody (Dr. Earl Brown) at a 1:250 dilution in the blocking buffer. The membrane was then washed 3x with TBS containing 0.2% Tween (TBST) and incubated for 1 hour at room temperature in the secondary antibody, anti-mouse IgG conjugated with horseradish peroxidase (HRP; Tago) or anti-human IgG conjugated HRP (Chemicon) at 1:5000 dilution in the blocking buffer. The membrane was then washed 4x with TBST and placed in the chemoluminescence developing solution (Perkin Elmer) for 1 minute and exposed, for various amounts of time to film (Kodak).

Luciferase assay

Luciferase assays were carried out using the Promega luciferase assay kit. Twenty-four hours after transfection, the cells were lysed as described above and 20 µl of the lysate were transferred to White 96-Well Polypropylene Assay Plates (Corning). The plate was placed in the SpectraMax® microplate auto-injection luminometer where 100

µl of the luciferase assay substrate (Promega) were automatically injected to each well containing the lysate and luminescence was detected over 10 seconds with a 2-second delay.

β-galactosidase assay

Twenty-five µl of lysate from cells transfected with a β-gal expressing plasmid were mixed with 25 µl Chlorophenol Red β-D-galactopyranoside (CPRG; Sigma Aldrich) in a clear 96-well flat-bottom polystyrene ELISA plate (Sarstedt). The plate was incubated for 10 minutes in the dark at room temperature and β-galactosidase activity was measured spectrophotometrically at a wavelength of 575 nm with a Spectra Shell ELISA plate reader (Tecan).

Preparation of constructs for rescue

Construction of expression plasmid for L gene

The L gene was cloned in 2 overlapping fragments (Fr1+ Fr2) with a unique site, *PacI*, in the overlapping sequence (the whole process of cloning has been shown in Figure 2.1). The 2 fragments were obtained by RT-PCR using primers corresponding to sequences 8436 to 11692 [5'-GAATGGCGGGCCTAAATGAGAT-3', 5'-CAGGACCC AGATATCTAGA CAG-3'] and corresponding to sequences 11311 to 14562 [5'-CATA GGAGACCCATTAGTGTC T-3', 5'-TGCCAGAGGAGTCTTTAGTCGA-3']. The fragments were gel purified and subjected to T/A cloning into pDrive (Qiagen) as has been described above. The fragments in the appropriate orientation, Fr2 reverse relative to the T7 promoter, were then digested out with *PstI* and *SalI,* gel purified and cloned into pCite (Novagene) which had also been digested with *PstI* and *SalI.* Clones positive

for Fr2 were again digested with *PacI* and *PstI* and the small fragment was re-ligated into pCite containing Fr1, digested with *PacI* and *PstI*, to generate the full L-ORF for both 1004-10/2 and Gw7 under control of a bacteriophage T7 RNA polymerase promoter (Figure 2.1).

Cloning NP and P genes

The primers which would amplify the NP and P ORFs were used in RT-PCR (Table 2.1) and the resulting amplicons were cloned into pDrive. The plasmid containing the P gene was then digested with *PstI* and *SalI* in order to subclone the P gene into pCite. The sequencing results for the P gene showed the cloned gene was coding for V protein rather than P protein . Therefore 2 primers were designed to mutate the cloned gene into P-ORF by adding 2 non-templated "G" residues to the proper sites (Table 2.2, 2.4). The success of the mutagenesis was confirmed by sequencing.

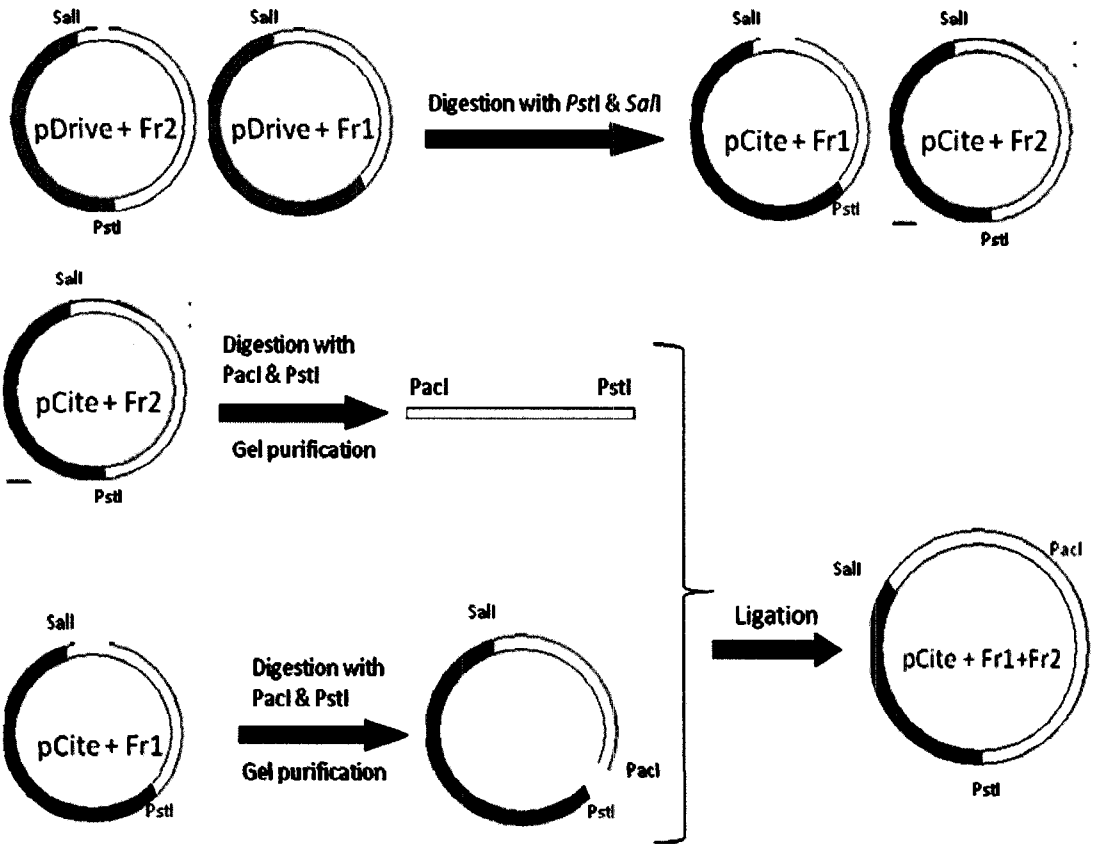

Figure 2.1 Construction of the full ORF clone of L gene. The first and the second fragments (Fr1 and Fr2) of the L gene were first obtained by RT-PCR and cloned into pDrive. The fragments in the appropriate orientation were then digested out with *PstI* and *SalI,* and cloned into pCite which had also been digested with *PstI* and *SalI.* Clones positive for inserted Fr2 were again digested with *PacI* and *PstI* and ligated into pCite containing Fr1, also digested with *PacI* and *PstI* and therefore the full L-ORF was generated.

Engineering the mini-genome construct

In order to assemble the Urabe mini-genome, all the coding and inter-cistronic regions of the viral genome were replaced by the Firefly luciferase (Luc) ORF. Luc-ORF was flanked by the 3' and 5' untranslated regions (UTR) of the 1004-10/2 virus, in anti-genomic sense, which are 145nt and 161nt long respectively (Figure 2.2). The approach was to generate 4 DNA fragments by PCR representing the 3' and 5' UTR regions, luciferase gene and the ribozyme sequence which would be ligated together to make the mini-genome. The primers designed for RT-PCR of the Trailer (5'UTR) region contained the T7 RNA polymerase promoter sequence and restriction sites for *BamHI* and *NotI.* The primer pairs used for RT-PCR amplification of the Leader (3' UTR) region included restriction sites for *BccI* and *AclI* (Table 2.4). The luciferase gene and ribozyme were amplified using a construct provided kindly by Dr. Ken Dimock (University of Ottawa) as the template. The hepatitis delta ribozyme (δ) and T7 RNA polymerase terminator (T7θ) were generated by using the primers which have restriction sites for *BamHI* and *BccI* (Table 2.5). The restriction sites were designed in a way to glue Luc-ORF to Leader and Trailer regions, δ-T7θ to Leader and ultimately the whole construct would be ligated into pUC19. All primers were designed to observe the "rule of six". To complete the mini-genome construct, a second approach was taken which was designing 4 long primers to add the 5'-UTR and the ribozyme sequences to already ligated luciferase and 3'UTR fragment (Table 2.6). For this purpose, gradient PCR was performed in the Mastercycler® gradient PCR machine (Eppendorf) as labeled "long" in Table 2.2.

Name	Sequence
θδ+	5'-GGATCCAACCCCTCAAGACCCGTT-3'
δ-	5'-TCCCATCTTCATTTTCCCCTTGGTGGGTCGGCATGG CATCTC-3'
Le+	5'-CCACCAAGGGGAAAATGAAGATGGA-3'
Le-	5'-CATAACGTTTCCAGGTAGTGTCAAA-3'
Luc+	5'-AAACGTTATGGAAGACGCCAAAAAC-3'
Luc-	5'-GATTGCGGCCGCAGAATTTCGTCATCGCTG-3'
Tr+	5'-TCTGCGGCCGCAATCGACTAAAGACTCCTC-3'
T7Tr-	5'-GGATCCATTATGCTGAGTGATATCCCACCAAGG GGAGAAAGTAA-3'

Table 2.5 Primers designed to make the four segments of the mini-genome construct

Name	Sequence
1st	5'-GGATCCGCTA GTTATTGCTC AGCGGGGGTC GGCATGGCAT CTCCACCTCC TCGCGGTCCG ACCTGGGCAT CCGAAGGAGG ACGCACGTCC-3'
2nd	5'-CCGAAGGAGG ACGCACGTCC ACTCGGATGG CTAAGGGAGA GCACCAAGGG GAAAATGGAG ATGGGATGTT GGTAGAACAA ATAGTGTAAG-3'
3rd	5'- GGTAGAACAA ATAGTGTAAG AAACAGTAAG CCCGGAAGTG GTGTTTTGCG ATTTCGAGGC CGGGCTCGAT CCTCACCTTT CATTGTCGAT-3'
4th	5'- CCTCACCTTT CATTGTCGAT AGGGGACATT TTGACACTAC CTGGAAACGT T[redacted]-3'

Table 2.6 Long primers used to add the leader and δ ribozyme sequences to the mini-genome construct. The highlighted sequences are the overlapping regions.

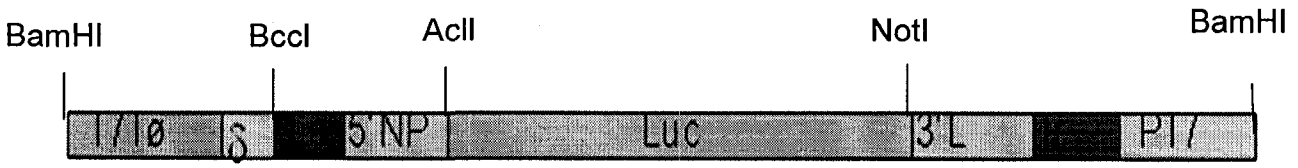

Figure 2.2 Schematic representation of the Urabe mini-genome which is comprised of the Luc-ORF flanked by 3' and 5' untranslated regions (UTR) of the Urabe virus under the control of T7 RNA polymerase promoter (PT7) at one end, and at the other end by a hepatitis delta virus ribozyme (δ) and T7 transcriptional terminator (T7θ). Also the restriction sites designed to ligate different parts of the mini-genome together.

When the entire mini-genome was constructed in the linear form, it was T/A cloned into pDrive (Qiagene) and then digested out using *BamHI*, gel purified and ligated to pUC19, which was already digested with *BamHI.* The whole sequence of the Urabe mini-genome is shown in Appendix 1.

Chapter Three: Results

The overall objective of this research was to determine whether there were differences in polymerase activity between 1004-10/2 and Gw7 which could contribute to the observed differences in growth and attenuation. The first objective was to confirm the sequence differences in the two polymerases which had been found in the original sequencing and the next objective was to compare the activity of the two polymerases. There are several ways to measure the polymerase activity of negative sense single stranded RNA viruses, including *in vivo* incorporation of ^{3}H UTP in the presence of Actinomycin D, expression of a reporter gene from a mini-genomic construct and *in vitro* RNA synthesis from purified virus. The former two methods were used in this project.

DNA cloning and sequence analysis of P and L

The original sequencing of the genomes of the two viruses indicated that there were coding differences in the P and L proteins and therefore the first objective of my project was to confirm these differences. As the later experiments would require expression of P and L from each of the viruses, it was decided to sequence each gene after cloning into an expression vector. For this purpose, the P and L ORFs from Gw7 and 1004-10/2 were obtained by RT-PCR from total RNA extracted from Vero cells infected with each of the two viruses. For P, primers hybridizing to the first 18 nt of the coding sequence and the final 18nt of the P gene were used. Products were T/A cloned into pDrive prior to subcloning into pCite (Collins et al., 1991), which is a plasmid designed for enhanced transcription of cloned sequences. After cloning of the P genes for the two viruses was completed, one clone of each gene was completely sequenced and the result was compared to the sequence previously determined in the lab (unpublished

Shah et al. 2008). In the original sequencing, a K→R change at the aa 274 of the P gene of Gw7 had been reported but this change was not found in the clone of Gw7 P. Thus further sequencing of two additional independent RT-PCR products generated from Gw7 RNA was carried out, and it was confirmed that there was no sequence difference in the P gene of the two viruses. Therefore, all further experiments were conducted with a single P clone from 1004-10/2. The sequence comparison confirmed that the clones were of the full P gene, which would yield V protein rather than P protein upon transcription. Consequently one clone of the P gene of 1004-10/2 was mutagenized to add 2 non-templated "G" residues at 535nt of the gene to yield a transcript for P protein, and the success of the mutagenesis was confirmed by sequencing.

Due to the large size of the L gene, attempts to generate a cDNA of the entire gene were not successful and therefore RT-PCR was performed to generate 2 overlapping fragments (Fr1+ Fr2) with a unique restriction site for *PacI* in the overlapping region which could be used to ligate the two fragments together. Each fragment was then T/A cloned into pDrive and the complete L gene for both viruses was assembled in the second plasmid, pCite as described in the Material and Methods (Figure 2.1). The original sequencing analysis had indicated that the L gene of Gw7 had unique sequences at aa163, aa320 and at nt 15328, located in the 5' noncoding region of the genomic RNA, while the L gene of 1004-10/2 was different from Gw7 and from consensus Urabe sequence at aa1085 and aa1871. The result of sequencing of the polymerase genes of the two viruses cloned by me confirmed these changes. However, my clones contained some mutations that did not exist in the initial sequencing. Therefore, further analysis was performed by comparison of these sequences to the published and banked sequences of other Urabe

viruses, including the Urabe P-AM9, a vaccine isolate, Urabe P6-CEF and Urabe P6-Vero, which were isolated after 6x passes the vaccine in CEF and Vero cell lines respectively (Sauder et al., 2006), 87-1004 (AF314560), and 87-1005 (AF314562), which are both post-meningitis isolates, and AM9-A (AB000386), AM9-B (AB000387), AM9-parent (AB000388), Biken (AF314561) and Smithkline Beecham (AF314559), which are all Urabe vaccine viruses. Those mutations in my clones which had not been reported in any other Urabe virus were considered to be introduced by Taq polymerase and therefore needed to be reversed. One coding change found by me that had not been reported for 1004-10/2 in the original sequencing was a S→F change at aa 512 (nt1536 of the L gene, c to t) of the L protein. This change was reported for 1005 (AF314562), another Urabe post-meningitis isolate (Wright et al., 2000). Hence this sequence was accepted as real. The L^{512} mutation in 1004-10/2 is within domain II of L protein and close to the region defined to be important for P binding (aa516-aa522) in Sendai virus (SV) (the equivalent of this sequence in mumps is aa526-aa532 (Sidhu et al., 1993;Cevik et al., 2004). This left 6 mutations in the L gene of 1004-10/2 and 6 mutations in L Gw7 which were considered artifacts (Table 3.1). The process of reversing the mutations was as follows; mutagenesis reactions were performed successively for each site, and then the final clone was sent for sequencing. Those mutations that were not reversed would be subjected to another round of mutagenesis and sequencing until every mutation was reversed. All the mutations for Gw7 L were successfully reversed and the success was confirmed by sequencing of the regions containing the mutated nucleotides. After all the mutations were reversed for L Gw7, the L gene of another Urabe G virus, G7, was sequenced by another student in the lab, and the results of sequencing revealed that 3 of the changes

1004-10/2	Gw7
aa 136 S-->G	aa 135 I-->T
aa 174 E-->G	aa 440 N-->D
aa 1334 D-->G	aa 839 S-->G
aa 1514 I-->T	aa 1066 L-->F
aa 1586 V-->A	aa 1458 I-->T
aa`1909 D-->G	aa 1852 A-->V

Table 3.1 Mutations caused by Taq polymerase and were subjected to reversal.

Aa1586 V→A

IDAVMWGTKQ[ ]LTNISQGIDYEIVV 87-1004
IDAVMWGTKQ[ ]LTNISQGIDYEIVV mumps
VDAILWGCKR[ ]INVLSNGGDLELVV sv5
CNMVYTCYMT[ ]LDLLLNEELEEFTF measles
CNLIYNCYMI[ ]LDLLLNDELDDFSF CDV

Aa174 E→G

ELSQIWFNN[ ]WSGSVKTWLM 87-1004
ELSQIWFNN[ ]WSGSVKTWLM mumps
TVVAAWHD[ ]DWKRISDFWIM sv5
NLGVYMHS[ ]QWFEPFLFWFT measles
TDPAFWFH-[ ]KWSTAKFAWLH NDV

Figure 3.1 Sequence alignment of mumps virus (Sidhu et al., 1993) and 87-1004 Urabe virus (AF314560), with closely related viruses such as SV5 gb|AAC95518.1|, measles gb|ABD34018.1|, CDV and NDV (Sidhu et al., 1993) to show the location of unreversed mutations for L 1004-10/2. The highlighted bases are where the mutated nucleotides are located.

which I had found and reversed for L Gw7 existed in G7 as well. The changes were at positions aa 135 I→T (nt405, t to c), aa440 N→D (nt1319, a to g) and aa839 S→G (nt2516, a to g). Therefore it was tempting to think that these changes were real but were missed in the initial sequencing. To confirm this, two more independent RT-PCR reactions were completed for the segments of Gw7 L containing these sites and the products were sent for sequencing. None of these changes were confirmed, although, in one of the PCR products, a mutation at a different nucleotide (nt1320) was found which would result in a N→K change at the same amino acid, aa440. This suggests that aa440 might be prone to mutations that can occur naturally but are unlikely to have a severe effect on the polymerase activity.

In spite of several attempts, including altering the PCR conditions and designing longer primers which would hybridize better, 2 mutations, at aa174 (nt522) and aa1586 (nt4757), out of the 6 in the L gene of 1004-10/2, could not be reversed. Amino acid 174 is upstream of domain I and aa1586 is upstream of domain VI and therefore their possible effect on the polymerase activity is unknown. Since these mutations are not located in any of the conserved motifs of the mumps L protein (Figure 3.1), it was decided to move ahead and use this clone of L1004-10/2 in the experiments.

In summary, P and L genes from Gw7 and 1004-10/2 were cloned into pCite and sequenced entirely. Also the four original differences in the L gene of both viruses were confirmed together with another difference in the L of 1004-10/2 which exists in 1005 as well, while the reported difference in the P gene of Gw7 was not verified.

In vivo incorporation of ^{3}H UTP in the presence of Actinomycin D

The next objective was to determine whether there is an inherent difference in the polymerase activity between 1004-10/2 and Gw7. One of the methods to determine the polymerase activity was to measure the amount of ^{3}H UTP incorporated into the viral RNA in Vero cells infected with either virus. A previous study of growth kinetics of the two viruses showed that at low MOI (0.05), replication of both viruses increased rapidly after day 1 p.i., peaked and plateaued between days 2 and 4, and decreased by day 5 p.i. (Wright et al., 2000). Also in Vero cells, Gw7 consistently grows to a titer 1 log lower than 1004-10/2 (Table 1.2). It was decided to do a preliminary experiment under similar conditions in order to get a general understanding of how this type of experiment would work. Thus for the first experiment, the polymerase activity was measured at 24, 48, 72 hrs post infection at relatively low MOI. However, it was decided to infect the cells with 10 fold more Gw7 than 1004-10/2 to compensate for the growth difference. For this purpose, Vero cells were cultured in 6-well plates, each well containing approximately $1x10^6$ cells, and infected at MOI 0.5 for Gw7 and 0.05 for 1004-10/2. At 15, 39 and 63 hours post infection cells, were treated with 5μg/ml Actinomycin D for 1 hour and then labeled with 50μCi of ^{3}H UTP for 8 hours also in the presence of Actinomycin D. The cells were then harvested for RNA extraction, which corresponds to 24, 48 and 72 hrs post infection. Theoretically, all the RNA synthesized in the presence of Actinomycin D should be viral RNA because Actinomycin D inhibits cellular RNA synthesis by binding to DNA at the transcription initiation complex and preventing RNA elongation (Sobell, 1985). The controls for this experiment included mock infected cells with or without Actinomycin D to confirm that Actinomycin D was inhibiting cellular RNA synthesis.

Both controls were labeled as described above. Since the newly synthesized RNA should have ^{3}H UTP incorporated, an increase in the radioactivity over the background (mock infected cells treated with Actinomycin D) represents the quantity of the viral RNA and therefore the polymerase activity. There is at least 50 fold difference in the counts between the mock infected controls with or without Actinomycin D (data not shown), which shows Actinomycin D has shut down the cellular RNA synthesis. Five micro-liters, out of 20 µl of the total RNA extracted for each sample were run on an agarose-formaldehyde gel and the gel was stained with Ethidium bromide to visualize any intact RNA. The only bands observed were ribosomal bands (Figure 3.2 B). Several attempts were made to visualize newly synthesized viral RNA on the radiogram by exposing the fixed and dried gel to an X-ray film, but no virus specific bands were detected (data not shown).

Another 5µl of the total RNA solution were used for determining the radioactivity of the sample by scintillation counting. The best way to compare the polymerase activity between the viruses would be to normalize incorporated ^{3}H UTP to the amount of polymerase complex in the lysates. Thus, attempts were made to detect the viral polymerase proteins in lysates of cells treated exactly the same as the cells used for the RNA extraction by Western blot. For this purpose ¼ of the total lysate for each sample was electrophoresed in an SDS-PAGE gel, transferred to membrane and probed with one or the other of two polyclonal antibodies specific for mumps virus available in the lab; one is a human serum and the other from mice vaccinated with Urabe virus (Wright, et al., 2000). Despite several attempts using various concentrations of the

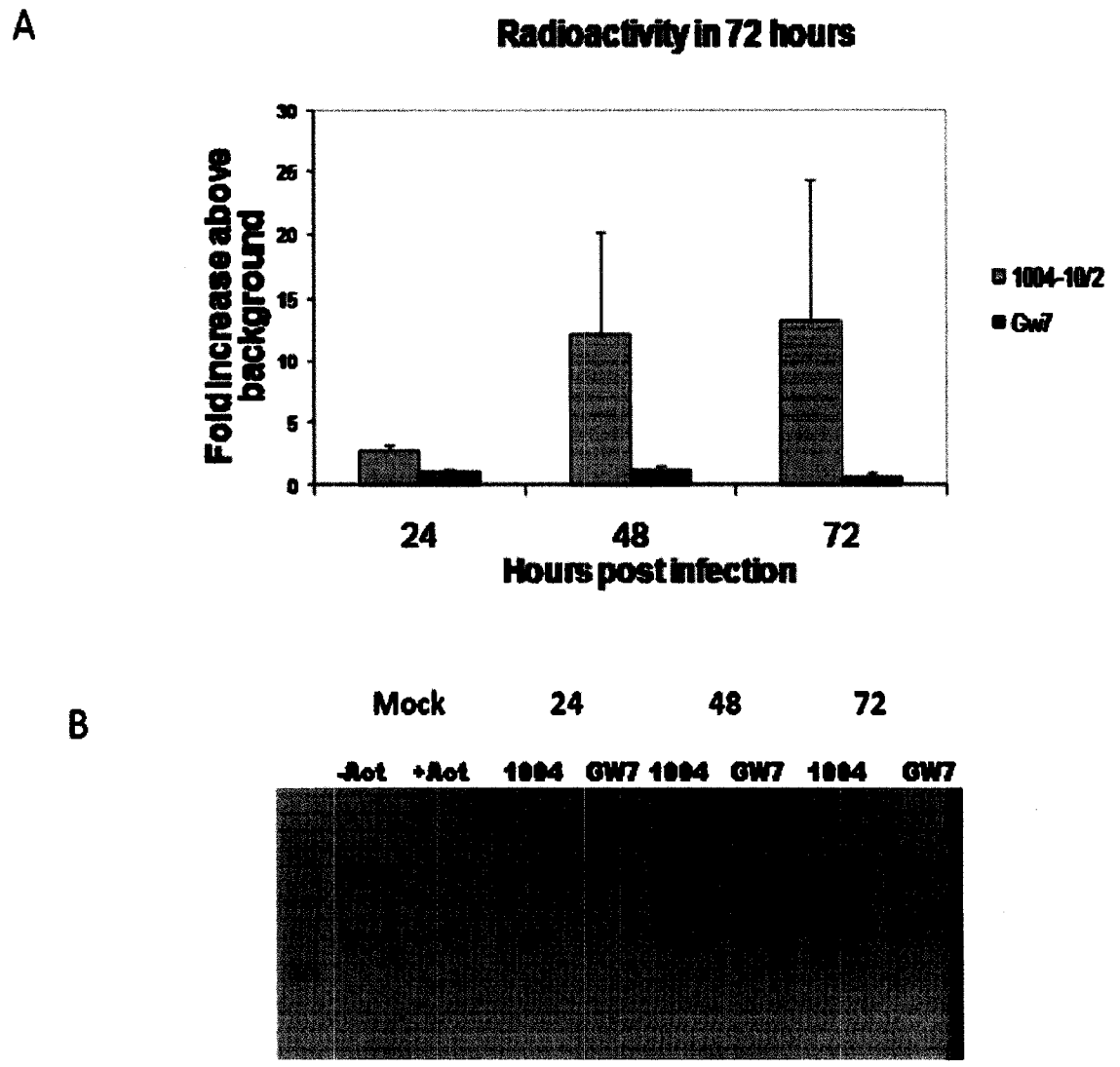

Figure 3.2 Incorporation of ^{3}H UTP in Vero cells infected with Gw7 or 1004-10/2. Radioactivity of viral RNA was analyzed at 24, 48 and 72 hrs p.i. in the presence of Actinomycin D (A). Vero cells were infected at MOI of 0.5 for Gw7 and 0.05 for 1004-10/2 and first treated with Actinomycin D and then labeled with ^{3}H UTP. Values are expressed as fold increase over the background; which is non-infected cells treated with Actinomycin D and labeled as described. Data are means of four independent experiments. Error bars represent the standard error of the mean. (B) EtBr stained gel electrophoresis of RNA purified and isolated from infected and labeled Vero cells as described under Materials and Methods, showing ribosomal RNA bands. This is a representative gel from one of 4 experiments.

antibodies and incubation times, no viral proteins could be detected under the conditions used in the experiment. Therefore the total counts of each sample were normalized to the amount of ribosomal RNA, which was determined by densitometry. The ribosomal RNA represents undegraded RNA extracted from viable cells; as Actinomycin D causes severe cytopathic effect (CPE). Normalization to ribosomal RNA at least allowed results to be compared per viable cell equivalents.

The result of this experiment is shown in Figure 3.2A, 1004-10/2 consistently showed higher counts compared to Gw7 at all three time points; but interestingly, while at 24 hrs p.i. 1004-10/2 shows only a slight increase, the difference becomes more drastic in the later time points, 48 and 72 hrs p.i. This can be explained by the accumulation of more polymerase complex for 1004-10/2 given the higher replication of the virus. It is also noteworthy to mention that the counts incorporated into RNA in Gw7 infected cells did not change from 24 to 72 hrs p.i. and this is unexpected as the virus yield for Gw7 increases to 6 (log pfu/ml) by 72 hrs p.i. (Wright, et al., 2000). Therefore this experiment suggests that the virus yield might not correlate with the polymerase activity. This experiment has been carried out four times and the differences in polymerase activity were not statistically significant (paired t-test).

The results of the first experiment were not normalized to the amount of polymerase protein and were conducted at times when secondary transcription occurred, therefore may not reflect a fair comparison of the activity of the polymerases versus the effect of increased accumulation of 1004-10/2 polymerase compared to Gw7. In order to have equal polymerase complexes for both viruses at the beginning of the experiment, it was decided to switch to a higher MOI and examine the polymerase activity at earlier

time points before the viruses undergo multiple cycles of replication. Generally a single replication cycle for paramyxoviruses takes about 14 to 30 hours (Lamb and Parks, 2007), unfortunately, the exact duration of a single replication cycle for mumps virus in cultured cells is unknown, but according to a one-step growth curve completed at MOI of 7, it is estimated that a single replication cycle for mumps should take about 15hrs (Wright et al., 2000). There was an issue with the amount of Gw7 available, as this virus does not grow to high titer (the titer of the virus is $2x10^6$ pfu/ml) and as it was preferable to work with the same stock of Gw7 used to determine the sequence, the highest MOI that could be achieved for this experiment was 2, which was not ideal, but still 4 times higher than the one used for the previous experiments.

Vero cells were cultured in 6-well plates and infected with 1004-10/2 or Gw7 at MOI of 2. At 1, 8 and 16 hours post infection, cells were treated with Actinomycin D for 1 hour and then labeled with 50μCi/ml of ^{3}H UTP for 8 hours, with Actinomycin D present during the entire period. Cells were then harvested for RNA extraction. Total RNA extracted from each sample was diluted in 20μl of ddH_2O and 5μl were analyzed by agarose-formaldehyde gel electrophoresis, which was stained with Ethidium bromide. Again, the only observed bands were the ribosomal RNAs (Figure 3.3 B). The gel was then fixed and dried and exposed to X-ray film for one week at -80°C to visualize the viral RNA bands (Figure 3.3 C). Besides ribosomal RNA, another band smaller than the 18s rRNA is seen in both infected and mock infected samples and an additional three bands approximately 1.5 kb, 2.5 kb and 3.5 kb were observed in lysates from infected cells but not in mock infected cells. These species are especially visible in 1004-10/2 at

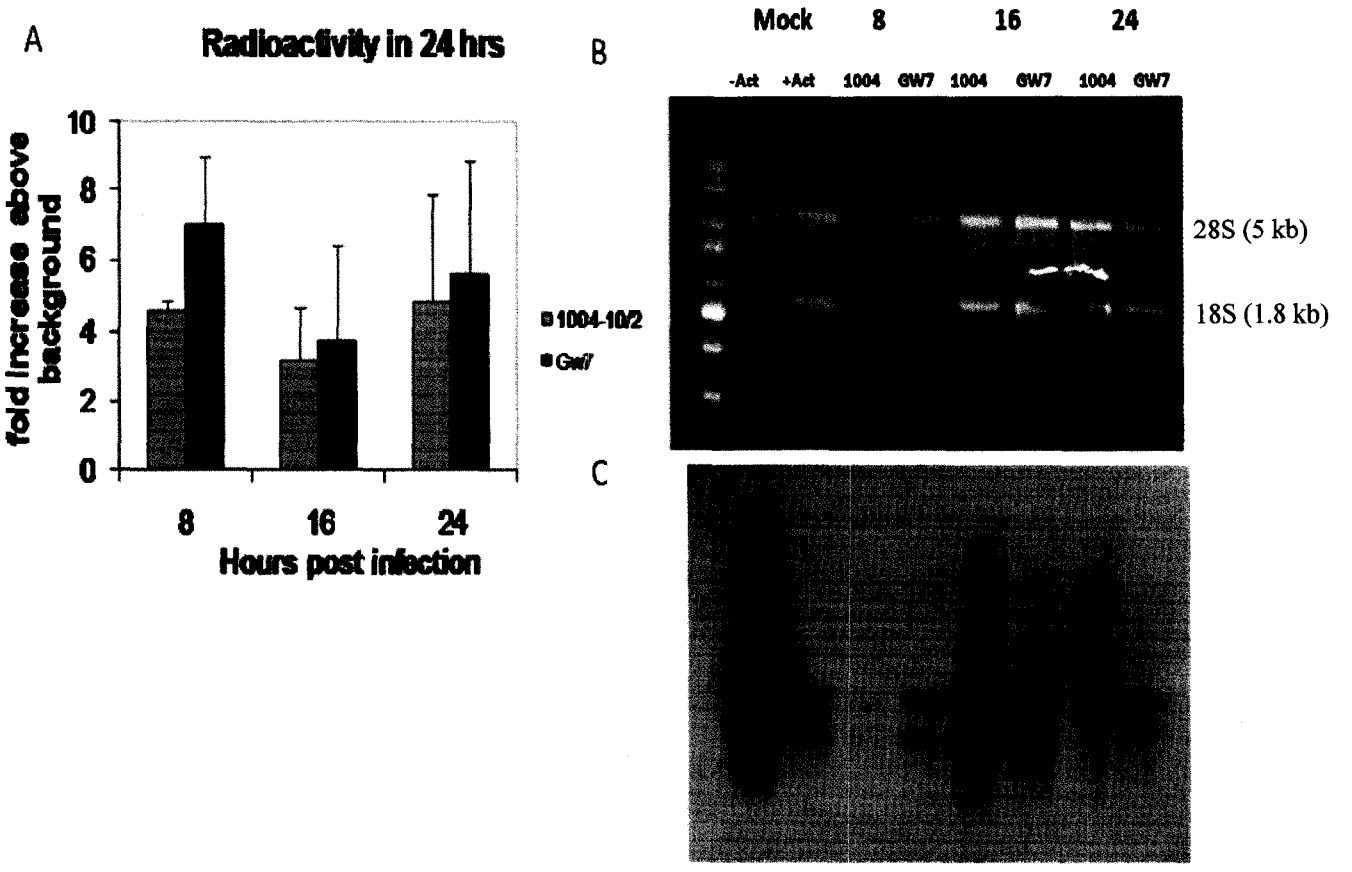

Figure 3.3 Incorporation of ^{3}H UTP in Vero cells infected with Gw7 and 1004-10/2 at MOI of 2. Radioactivity of ^{3}H UTP incorporated into the viral RNA was analyzed at 8, 16 and 24 hrs p.i. in the presence of Actinomycin D (A). Vero cells were infected at MOI of 2 for both viruses treated with Actinomycin D and then labeled with ^{3}H UTP. Values are expressed as fold increase over the background (mock infected cells treated with Actinomycin D). Values represent the means of three experiments and error bars are standard error of the mean. (B) Agarose gel electrophoresis of the isolated RNA stained with EtBr. (C) Radiogram made by exposing the fixed, dried gel to an X-ray film. Gels represent one of 3 independent experiments.

16hrs p.i.. The smallest RNA might be a viral RNA, as the sizes of several mumps mRNAs are around 1.5 kb, but there is no predicted RNA in mumps to correspond to the two latter bands. [The sizes of mumps RNAs are; 1.7kb for NP, 1.2kb for P, 1.15kb for M, 1.6 kb for F, 0.18kb for SH, 1.75 for HN, 6.75kb for L and 15.4kb for genomic and antigenomic RNAs (Carbone and Rubin, 2007).]

Another 5µl of RNA solution was used to examine the radioactivity of each sample and the results were normalized to the Ribosomal RNA (Figure 3.3 B). In this experiment, at all three time points, viral RNA synthesis for both viruses was observed, with at least a 3 fold increase in radioactivity over background, which is mock infected cells treated and labeled as described (Figure 3.3 A). Lysates of the cells infected with Gw7 showed slightly higher radioactivity in comparison to 1004-10/2 at all the three time points. This experiment was carried out three times and the differences were not statistically significant (paired t-test).

Establishing a mini-genome system for Urabe

For a long time the recovery of the negative stranded RNA viruses from cloned cDNA was impossible since, in contrast to positive stranded RNA viruses, the replicative form of RNA, genome or anti-genome cannot be used as mRNA to direct the synthesis of viral proteins (Garcia-Sastre, 1998). Many of the important pathogenic viruses have negative strand RNA genomes and their recovery was quite essential to the study of the different aspects of replication, attenuation and for production of recombinant viruses. This was first achieved by ground-breaking work by Schnell and colleagues for Rabies virus by transfecting four plasmids encoding the full-length anti-genomic viral RNA,

including the 3' and 5' untranslated regions (UTR) and intergenic regions, with a self-cleaving ribozyme at the 3' end, together with the rabies-virus nucleoprotein (NP) and RNA polymerase proteins (L and P) under the transcriptional control of T7 RNA polymerase, provided by a recombinant vaccinia virus (vTF7–3) (Schnell et al., 1994). In this model, transcription and translation from the transfected plasmids result in encapsidation of the anti-genomic RNA which then becomes a template for the viral RDRP, leading to generation of rescued infectious virus. The use of anti-genomic RNA, rather than genomic RNA, was to prevent hybridization between the negative sense viral RNA and the NP, P and L mRNAs (Roberts & Rose, 1998).

The strategy of constructing synthetic RNA genome analogues is an alternative approach to rescuing whole virus to study the different aspects of the viral replicative cycle. In this case the 3' and 5'untranslated regions (UTR) of the genome of a non-segmented, negative sense RNA virus are fused to a reporter gene, such as bacterial choloramphenicol acetyl transferase (CAT) or firefly luciferase, thus replacing all the viral genes (Freiberg et al., 2008;Lin et al., 2005;Dimock & Collins, 1993). These chimeric vRNA analogs or mini-genomes, along with plasmids encoding the proteins of the polymerase complex (NP, P and L) or a helper virus should be transfected into permissive cells to participate in all aspects of the viral replicative cycle (Brown et al., 2005;Clarke et al., 2000;Dimock & Collins, 1993;Park et al., 1991).

It was decided to construct a mini-genome system to compare the activities of the polymerases from 1004-10/2 and Gw7. The designed Urabe mini-genome consists of the firefly Luciferase ORF flanked by the authentic 5' and 3'untranslated regions derived

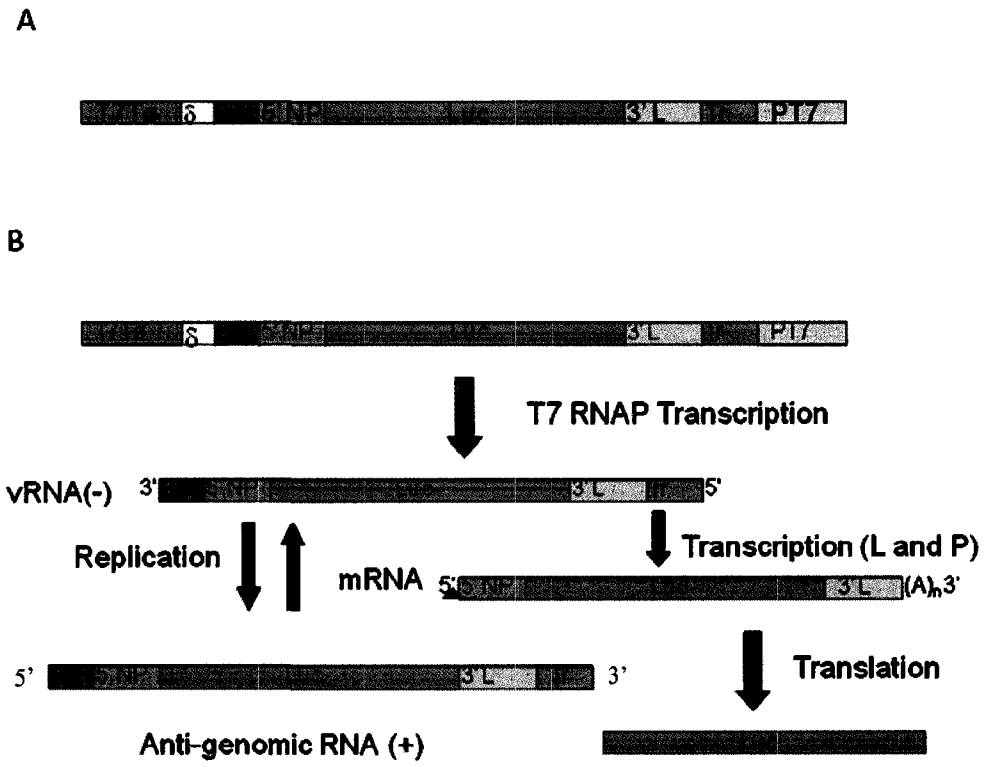

Figure 3.4 Establishment of the Urabe mini-genome system. (A) Schematic of the mini-genome system (not to scale) which is flanked at one end by a bacteriophage T7 RNA polymerase (T7 RNAP) promoter (PT7) and at the other end by a hepatitis delta virus ribozyme (δ) and T7 transcriptional terminator (T7θ). The Luc ORF replaces all of the coding and intercistronic sequence of the Urabe Muv genome; the remaining essential Muv specific sequence comprises the 3' leader (55 nt) with adjacent 90-nt NP gene untranslated region (UTR) and the 5' trailer (24 nt) adjacent to the 137-nt L gene untranslated region. (B) T7 RNA transcripts can be synthesized under the control of the T7 promoter to generate viral negative sense Urabe RNA. The Urabe mini-genome also contains three extra G residues after the T7 RNAP promoter and prior to the trailer sequence (Tr) in order to increase T7 RNAP transcription efficiency. The exact leader sequence (Le) is generated by cleavage with hepatitis delta virus ribozyme. In the presence of the Urabe L, P, and NP proteins this template is used for both replication and transcription to give rise to the reporter gene mRNA, resulting in Luc activity.

from the 1004-10/2 virus, adjacent to the bacteriophage T7 RNA polymerase (T7 RNAP) promoter (PT7) at one end and the hepatitis delta virus ribozyme (δ) and T7 transcriptional terminator (T7θ) at the other end (Figure 2.2 and 3.4 A). I used the 5' and 3' UTR from 1004-10/2 for two reasons; one is that 1004-10/2 has the consensus sequence for these regions while there is a nucleotide change (a→g) at the position nt15328 of Gw7, and the other reason is the fact that 1004-10/2 grows to higher titers which makes it easier to generate the fragments by RT-PCR. The reason for adding δ to the construct was to have an authentic end generated for transcribed RNA; the self-cleavage by the ribozyme will result in the generation of the exact leader sequence. It is also important that the transcription by T7 RNAP ends at the last nucleotide of the leader to maintain the "rule of six", which was explained earlier. The mini-genome system was engineered in a way that transcription from the T7 promoter will give rise to a negative strand mini-genome, which cannot be translated but in the presence of NP and the polymerase complex (P and L), will become the template for replication and transcription. The transcription gives rise to Luc mRNA which results in measurable Luc activity, while the replication results in synthesis of a positive strand or anti-genomic RNA that becomes the template for further replication (Figure 3.4 B). The advantage of this design is that the Luciferase activity is only observed in the presence of the viral polymerase complex; without a complete polymerase complex, no Luciferase mRNA should be synthesized. Therefore, in this model, the Luciferase activity represents the polymerase activity.

The approach to make the mini-genome construct was to generate 4 DNA fragments by PCR that would be ligated together (Figure 3.5 A); 1) a fragment

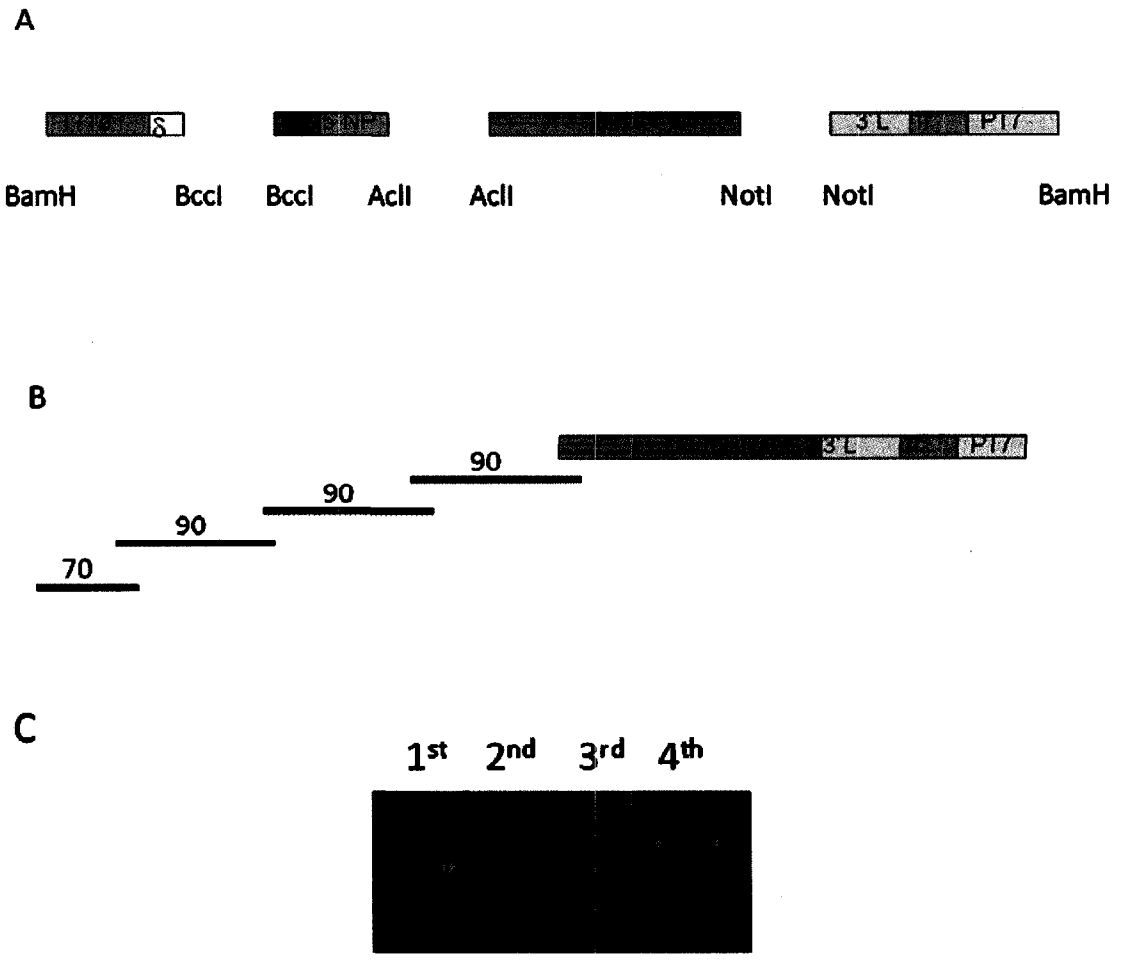

Figure 3.5 Diagram showing the steps to make the Urabe mini-genome. (A) Restriction sites were designed to ligate different parts of the mini-genome. (B) Long overlapping primers to make the rest of the mini-genome. (C) Agarose gel electrophoresis of the DNA products after each round of PCR with the above mentioned long primers.

incorporating the T7 promoter added to the Urabe trailer (Tr; 24nt) and 3' UTR sequences (137nt), 2) a fragment with the Luciferase ORF (Luc; 1685nt), 3) a fragment with the Urabe leader (Le; 55nt) and 5' UTR (90nt) sequences, 4) a fragment with the T7 terminator (T7θ) added to the hepatitis delta ribozyme (δ; 87nt). The first step to make the construct was to amplify the leader and trailer sequences individually by performing RT-PCR on RNA isolated from Vero cells infected with 1004-10/2 to amplify luciferase and ribozyme sequences using a plasmid containing the Luciferase and ribozyme sequences as the template. All the primers were designed to incorporate restriction sites to be used for ligation of the segments together, and to maintain the rule of six. Also, PT7 and T7θ were included in T7Tr- and θδ+ primers respectively (Figure 3.4A and Table 2.5). Then the luciferase (Luc) and the trailer (Tr) fragments were digested with *NotI* and ligated to each other. The ligation was confirmed both by gel electrophoresis and performing PCR using Luc+ and T7Tr- primers. The leader and the ribozyme were also digested with *BccI* and ligated to each other and the ligation was confirmed by PCR using (θδ+ and Le-) (Figure 3.5 A). Attempts to ligate T7Tr-Luc to θδ-Le after digesting them with *AclI* were unsuccessful. Further attempts were made but the ligation of these two fragments was not achieved; therefore another approach was chosen to make the rest of the construct.

Long primers each comprising of 70 to 90 nucleotides incorporating the sequences for Luciferse, virus 5' UTR, ribozyme and T7 terminator were designed. Each primer had 20 overlapping nucleotides with the next primer and thus the rest of the mini-genome was constructed with four rounds of PCR (Figure 3.5 B, 3.5 C). The final PCR product was cloned into pDrive and 2 clones were sent for sequencing. The result of.

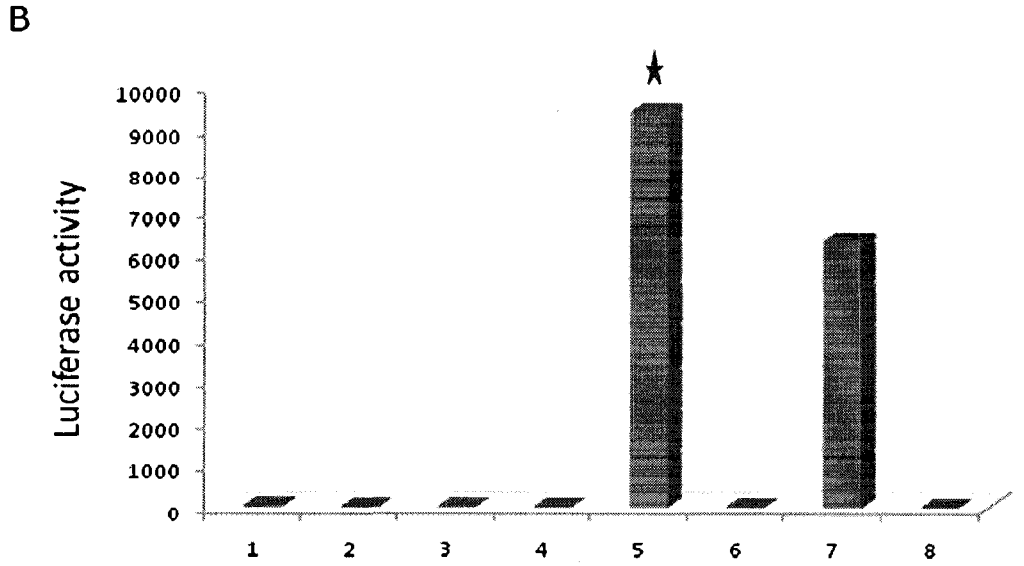

Figure 3.6 Reporter gene Luc expression from the mini-genome system. Plasmids containing different clones of the Urabe mini-genome were transfected into Vero cells that has already been infected with 1004-10/2 and the luciferase activity was measured. Clone number five, which shows the highest activity, was selected and sent for sequencing.

sequencing showed that there were mutations in different parts of the constructs (3 mutations in clone #1 and 4 in clone #2), perhaps caused by Taq polymerase during PCR Instead of trying to reverse the mutations by site directed mutagenesis, more colonies were screened for size and tested for Luciferase activity. For this purpose, Vero cells were cultured in a 12-well plate and inoculated with 1004-10/2 at MOI of 2. At 1 hour post infection, cells were transfected with one of 8 different clones of the mini-genome construct and 24 hrs post transfection the luciferase activity was measured (Figure 3.6). The clone with the highest luciferase activity (No.5) was selected and sent for sequencing, which confirmed that the sequence of the entire construct was correct. Therefore clone No.5 was subjected to digestion with BamHI and the mini-genome was sub-cloned into pUC19 vector, which does not have its own T7 promoter, thus ensuring that transcription from the mini-genome would only occur from the T7 promoter inside the construct.

Measuring the polymerase activity with the help of mini-genome and the polymerase complex

As it has been explained earlier, in order to drive the expression of the luciferase gene from the mini-genome system, the mini-genome plasmid should be co-transfected with NP and the polymerase complex (P and L). L expression plasmids for both viruses were constructed and a single P clone was mutated to express the P protein, as was elaborated before. The NP ORF, which is identical in the two viruses according to the original sequencing, was amplified by RT-PCR and T/A cloned into pDrive between the T7 RNA polymerase promoter and the T7 RNA polymerase transcription termination

sequence. Then the plasmid was sent for sequencing. The sequencing result was identical to the initial sequencing result for NP.

To compare the polymerase activity of 1004-10/2 and Gw7, using the mini-genome system, Vero cells were cultured in 12-well plates with each well containing approximately 500,000 cells, and transfected with all components of the RDRP complex: 500ng of NP, P and L plasmids of either virus, at various combinations, together with 500ng of the mini-genome (Lin et al., 2005) and 200ng of β-galactosidase plasmid for measurement of transfection efficiency. The expression of all these plasmids is under the control of the T7 promoter, thus recombinant T7 vaccinia virus was used to provide T7 polymerase. Cells were harvested at 24hrs post transfection by adding 100 μl of the proper lysis buffer and cell lysates were subjected to different assays as will follow.

In order to make sure that the plasmids encoding for the polymerase complex proteins were expressed in each sample, ¼ of the cell lysate for each sample was used in Western blot analysis. Here again, despite several attempts, NP, P and L proteins could not be detected by Western blot analysis using the above mentioned polyclonal antibodies. Although the expression of NP, P and L could not be confirmed by Western blot, the fact that the mini-genome system was functional and luciferase expression was detected shows that these proteins were expressed and their expression was sufficient to drive transcription of Luciferase mRNA from the mini-genome plasmid. Being confident about the expression of the plasmids, a β-galactosidase assay was performed on 10μl of cell lysate of each sample to control for transfection efficiency (Figure 3.7B).

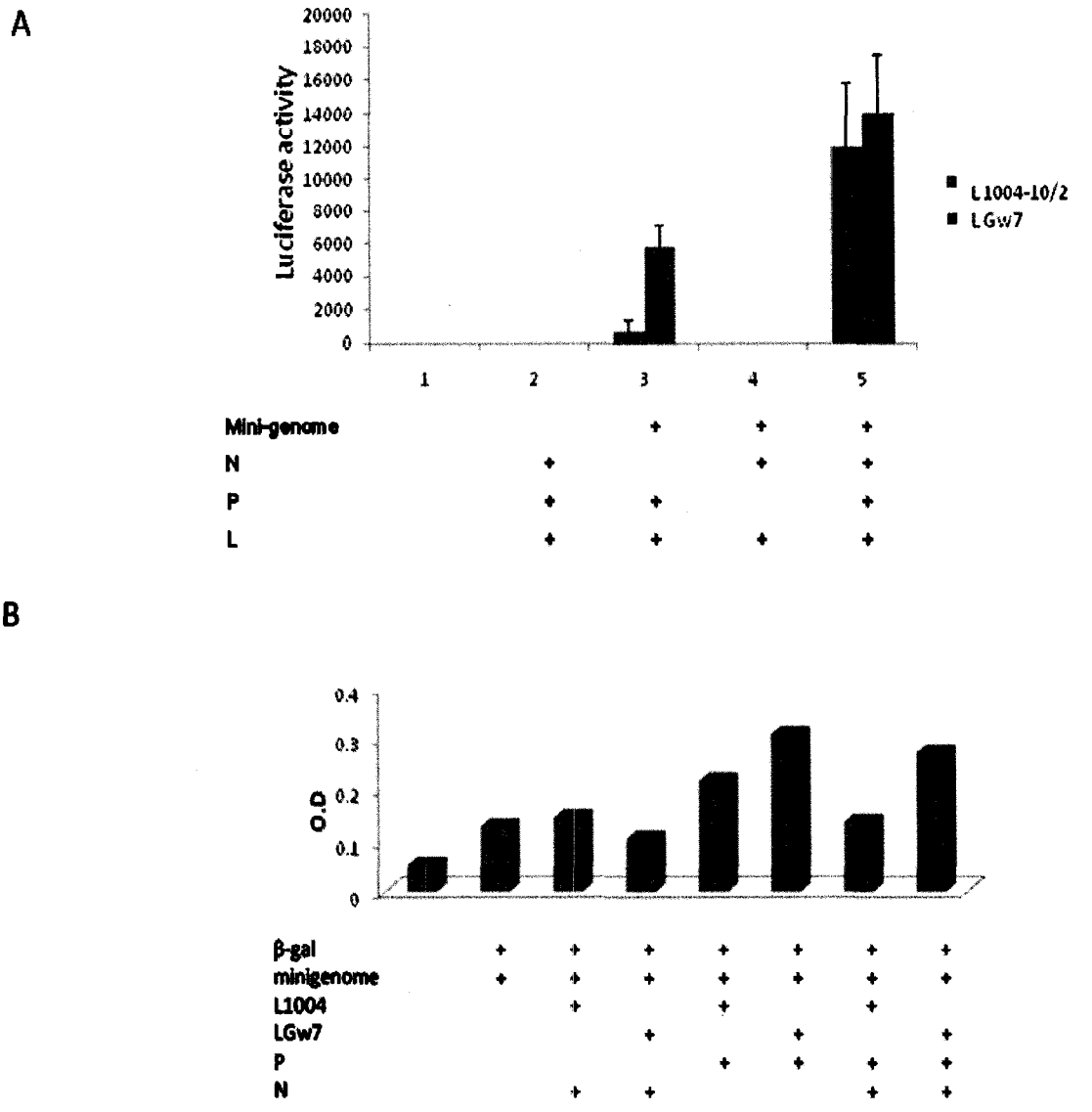

Figure 3.7 Luciferase expression driven by viral polymerase in a mini-genome system. (A) Plasmids encoding NP, P, L, and the mini-genome were transfected into Vero cells in 12-well plates and Luc activity was measured. Cells were also transfected with a plasmid expressing β-gal to normalize for transfection efficiency. Data represent the mean of three independent experiments and the error bars are standard error of the mean. (B) β-gal expression from the cells transfected with NP, P, L, mini-genome and β-gal. Data represent one of three independent experiments.

Twenty μl of cell lysate of each sample were used for luciferase assay. As the negative control, one well was transfected only with the mini-genome and β-galactosidase plasmids and this background luciferase activity was subtracted automatically from the activity of the rest of the samples (Figure 3.7A lane 1). An additional negative control was a pair of wells transfected with the components of the polymerase complex of either virus without being co-transfected with the mini- genome plasmid and as shown in Figure 3.7A lane 2, no luciferase activity was observed. As expected, almost no luciferase activity was detected in the absence of P plasmid (Figure 3.7A lane 4) or L plasmid (data not shown). After normalization of all samples for transfection efficiency, the activities of the two polymerases were essentially equal (Figure 3.7 A lane 5). Interestingly, luciferase activity was observed, to some extent, in the absence of NP for both viruses (Figure 3.7A lane 3) which was unexpected and in the presence of the Gw7 L plasmid this activity was higher than for the 1004-10/2 L plasmid. The experiment was carried out three times and the differences were not statistically significant (paired t test).

Measuring the polymerase activity with the mini-genome and helper viruses

Another way to compare the polymerase activity of 1004-10/2 and Gw7 was to use infected cells to drive the mini-genome transcription and replication, rather than NP, P and L expressed from plasmids so if there are genetic differences that might exist in the quasispecies or were missed by sequencing, these will be included by performing the experiment using the actual viral particles. Also, as the clone for L 1004-10/2 had two

mutations which could not be reversed, and because the non-coding difference in the trailer of Gw7 was not incorporated into the mini-genome, there was the possibility that failure to observe a difference could be because these differences were not accounted in the construct. Also, other viral proteins which have been shown to have an effect on the polymerase activity are not part of the polymerase complex, For example V protein has been demonstrated to reduce the viral RNA synthesis (Lin et al., 2005;Horikami et al., 1996). Even though no differences were found in the V protein of the two viruses, the interactions of the polymerase complex of the viruses with the V protein or possibly other viral proteins might differ. Because of all these reasons, it was decided to use 1004-10/2 and Gw7 as helper viruses to drive expression from the mini-genome.

For this experiment, the Vero cells were cultured in 12-well plates and first infected with 1004-10/2 or Gw7 at MOI of 2. One hour post infection, cells were transfected with 500ng of the mini-genome and 200ng of β-gal plasmid. Twenty-four and 36 hours post transfection, cells were harvested and equal amounts of cell lysate for each sample were subjected to the luciferase assay. This experiment has been repeated 3 times and the results have been normalized to β-gal expression (Figure 3.8B). As demonstrated in Figure 3.8A lane 1, lysates of cells infected with 1004-10/2 and Gw7 showed no luciferase activity on their own. As the negative control, one well was transfected with the mini-genome alone and the luciferase activity in this sample was considered the background and was subtracted from the rest of the samples. Results obtained from the expression of the mini-genome construct reveal that luciferase activity in 1004-10/2 infected cells was slightly higher at 24 hours p.i (Figure 3.8A lane 2), while at 36 hours

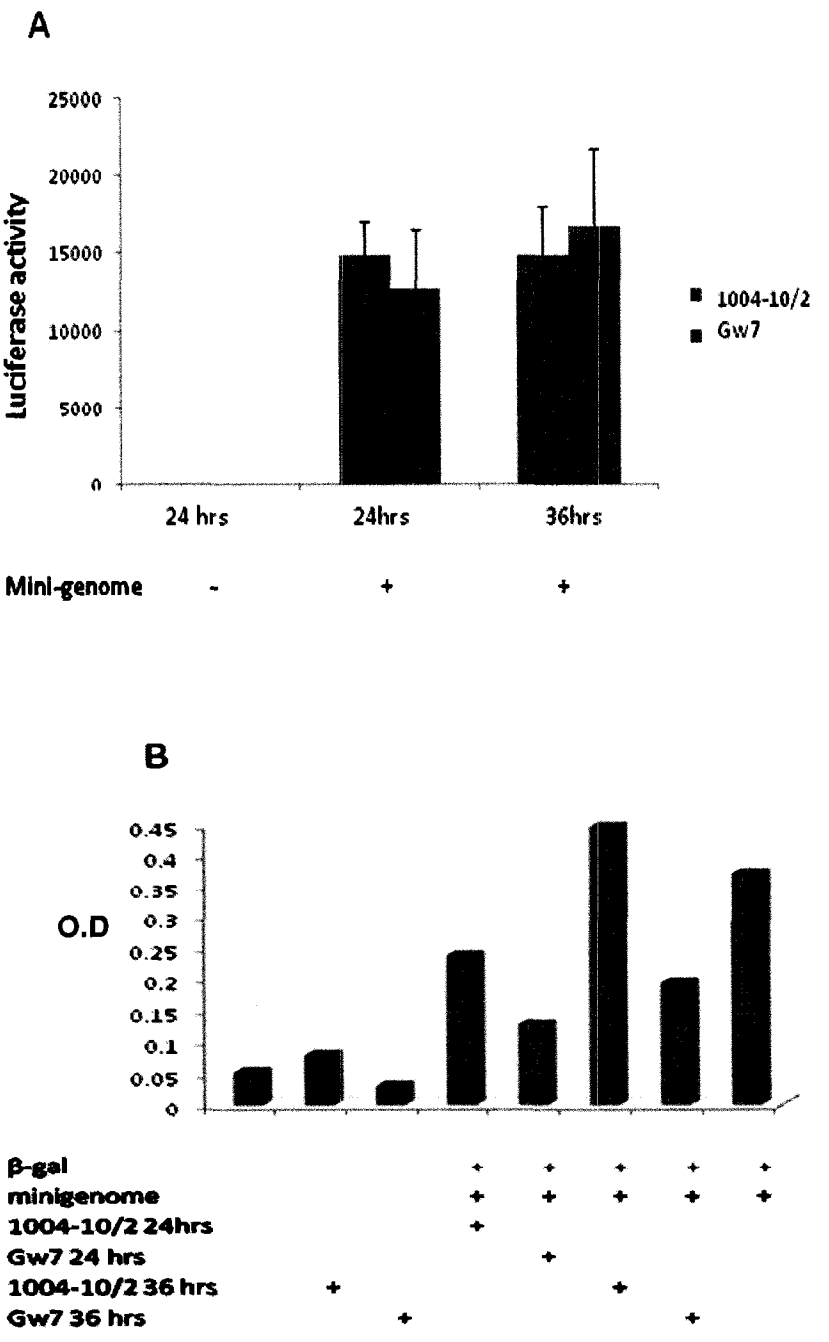

Figure 3.8 Luc expressions from the mini-genome system. (A) Plasmid encoding the mini-genome was transfected into Vero cells in 12-well plates which had already been infected with 1004-10/2 or Gw7 and Luc activity was measured 24 or 36 hrs post transfection. β-gal was added to normalize the transfection efficiency. Data represent the mean of 3 experiments and the error bars are standard error of the mean. (B) β-gal expression from the cells infected with either viruses and transfected with mini-genome and β-gal. Data represent one of three independent experiments.

p.i (Figure 3.8A lane 3), Gw7 infected cells show slightly higher activity. None of these differences are statistically significant.

The only weakness of this experiment is that, like the previous experiment, the luciferase activity was not normalized to the amount of the polymerases complex of each sample. Here again, the problem was the inability to detect the L protein by Western blotanalysis. However, the results from this experiment are consistent with the results obtained from rescuing the mini-genome with the polymerase complexes and with the results from incorporation of ^{3}H UTP in cells infected with either viruses at MOI of 2. Therefore, it can be concluded that no inherent difference in the polymerase activity between 1004-10/2 and Gw7 was observed by the experiments performed in this study.

Chapter Four: Discussion

The ultimate goal of this project was to find out if the genetic differences between the polymerases of the two isolates of Urabe virus, 1004-10/2 and Gw7, would affect the function of the enzyme. Therefore, the first objective was to confirm all the differences which had already been found in the polymerase complex genes. Initial sequencing had identified four coding differences between the polymerase proteins of these isolates (Table 1.4) and a coding change in the P protein of Gw7, at position of aa 274 (K→R).

As part of this project I cloned the P and L genes for both viruses in order to confirm their sequences and express the proteins. After sequencing three independent RT-PCR products of the P gene, I could not confirm the K274R difference in the P gene of Gw7 which had been reported in the initial sequencing. Therefore it was concluded that this mutation was either introduced during PCR or was represented in a minor population of Urabe viruses.

Because the L protein is very large, and is encoded by a gene of 6810 nucleotides, cloning the entire gene was achieved in several steps. After both L genes were successfully cloned, only one clone for each virus was sequenced and the results were compared with the original sequences. The original sequencing of the genomes of the two viruses was done by sequencing three clones of single PCR products and this sequencing identified 4aa differences in the L proteins of the viruses (Table 1.4). My sequencing results confirmed the reported differences at aa163 and aa320 of the L protein of Gw7 and the differences at aa1085 and aa 1871 of the L protein of 1004-10/2 relative to the Urabe consensus. Several additional mutations were found and were compared with the published sequences of other Urabe viruses. Those mutations which had not been

reported in other Urabe viruses were considered to be introduced by Taq polymerase and were reversed. One of the coding changes at aa 512 of the 1004-10/2 L protein (S→F) had already been reported for 1005, another Urabe A virus which was isolated from a patient with meningitis associated with vaccination. Therefore this change was considered as real. All those coding mutations considered to be introduced by Taq polymerase in Gw7 L were successfully back mutated to reflect the original sequence but only four mutations out of six were reversed for L 1004-10/2. Further attempts to reverse the other two sites were unsuccessful. One possibility for these failures is that in spite of using the *DpnI* enzyme which digests the methylated parental plasmids, parental plasmids, instead of mutated ones, were constantly isolated. Or the gene product may be toxic for *E.coli* upon mutagenesis. The two sites, aa174 E→G and aa1586 V→A, were not located in any of the recognized functional motifs of the L protein. Therefore their possible effects on the polymerase activity are unknown and were assumed to be minimal.

The second question was whether the genetic differences in the L protein result in a significant difference in the polymerase activity between the two viruses which would contribute to their biological differences in cell tropism and attenuation. Sequence comparison of 1004-10/2 and Gw7 with other Urabe viruses shows that the three unique mutations in the l004-10/2 L, i.e S512F, L1085P and A1871V may not be determinants of virulence since they do not exist in 1004-4/1, a virus isolated from the same stock of 1004 as 1004-10/2. Also, another study has shown that passage of the parental Urabe vaccine (P-AM9) in Vero and CEF cell lines selects for 1004-10/2 sequence at aa512 and aa1085 but these viruses showed attenuated phenotype in RNVT (Sauder et al., 2006). On

the other hand, the two unique mutations of Gw7 L, i.e T163N and I320M are also seen in another "G" Urabe virus, G7. Therefore these mutations might be associated with the attenuated phenotype of these viruses.

In many paramyxoviruses including mumps, the HN and F proteins are known to play an important role in cell tropism and virulence of the virus (Wright et al., 2000;Afzal et al., 1998);(Malik et al., 2007;Amexis et al., 2003), however, as it has been mentioned previously, no marked difference in cell binding and fusion between 1004-10/2 and Gw7 has been observed (unpublished Shah et al. 2008). On the other hand, other data have demonstrated that the amino acid changes at position aa 1165 (Glu→Asp) and aa 736 (Ile→Val) of L protein might be associated with mumps virulence in the RNVT (Rubin et al., 2003) but these changes do not exist in the viruses under study here. None of the mutations in the polymerases of 1004-10/2 and Gw7 correspond with mutations found in the L genes of other paramyxoviruses which have been shown to affect attenuation/virulence, such as aa942, 992, 1710 in HPIV1 L (Bartlett et al., 2007;McAuliffe et al., 2004), aa1248, 1800 in measles L (Takeda et al., 1998), aa1558 in HPIV3 L (Skiadopoulos et al., 1999), aa248, 1030 in RSV L (Collins & Murphy, 2005), aa626, 1791, 2024 in SeV L (Fujii et al., 2002b). However, these findings do support the importance of the polymerase protein in virulence of mumps virus.

One of the approaches to examine and compare the polymerase activity of the two viruses was to compare the amount of ^{3}H UTP incorporated into the viral RNA after infection of Vero cells with either viruses. The polymerase activity was examined under two different conditions; 24, 48, 72 hrs p.i at low MOI and 8, 16, 24 hrs p.i at higher MOI. In the first set of experiments, at 48 and 72 hours p.i, the radioactivity detected

from 1004-10/2 infected cells is respectively 12 and 13 fold more than Gw7, which is a remarkable difference and even compensating for input Gw7 made no difference in the outcome of the experiment. The reason that can explain this finding is the fact that by 48 hrs p.i both viruses have undergone multiple cycles of replication and since 1004-10/2 grows faster and to higher titers in Vero cells than Gw7, there will be more polymerase which would produce more RNA than Gw7. In other words, the observed difference at time points later than 24hrs p.i. is not necessarily due to greater polymerase activity of 1004-10/2 L, but rather because 1004-10/2 has more polymerase complex. To be sure that the assay was measuring primary RNA synthesis by equal amounts of polymerase complex for each virus, the experiment could be done at earlier time points using much higher MOI, as high as MOI of 10. However, due to the lack of Gw7, the experiment was performed at MOI of 2 for both viruses and a higher polymerase activity from 1004-10/2 could not be detected under this condition at 8, 16 and 24 hours p.i.

The problem which more or less affects most of the performed experiments is the lack of ability to normalize polymerase activity to the amount of polymerase complex. Several attempts were made to detect the polymerase protein by Western blot, including altering the concentration of antibodies and the incubation times, but all faced failure. For many Paramyxoviruses such as Sendai virus, labeling with Express-[^{35}S] was used to detect viral proteins including the L protein (Feller et al., 2000). Therefore there is a chance that the polymerase protein of the mumps virus could be detected in the presence of Actinomycin D and Express-[^{35}S]. However, this method has not been tried in this study. Since a successful Western blot could not be performed to normalize the experiment to the viral proteins, normalizing to ribosomal RNA was done. The ribosomal

RNA was used to normalize results because it is an indicator of intact RNA, but obviously the amount of ribosomal RNA, like other cellular RNA, reduces as the virus growth proceeds; on the other hand, detecting the ribosomal RNA bands on the gel is an indicator of viable cells and undegraded RNA. Moreover, ribosomal RNAs have been used to normalize results to the total RNA load in other studies examining RNA synthesis (Walpita & Peters, 2007;Lang et al., 2003).

Urabe mini-genome was successfully constructed and luciferase expression was achieved by co-transfecting the mini-genome plasmid with the plasmids expressing NP, P, and L proteins in Vero cells in the absence of mumps helper virus. Despite the failure to visualize these proteins, the results demonstrated that the NP, P, and L proteins were expressed and were able to drive the expression of luciferase from the mini-genome. For further experiments, tagged clones of these genes can be used to make their detection possible. However, again there was no difference in the ability of the L from 1004-10/2 and Gw7 to drive luciferase expression. There was a concern that the two unreversed mutations in L 1004-10/2 might alter the true activity of the enzyme. Although not much is known about the structure of the L protein of the paramyxoviruses, it has been discovered that, contrary to NP and P proteins, there is no structural disorder region (specific region of a protein that is biologically functional while lacking any constant secondary and tertiary structure) in the complicated structure of the polymerase protein (Bourhis et al., 2006). This finding suggests that a precise protein scaffold is necessary for various enzymatic activities of the L protein and therefore any coding change in this protein, such as the unreversed mutations for L 1004-10/2, may have a dramatic effect on the polymerase activity. To circumvent the possible problem with the sequence of L

1004-10/2, I decided to use viral particles to drive the expression of the mini-genome. Also there are always some genetic differences within a viral quasispecies which make the general phenotype of that quasispecies, thus by using the viral particles to rescue the mini-genome, those changes are included, which makes it possible to study the polymerase activity of the whole viral population. Even though the actual virus particles were used to drive the mini-genome, a significant difference in the polymerase activity of the two viruses could not be detected. It is notable that at 24hrs p.i in Vero cells, Gw7 grows to a titer 1 log lower than 1004-10/2, however, the experiments show that this difference in growth did not result in a lower polymerase activity by Gw7 than 1004-10/2. Again it was not possible to normalize the experiment to the amount of the polymerase, as the polymerase complex could not be detected under the conditions used in the experiment, and the experiment were carried out at MOI of two rather than a higher MOI which may not have been the optimal condition to measure primary transcription. However, it is worth reiterating that the conditions under which this experiment was carried out, such as cell equivalence, multiplicity of infection, transfection master mix, incubation times, etc. were the same for both viruses. Also, the β-gal assay was performed to normalize transfection efficiency, therefore any possible differences, in the amount of polymerase complex between the viruses should be minor.

Recent studies have demonstrated that the *cis*-acting elements within 3' and 5' UTRs of paramyxoviruses can be important determinants of attenuation. For example mutations in the leader sequence of Sendai virus can lower its pathogenicity (Walpita & Peters, 2007;Fujii et al., 2002a) and mutations in the leader sequence of human parainfluenza virus type 3 (PIV3) result in temperature sensitivity and attenuation

(Skiadopoulos et al., 1999). Similarly, *cis*-acting sequence mutations have been identified to control virulence in measles virus (Parks et al., 2001). As mentioned before, there is a change at nt 15328 of Gw7 (g→a) which falls in an enhancer region at the 5' UTR where *Paramyxovirinae* anti-genomic promoter essential for replication is situated (Cordey & Roux, 2007;Keller & Parks, 2003;Keller et al., 2001). This enhancer region functions as a positive-acting signal for RNA replication, and therefore this nucleotide change might have an effect on the polymerase activity of Gw7. Hence the fact that the 3'and 5' UTR of 1004-10/2 was used to make the mini-genome construct might have eliminated the possible importance of this factor in making a difference in the polymerase activity. One way to determine the possible effect of this mutation on viral replication is to mutagenize this nucleotide in the mini-genome sequence and perform two sets of experiments; one with the mini-genome which has the consensus sequence in the trailer region and the other one with the mini-genome which has the Gw7 trailer sequence. Any difference in luciferase activity between the two sets would be because of this mutation.

It is also noteworthy to mention that some of the cellular RNA-binding proteins, such as human La protein have been shown to be associated with viral IRES elements in positive sense RNA viruses and also with 3' and 5' *cis*-acting elements of genomic RNA in some negative sense RNA viruses. This association, enhances translation and replication *in vitro* and *in vivo* (Cordes et al., 2008;Raha et al., 2004). There are two differences between 1004-10/2 and Gw7 which might fall within La protein binding sites which are capable of altering the transcription and replication.; one which was mentioned above is the change at nt 15328 of Gw7 (g→a) which falls in the trailer region and the other is the non-coding change at nt 1880 of 1004 (c→t) in the intergenic region between

NP and P located 85 nt downstream of the NP stop and 99 nt upstream of the P start. It is notable that there is no evidence that La protein plays a role in reinitiation of transcription in negative sense RNA viruses. The phenomenon of enhancement of viral replication and mRNA translation by La protein has only observed in human cell lines such as A549 and Hela (Wolin & Cedervall, 2002). So it is possible that this protein plays a role in the higher growth of 1004-10/2 in A549 and Hela cells in comparison to Gw7.

When the project was started, it was expected that 1004-10/2 would display a more polymerase activity than Gw7, but for most of the experiments this was not the case. In fact, Gw7 showed a low but consistent trend to higher activity. Elevated polymerase activity of attenuated viruses has been reported in two separate studies on mumps and measles viruses (Malik et al., 2007;Bankamp et al., 1999). In these cases the hypothesis is that increased polymerase activity leads to production of more viral antigens during the early stages of infection, which in turn may improve the stimulation of antiviral immune responses resulting in a more rapid clearance of the attenuated virus (Bankamp et al., 1999). However, how increased polymerase activity would result in less replication of an attenuated virus in cell culture, where there is no immune response, is less clear.

It is also known that increased viral transcription raises the amount of double-stranded RNA within the cell, which triggers a more effective RIG I-mediated beta interferon (IFN-β) response which leads to a more rapid viral clearance. Also, it has been recently shown that cytosolic 5'-triphosphate ended viral leader transcript of measles virus is an efficient activator of the RIG I-mediated IFN-β response (Plumet et al., 2007). If this were the case, then the fact that 1004-10/2 replicates well in A549 cells implies a

reduced sensitivity of this virus to IFN compared to Gw7 since A549 cells produce interferon while Vero cells do not (Steward et al., 1972). However, unpublished work in the lab showed that there was not a remarkable difference in the response of 1004-10/2 and Gw7 to interferon and A549 cells do not produce a high level of interferon which would account for the 4log reduced growth of Gw7 in A549 cells (Wright. et al., 2000).

Even though no inherent difference in the polymerase activity of the two viruses was found in this study, there is still the possibility of difference in stability of the structure of the polymerase proteins between 1004-10/2 and Gw7 which may allow more accumulation of 1004-10/2 L. Differences in the stability of the polymerases from the two virus could also contribute to the differences in tropism. There are common methods to assess protein stability such as the use of protein tags to monitor the protein turnover by western blot or employing autofluorescent protein tags, like green fluorescent protein (GFP) to examine the speed of degradation of the polymerase protein *in vivo* (Ignatova, 2005). Recent studies have shown that Hsp90 is required for the proper folding of the polymerase protein of paramyxoviruses (Connor et al., 2007). In other words, chaperones like Hsp90 stabilize the L protein. There is 71% similarity at the amino acid level between the Hsp 90 proteins of Vero (gi.109071325) and human cell lines such as A549 and Hela (gi.153792590). Therefore, the differences in amino acid sequence or possible differences in the amount of Hsp 90 between these cell lines, might explain the difference in cell tropism between these two viruses. Additionally, it has been shown that over-expression of Hsp72 increased the level of reporter gene expression from a measles mini-genome (Parks et al., 1999). Although not completed as part of my project, the plan is to perform the mini-genome assay in A549 or Hela cell lines to study the polymerase

activity in relatively non-permissive cell lines for Gw7, and compare the results with the polymerase activity in Vero cells. Also, to examine the possible effect of Hsp proteins on polymerase activity of the viruses used in this study, first the possible difference in quantity of Hsp proteins should be examined by western blot and then the mini-genome assay can be performed either in the presence of siRNAs which knock down Hsp90 or hsp72, or the plasmids which over-express them. It might be interesting to purify the tagged polymerase and by using mass spectrometry, examine other cellular proteins which might interact with this protein.

Also, to further assess the polymerase activity in transcription and replication, it will be very helpful to carry out Northern blot analysis of labeled mRNA and anti-genomic RNA in the presence of Actinomycin D or micrococcal nuclease, which destroys un-encapsidated RNA; or performing real-time RT-PCR to measure the polymerase activity. Also, an alternative approach to study the polymerase activity would be the *in vitro* polymerase assay in which RNA synthesis is performed in the presence of purified virus or NP, P and L proteins *in vitro* (Moyer et al., 1991). Lastly, in order to better analyze and compare different aspects of the polymerase activity, development of a rescue system for full cDNA of both viruses is suggested.

I compared the relative activities of two mumps viruses' polymerases by two methods and no significant differences in the polymerase activity were detected. However, for differences in growth observed between the viruses, there is still a possibility that differences in the other viral proteins including the polymerase, Fusion and Hemagglutinin-Neuramidase have an effect. The same is likely true of *in vivo* virulence and attenuation. There is no solid evidence that the virulent phenotype of

1004-10/2 is dependent on growth in tissue culture, as there are some examples where higher growth in Vero and CEF cells was not associated with virulence of mumps viruses (Sauder et al., 2006;Rubin et al., 2003). On the other hand, another group has shown that a "G" Urabe virus which replicates to lower titers in rat brain than an "A" Urabe virus, also grows lower in human neuroblastoma SH-SY5Y cells (Santos-Lopez et al., 2006). To date, no single mutation has been found to be totally responsible for the virulence or attenuation of any specific RNA virus and therefore, genetic basis of mumps virus virulence and cell tropism is unlikely to lie within one gene (Ivancic et al., 2005;Amexis et al., 2003).

In summary, sequence differences in the polymerase proteins of 1004-10/2 and Gw7 have been confirmed but an inherent difference in the activity of these proteins has not been detected; however, the Urabe mini-genome system has been established by which further studies can be performed on other factors which might have an effect on virus replication.

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BamHI
T7 terminator (θ)
δ
5′ GGATCC GCTAGTTAT TGCTCAGCGG GGGTCGGCAT GGCATCTCCA CCTCCTCGCG
GTCCGACCTG GGCATCCGAA GGAGGACGCA CGTCCACTCG GATGGCTAAG GGAGAGC
Le
accaagggga aaatggagat gggatgttgg tagaacaaat agtgtaagaa acagtaagcc
cggaagtggt gttttgcgat ttcgaggccg ggctcgatcc tcacctttca ttgtcgatag
gggacatttt gacactacct ggaaa
AclI
Luc
ATGGAAGACG CCAAAAACAT AAAGAAAGGC CCGGCGCCAT TCTATCCTCT AGAGGATGGA
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241 TGCAGTGAAA ACTCTCTTCA ATTCTTTATG CCGGTGTTGG GCGCGTTATT TATCGGAGTT
301 GCAGTTGCGC CCGCGAACGA CATTTATAAT GAACGTGAAT TGCTCAACAG TATGAACATT
361 TCGCAGCCTA CCGTAGTGTT TGTTTCCAAA AAGGGGTTGC AAAAAATTTT GAACGTGCAA
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841 AAAATTCAAA GTGCGTTGCT AGTACCAACC CTATTTTCAT TCTTCGCCAA AAGCACTCTG
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1261 GGAGACATAG CTTACTGGGA CGAAGACGAA CACTTCTTCA TAGTTGACCG CTTGAAGTCT
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1681 ATTCT
NotI
Tr
atcgact aaagactcct ctggcatgat acgtcaccaa aaggttccac accagcatcc aaattcttct
agaccgtaca cgacctcgaa caatcataac cacatcaata ttaaatccat aatatcattt taagaaaaaa
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GGGATATCAC TCAGCATAAT GGATCC 3′