THE EFFECT OF PUTATIVE VESICULAR STOMATITIS VIRUS METHYLTRANSFERASE MUTANTS ON TRANSCRIPTION AND REPLICATION

By

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The vesicular stomatitis virus (VSV) mRNAs are methylated at the guanine-N7 and 2'-O-adenosine positions. The RNA-dependent RNA polymerase synthesizes the viral mRNAs which are modified at their 3' and 5' ends by polyadenylation, capping, and methylation. Two host range mutants, *hr*1 and *hr*8, isolated by Simpson and Obijeski are defective in methylation. WT mRNA caps are dimethylated by 7-methylguanosine and 2'-O-methyltransferase yielding the cap structure 7mGpppAmpAp. The cap on an mRNA from *hr*1 has no methylation (GpppApAp) and the cap on an mRNA from *hr*8 was thought be 2'-O-mono-methylated or not methylated at all. Unmethylated mRNAs, like those produced by *hr*1 and *hr*8, are deficient in protein synthesis. Further, *hr*1 and *hr*8 are host restricted as well as temperature sensitive. The N, P, and L proteins that form the polymerase and the respective genes of the *hr*1 and *hr*8 mutants were sequenced

and compared to WT N, P and L genes. *Hr*1 has two amino acid substitutions differing from WT in the L gene and *hr*8 has five amino acid substitutions, also found in the L gene. In this study, the *hr*1 and *hr*8 mutations were introduced separately, together, and combination into the L gene. The effects on the VSV L mutant and WT L proteins were studied in CAT minigenome assays. The assays were conducted in permissive and nonpermissive cell lines as well as permissive and nonpermissive temperatures. The second amino acid change at position 1671 in the *hr*1 mutant appears to be responsible for the temperature sensitivity. The data on the *hr*8 mutant proved to be inconclusive. Further studies performed in the Moyer lab confirm that amino acid 1671 is responsible for temperature sensitivity as well as the host restriction. Additionally, amino acid position 1481 is responsible for both host restriction and temperature sensitivity. Position 1671 may play a critical role within the S-adenosylmethionine binding domain and position 1481 could possibly be part of the catalytic site for 7mG methyltransferase.

CHAPTER 1
INTRODUCTION

General Background

Vesicular Stomatitis Virus (VSV) is a Vesiculovirus found in the order *Mononegavirales.* A number of viruses causing human diseases are contained in this order, including measles, mumps, rabies, Ebola, Marburg, Borna, Hendra, and Nipah. More specifically VSV is found in the family Rhabdoviridae, which also includes rabies. Over 200 rhabdoviruses have been identified. Vesiculovirus is one of six genera in the Rhabdoviridae family.

VSV contains two serotypes, Indiana and New Jersey, both of which infect a broad range of insects and mammals. The virus is present in North and South America and relatives of the virus can be found in Africa and Asia. Symptoms of the disease resemble foot and mouth disease with vesicular lesions on the tongue, gums, teats and hooves in livestock. Headache, nausea, malaise and general influenza-like symptoms are also possible. The virus is spread through vesicular fluid and saliva; it can also be transmitted by infected flies and mosquitoes. VSV can infect humans causing a mild illness.

VSV, like rabies, is a bullet shaped virus. It is approximately 180 nm long by 75 nm wide. The virus is enveloped with G-protein spikes on the surface. It has a single-stranded negative sense RNA genome of 11,200 nucleotides. RNA that is negative sense cannot be translated immediately after entry into the cell. Therefore, an antigenomic intermediate must be produced by the RNA-dependent RNA polymerase, which comes packaged in the virus, in order to make a viable mRNA.

Transcription

Before transcription can occur, the virus must attach to a receptor on a host which initiates the infection. Next, the virion is endocytosed by the host cell and the ribonucleoprotein (RNP) core with the attached RNA-dependent RNA polymerase (RdRp) is released into the cytoplasm. The RNP with N-encapsidated, negative strand RNA serves as the template for transcription. The RdRp, which consists of two viral subunits: the phosphoprotein (P) and the large protein (L), transcribes the template to produce (+) strand mRNAs which are modified at their 3' and 5' ends by polyadenylation, capping, and methylation, respectively.

The viral genes are transcribed sequentially in VSV, producing leader RNA and then the capped and methylated mRNAs with a gene order of 5'-N-P-M-G-L-3' (Ball and White, 1976) (Figure 1-1). VSV polymerase pauses between each gene and then begins synthesizing the next downstream gene (Iverson and Rose, 1981). Possibly, this is to generate the poly(A) tail on the mRNA.

Additionally, the polymerase is responsible for capping the mRNA (See Capping and Methylating Mechanism). It is thought that conserved sequences preceding and within each gene contain the capping and reinitiation signals (Stillman and Whitt, 1999). Cap methylation, involving the methyl donor S-adenosyl-L-methionine (AdoMet), is essential for translation of viral mRNAs but not mandatory in viral mRNA synthesis (Gingras et al., 1999, Horikami et al., 1984, Horikami and Moyer 1982).

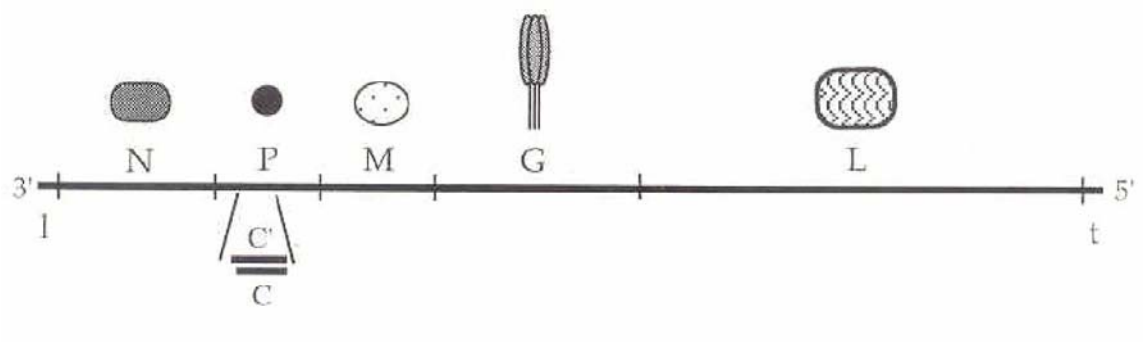

Figure 1-1. Schematic of the VSV gene order. Image taken from Rose and Whitt (2001).

Replication

Replication of VSV occurs after the synthesis of mRNAs and their translation. Replication requires ongoing protein synthesis. Genomic replication cannot occur until N protein is synthesized. Additionally, the P and L proteins, which form the polymerase catalyzing transcription, are also responsible for replication. Sufficient encapsidation of leader RNA by the N protein signals the polymerase to ignore mRNA initiation and termination signals and consequently produces full length positive-sense genome RNA (Blumberg et al., 1983). Genome RNA synthesis is always coupled to its encapsidation by N. The encapsidated positive-stranded genome RNA is replicated again to produce the encapsidated genomic negative-stranded RNA.

Assembly and Release

Encapsidated RNA (RNP) is now ready for assembly and release. The matrix protein (M) condenses the RNP and packages it along with the RNA-dependent RNA polymerase. The glycoprotein (G), which produces the spikes on the surface of the virus, is transported through the secretory pathways of the cell to the outer cell plasma membrane. In VSV, this is typically on the basolateral surface of cells (Stephens et al., 1986). The M protein brings the assembled RNP and viral polymerase complex to the

cell plasma membrane through an interaction of the G and M proteins. As the virus buds out from the cell membrane, the glycoprotein spikes become the new exterior of the virus.

Proteins

M Protein

The M protein, or matrix protein, is derived from the third mRNA to be transcribed from the viral genome. M is the smallest protein of the five VSV proteins. It condenses the nucleocapsid for packaging, disrupts the cytoskeleton, and aids the budding of the virus from the cell.

G Protein

The G protein, or glycoprotein, is derived from the fourth mRNA to be transcribed from the viral genome. It is a membrane protein responsible for the coat of spikes (~400) on the virion membrane. The G protein enhances the efficiency of budding 10- to 30-fold (Rose and Whitt, 2001). G protein attaches the virus to the susceptible cell and is the protein to which neutralizing antibody is directed.

N Protein

The N protein, or nucleocapsid protein, is derived from the first mRNA to be transcribed from the viral genome. N is a cytoplasmic protein with several functions. N is responsible for packaging the RNA into a compact core that serves as the template for transcription and replication. The P-L polymerase complex binds to N on the RNP during transcription and replication and N interacts with the M protein during virus assembly. As mentioned previously, N is thought to control the rates of transcription and replication as well as the transition between transcription and replication (Blumberg and Kolakofsky, 1981). The N protein probably has two domains: the highly conserved

(~80%) N-terminus and the poorly conserved (~20%) C-terminus (Parks et al., 1992). Deletion experiments by Buchholz et al. (1993) suggest that the highly conserved N-terminal region is required for RNA binding and nucleocapsid assembly. The P protein of the polymerase complex binds the C-terminus of N in RNP.

P Protein

The P protein, or phosphoprotein, is derived from the second mRNA to be transcribed from the viral genome. The P protein was initially thought to be a homotrimer, but more recent studies indicate that it is actually a tetramer (Tarbouriech, et al., 2000). P works in combination with the L protein in forming the RNA-dependent RNA polymerase. It alone is not responsible for enzymatic activity, but probably helps fold the L protein for activity (Kolakofsky et al., 1991). Additionally, P binds to N and preserves the soluble state of N in the cytoplasm until RNA genome synthesis requires N for encapsidation (Robbins and Bussell, 1979).

The coiled-coil oligomerization regions of the P protein in the C-terminal end are necessary for the interaction between P and L (Ryan and Portner, 1990). P protein oligomerization occurs in many viruses, such as respiroviruses, morbilliviruses, Rubulavirus, and RSV (Slack and Easton, 1998). Therefore, oligomerization is thought to be a general characteristic of the protein.

The N-terminus contains a small region that is essential for RNA encapsidation. The specific function of the region is unknown. However deletion of residues 1 to 78 of the P protein hinders RNA synthesis and encapsidation (Curran et al., 1994).

L Protein

The L protein, named for its large size, is derived from the fifth mRNA to be transcribed from the viral genome. Like P, the L protein is also an oligomer (Smallwood

et al., 2002, Cevik et al., 2003). Containing more than 2100 amino acids in a single polypeptide chain, it is the largest of the five VSV proteins. The L protein is the catalytic subunit of the RNA-dependent RNA polymerase. It is a multifunctional protein involved in transcription, replication, mRNA capping, methylation, and polyadenylation (Abraham et al., 1975, Hercyk et al., 1988, Schubert et al., 1980).

Due to its multifunctional characteristics, it was believed that the L protein consisted of several functional regions. Further experiments confirmed this hypothesis. When L protein sequences from several (-) sense RNA viruses were compared, six conserved regions were found and named Domains I to VI (Poch et al., 1990, Sidhu et al., 1993) (Figure 1-2). Domains II, III and VI contain conserved motifs that appear to be essential for RNA synthesis. Domain II contains a charged RNA binding motif (Smallwood et al., 1999). A template recognition/phosphodiester bond-forming motif, mandatory for VSV RNA synthesis, has been mapped to Domain III (Jin and Elliot, 1992, Sleat and Banerjee 1993, Schnell and Conzelman, 1995). In 1993, Canter et al. showed that a purine binding element essential for polymerization could be eliminated with one deletion in Domain VI of VSV L.

Additional studies by Feller et al., 2000, showed that when mutations are inserted in Domains IV and/or Domain VI a broad range of RNA synthesis defects occur. For example, Sendai virus L protein becomes heat sensitive and can no longer transcribe effectively. Some of the mutations resulted in a decrease in *le*+ RNA synthesis and even a lack of RNA synthesis initiation. However, mutations in Domain IV and VI of the L protein in Sendai virus did not result in the inability of L to bind P. Mutations in Domain

V also gave multiple defects in RNA synthesis, however the majority of the mutants were defective in replication but not transcription (Cortese et al., 2000).

The original thought was that each of the six conserved regions of the L protein corresponded to a separate activity (Poch et al., 1990, Sidhu et al., 1993). However, when Smallwood et al. (2002) and Cevik et al. (2003) used site-directed mutagenesis to create mutants in each of the domains, they noticed a commonality of RNA synthesis defects between each of the domains. Therefore, similar phenotypes can be attained through multiple mutations across the domains suggesting multiple independent domains. This was supported by the finding that two L proteins that are deficient in RNA synthesis alone can complement each other and restore RNA synthesis *in vitro*, and this complementation is dependent on cotranslation of the L mutants, suggesting that L is an oligomer in the polymerase complex (Smallwood et al., 2002).

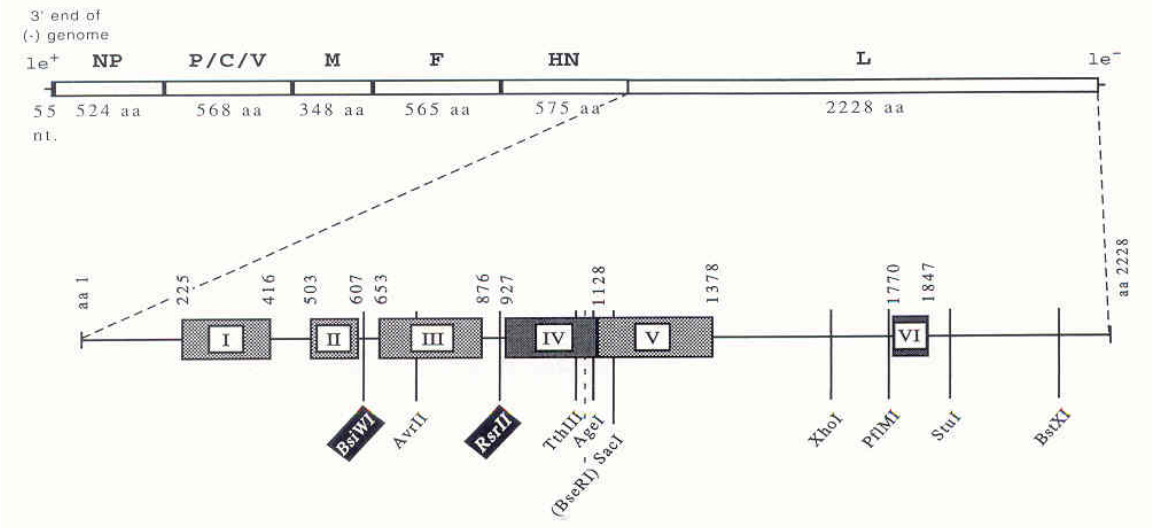

Figure 1-2. Conserved regions in Sendai L protein. Amino acid positions are indicated above the beginning of the conserved region. Image taken from Feller et al. (2000).

Capping and Methylating Mechanism

Abraham et al. (1975) suggest that capping is mediated by the polymerase complex. But, so far no mutations located in the L protein have affected capping. However, defects in viruses with a methyltransferase deficiency have been mapped to the L protein (Hercyk et al., 1988). Cellular mRNA caps are formed by essentially three enzymatic steps. First, RNA triphosphatase removes the γ phosphate from the 5' end of the nascent RNA. Then, guanylyltransferase adds a GMP in a 5'ppp5' linkage to the nascent RNA. Lastly, methyl groups are added at G and the penultimate base forming the 7mG and 2'O base. However, 5' capping in non-segmented negative strand (NNS) RNA viruses is different. The α and β phosphates in the GpppA phosphate bridge are derived from a GDP donor (Abraham et al., 1975) which is added to GMP terminated RNA. Then, the 5' terminus is methylated by guanine-N7-methyltransferase and nucleoside-2'-O-methyltransferase activities of L (Moyer and Banerjee, 1975) to yield m7GpppAmpNpNpNp.

Project Background

In the 1970's, Simpson and Obijeski were chemically mutagenizing VSV to determine functional and structural properties of the viral genus (Simpson and Obijeski, 1974). Since VSV normally has such a broad host range, they attempted to isolate mutants that exhibited host restriction (*hr*). VSV *hr* mutants share the same structural proteins as WT VSV. Many of the mutants were host restricted in cells of human origin, like HEp-2, or HeLa cells, but not in chicken embryo fibroblasts or hamster cells. *Hr*1, for example, had deficient protein synthesis in nonpermissive cells. Some of the *hr* mutants appeared to be temperature sensitive as well.

Normal viral mRNAs that are synthesized *in vitro* or *in vivo* are polyadenylated at the 3' end and guanylated and methylated at the 5' end. However, further studies on the *hr* mutants have shown that *hr*1 is totally defective and *hr*8 is partially defective *in vitro* in methylating the mRNA (Horikami and Moyer, 1982). A WT mRNA cap structure is presumed to be methylated by two activities, 7-methylguanosine and 2'-O-methyltransferase in L, yielding a dimethylated mRNA: 7mGpppAmpAp. The cap on an mRNA from *hr*1 has no methylation, GpppApAp, and the *hr*8 mRNA cap can be 2'O-mono-methylated or not methylated at all. 7mG methylated mRNAs are required for protein synthesis in mammalian cells, so unmethylated mRNAs give the deficient protein synthesis described by Obijeski and Simpson (Simpson and Obijeski, 1974, Horikami and Moyer, 1982).

The L protein was shown to possess the methyltransferase activities (Hercyk et al., 1988) and sequencing of the VSV L, N, and P genes has shown that these *hr* mutations are in fact in the L protein. *Hr*1 has two amino acid changes from WT L and *hr*8 has five changes (Table 1-1). The goal of my work is to determine which one amino acid change, or combination of changes, is actually responsible for the host restriction and temperature sensitivity of the viruses by measuring the effect each mutation in L has on transcription and replication when compared to WT L. Each amino acid change has been constructed separately in the WT L gene, and named as shown in Tables 1-2 and 1-3. Additionally, several combinations of changes have also been made in the WT L gene. The original L mutants of *hr*1 and *hr*8 have also been reproduced by the combination of all the mutations.

Table 1-1. Amino acid changes of *hr*1 and *hr*8 from Simpson WT.

Amino Acid	Simpson WT	*hr*1	*hr*8
148	THR	-	ALA
505	ASN	ASP	-
1097	THR	-	ILE
1356	ALA	-	ASP
1375	TYR	-	SER
1481	GLY	-	ARG
1671	ASP	VAL	-

Table 1-2. Nomenclature of *hr*1 mutants.

Nomenclature	Amino Acid	Simpson WT	*hr*1*
hr1-0	505	ASN	ASP
hr1-1	1671	ASP	VAL

*The original *hr*1 mutant contains both amino acid changes from Simpson WT.

Table 1-3. Nomenclature of *hr*8 mutants.

Nomenclature	Amino Acid	Simpson WT	*hr*8*
hr8-0	148	THR	ALA
hr8-1	1097	THR	ILE
hr8-2	1356	ALA	ASP
hr8-3	1375	TYR	SER
hr8-4	1481	GLY	ARG

*The original *hr*8 mutant contains all five mutations. Each individual mutation has a name that corresponds with its amino acid change from Simpson WT.

CHAPTER 2
MATERIALS AND METHODS

Clones

In general, all the clones were produced by making a PCR product where the WT VSV L gene or sequential *hr* mutant L genes were used as the template and two complementary mutagenic primers (Table 2-1) with a silent restriction site created the desired mutation. The pBS-SK-L plasmid (Figure 2-1) was used as the WT VSV L template. The pBS-SK-L plasmid, as well as the pBS-N and pBS-P plasmids, was kindly provided by J. Rose. The Stratagene QuikChangeXL Site-directed Mutagenesis Kit was used to direct the PCR. The PCR product was digested with DpnI and then transformed into the ultracompetent cells provided by the kit. The cells were plated on LB-Amp+ plates and colonies were then screened by PCR with primers bracketing the mutation site and that PCR product was digested with an enzyme that corresponded to the introduced silent restriction site. The silent restriction site was designed so as not to change the amino acid sequence, but allow a cut site near the desired mutation to suggest that the mutation was also present (Table 2-2). Colonies that contained the cut site were grown up, and the DNA was isolated by a Qiagen Midi-prep or Maxi-prep. A TNT coupled transcription/translation (see below) was performed on the DNA to make sure that it produced a full length protein and the DNA was sequenced by UF's DNA Sequencing Core to determine that it was actually correct.

All clones produced this way were then subcloned back into the wild-type L gene. Instead of sequencing the entire mutant DNA, only a region containing the mutation site

between the restriction sites to be used for subcloning was sequenced. Therefore that region, or insert, was cut out in a double digestion (Figure 2-1) and ligated back into the WT L gene at those sites to ensure that no other mutations were present (Table 2-3). Some of the subclones were then tested for the silent restriction site and the clones were tested again by TNT for expression of full length L protein, while others were sent to sequencing to test for presence of the insert DNA subcloned into WT L DNA.

Some of the clones were produced solely from subcloning. Once many of the mutant clones had been made it was easier to subclone one into another than to start from the beginning with the QuikChange Kit. This procedure was performed the same as described above, however, instead of using WT L DNA as the vector another mutant clone was used as the vector. For example, *hr*1-0,1 was made by subcloning the insert *hr*1-1 into the vector *hr*1-0. This mutant was then sequenced to confirm the presence of both vector and insert.

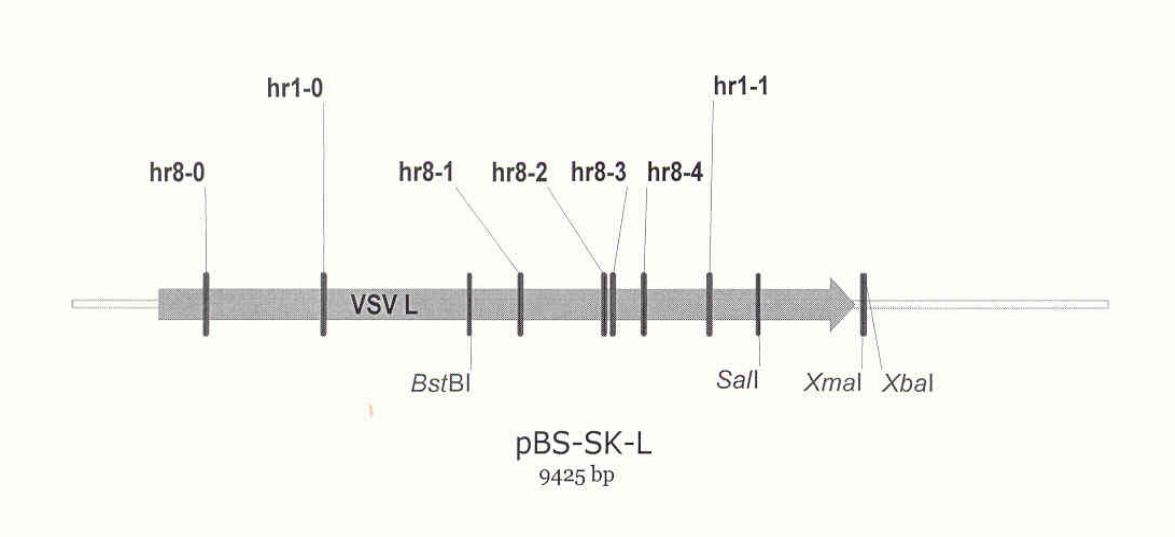

Figure 2-1. The pBS-SK-L plasmid was used as the WT-L template for cloning through mutagenesis. The inserted mutations were sequenced and then subcloned by cutting out the mutation in a double digestion, using *Bst*BI and *Sal*I, *Xma*I and *Bst*BI, or *Xba*I and *Bst*BI, and ligating it back into WT-L.

Table 2-1. Primers used to incorporate the mutations of *hr*1 and *hr*8.

Primer	**Primer Sequence**
SM 577	CAAAAAGGAAAGATGGGCCGACTCATTCAAAATTC
SM 578	GAATTTTGAATGAGTCGGCCCATCTTTCCTTTTTG
DH-1	CAAATCCTGGGGCCGTACAGTTATTGGGACAACTG
SM 533	CAGTTGTCCCAATAACTGTACGGCCCCAGGATTTG
DH-2	GGAAGAATTTAGCACCTGCTGAGCAATCC
SM 534	GGATTGCTCAGCAGGTGCTAAATTCTTCC
SM 538	GGAGACTTGGCCTCGAGAAAATCTACTC
SM 535	GAGTAGATTTTCTCGAGGCCAAGTCTCC
SM 617	CCTCCTATCCGACTAGTAACCGTGATATGAGGGTGATTGTCAG
SM 618	CTGACAATCACCCTCATATCACGGTTACTAGTCGGATAGGAGG
SM 580	GACACAAAGGCTACCGACTGGAAAGAATTTC
SM 581	GAAATTCTTTCCAGTCGGTAGCCTTTGTGTC

Table 2-2. Mutagenic primers and incorporated silent restriction sites.

For Mutagenesis:				
Mutant*	**Template**	**Sense Primer**	**Antisense Primer**	**Silent Restriction Site**
Hr8-0	pBS VSV-L	SM 577	SM 578	HaeIII
Hr8-1	pBS VSV-L	DH-1	SM 533	BcgI
Hr8-2	pBS VSV-L	DH-2	SM 534	HaeII
Hr8-3	pBS VSV-L	SM 538	SM 535	XhoI
Hr8-4	pBS VSV-L	SM 617	SM 618	SpeI
Hr8-1,2	hr8-1	DH-2	SM 534	HaeII
Hr8-1,2,3	hr8-1,2	SM 538	SM 535	XhoI
Hr8-1,2,3,4	hr8-1,2,3	SM 617	SM 618	SpeI
Hr8-0,1,2,3	hr8-1,2,3	SM 577	SM 578	HaeIII
Hr8-0,1,2,3,4	-	-	-	-
Hr1-0	pBS VSV-L	SM 580	SM 581	BsrI
Hr1-1	Made by Rich Hall			
Hr1-0,1	-	-	-	-

*Mutagenesis was performed using the primers and a silent restriction site was added. *Hr*1-1 was made by Rich Hall, *hr*8-0,1,2,3,4 and *hr*1-0,1 were produced solely by subcloning.

Table 2-3. Subcloning enzymes, vectors, and inserts.

For Subcloning:			
Mutant	**Vector**	**Insert**	**Enzymes**
hr8-0	hr8-0	pBS VSV-L	XbaI & BstBI
hr8-1	pBS VSV-L	hr8-1	SalI & BstBI
hr8-2	pBS VSV-L	hr8-2	SalI & BstBI
hr8-3	pBS VSV-L	hr8-3	SalI & BstBI
hr8-4	pBS VSV-L	hr8-4	SalI & BstBI
hr8-1,2	pBS VSV-L	Hr8-1,2	SalI & BstBI
hr8-1,2,3	pBS VSV-L	Hr8-1,2,3	SalI & BstBI
hr8-1,2,3,4	pBS VSV-L	hr8-1,2,3,4	SalI & BstBI
hr8-0,1,2,3	hr8-0,1,2,3	pBS VSV-L	XmaI & BstBI
hr8-0,1,2,3,4	hr8-0	hr8-1,2,3,4	SalI & BstBI
hr1-0	pBS VSV-L	hr1-0	SalI & BstBI
hr1-1	Made by Rich Hall		
hr1-0,1	hr1-0	Hr1-1	SalI & BstBI

*Subcloning was performed by using two enzymes to cut the DNA and an insert was then ligated into the vector.

Cells and Virus

A549, baby hamster kidney (BHK), and HEp-2 cells all from ATCC were used to express VSV proteins from plasmids *in vivo*. The cells were maintained using F11medium. For BHK cells it was supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2% glutamine, 1% penicillin-streptomycin, and 0.35% glucose. The medium for A549 and HEp-2 cells was supplemented with 8% fetal bovine serum, 1% nonessential amino acids, 1% glutamine, 1% penicillin-streptomycin, and 1% sodium pyruvate. Cells were grown to ~80% confluency on 35 mm dishes for infection and transfection. The vaccinia virus-T7 polymerase recombinant and the derivative MVA-T7 virus were the gift of B. Moss (NIH).

TNT Coupled Transcription/Translation Reaction System

The TNT Coupled Transcription/Translation Reaction System (Promega) was used to verify that the mutant clones produced full length L proteins. Approximately 1ug of DNA was mixed with 6.25 ul TNT Rabbit Reticulocyte lysate, 0.5 ul TNT buffer, 0.25 ul T7 RNA polymerase, 0.25 ul amino acids minus leucine, 0.5 ul RNasin, and 4.25 ul ^{3}H-leu. The reaction was mixed gently and incubated at 30°C for 2 hours. The product was separated on a 7.5% SDS polyacrylamide gel next to wild-type L from WT L DNA to determine if the mutant was producing full length L protein. The gel was washed, impregnated with PPO, dried, and incubated with x-ray film at -80°C overnight.

In vivo Expression of L Mutant Proteins

A549 cells were split 1:30 the day prior to infection and grown to ~80% confluency in 35 mm dishes. The cells were then infected with VVT7, a vaccinia virus recombinant encoding T7 RNA polymerase, at an m.o.i. of 2.5 pfu/cell using F11 infection medium. The infected dishes were rocked for 1 hour at 37°C. The infection medium was then aspirated and the cells were transfected with 2 ug VSV P and 2 ug VSV L (WT and mutants) plasmids in Opti-MEM and 3 ul lipofectamine per 1 ug of DNA for four hours at 37°C.

Four hours after transfection, the transfection media was aspirated and ^{35}S methionine overnight label (1 ug) media (2 ml) was added (100 uCi/ml or ^{35}S methionine, 1 ml F11, 0.1 ml penicillin-streptomycin, 0.1ml glutamine, 0.1ml HEPES, pH 8.0, and 9 ml cysteine/methionine-free MEM). The following day the media was aspirated and the cells were washed with cold PBS. The cells were scraped into a sterile microfuge tube with 100 ul of 1% NP40 lysis buffer using sterile rubber policemen. The cells were then spun down for 30 min at 13,000 rpm at 4°C. The supernatant (10 ul) was analyzed on a

7.5% SDS polyacrylamide gel. The gel was washed, dried, and incubated with film at -80°C overnight.

CAT (Chloramphenicol Acetyltransferase) Assay

The DI-CAT plasmid (Figure 2-2), constructed by Dr. Michael Baron, contains a gene encoding minus sense chloramphenicol acetyltransferase flanked by the 3' and 5' termini of VSV genome RNA downstream of a T7 promoter. Cells are infected with MVA expressed T7 RNA polymerase which drives the synthesis of VSV polymerase complex plasmids (N, P, and L) as well as DI-CAT. N, P, and L mRNAs are then translated by the cellular machinery. DI-CAT is synthesized by the T7 polymerase to produce an antisense copy of the VSV minigenome with the minus sense CAT ORF between the VSV 5' and 3' termini. The ribozyme contained in the DI-CAT plasmid cleaves the 3' end of the RNA after transcription leaving only the CAT RNA which is inefficiently and nonspecifically encapsidated. This creates a template for the viral polymerase to replicate the RNA to a positive sense minigenome. Then transcription of the template occurs to produce a functional mRNA (Figure 2-3). The mRNA can then be translated into protein (CAT) by the cellular translation machinery.

A549 cells were split 1:30, BHK cells were cut 1:24, or HEp-2 cells were cut 1:24 the day prior to infection and grown to ~80% confluency in 35 mm dishes. The cells were then infected with MVA-T7, a modified vaccinia virus expressing T7 RNA polymerase, at an m.o.i. of 2.5 pfu/cell using F11 infection medium. The infected dishes were rocked for 1 hour at 37°C. The infection medium was then aspirated and the cells were transfected with plasmids containing 1 ug N, 0.3 ug L (WT or mutant), 0.5 ug P, 0.5 ug DI-CAT along with Opti-MEM and 3 ul of lipofectamine per 1 ug of DNA. The cells were transfected for 24 hours at either 34°C, 37°C, or 40°C.

Twenty four hours post-transfection, the transfection media was aspirated and fresh supplemented F11 media was added. The following day the media was aspirated and rinsed with cold PBS. 200 ul of 0.25 M Tris-HCl, pH 7.8 and 0.5% Triton X-100 was added to the dishes and the cells were scraped with sterile rubber policemen. The scraped cells were spun down in sterile microfuge tubes for 10 min at 13,000 rpm at 4°C. The CAT-ELISA Kit (Roche) was used to test the abilities of the L mutants to direct replication and transcription compared to WT VSV L. This was done by ELISA for CAT protein. First, 50 ul of the supernatant was added to wells containing 150 ul of sample buffer (provided by the kit). Incubating the wells for 1 hour at 37°C allows the CAT protein to bind to the walls of the wells. The wells were washed five times with 200 ul of 10X Wash solution diluted 1:10. Then, 200 ul of α-CAT-DIG antibody (1:100 in sample buffer) was added to the wells. After the second 1 hour incubation, the α-CAT-DIG has bound to the proteins. Again, the wells are washed five times. Finally, 200 ul of α-DIG-POD antibody (3:400 in sample buffer) was added to the wells. This binds to the α-CAT-DIG during the 1 hour incubation. The wells are washed five more times and then 200 ul of POD substrate is added (Figure 2-4). The substrate turns green in the presence of bound α-DIG-POD. A plate reader determines the ratio of protein present compared to mock and WT. All mutants were tested in triplicate in a minimum of two separate experiments and mock was subtracted from each sample. Therefore, each mutant has at least six results.

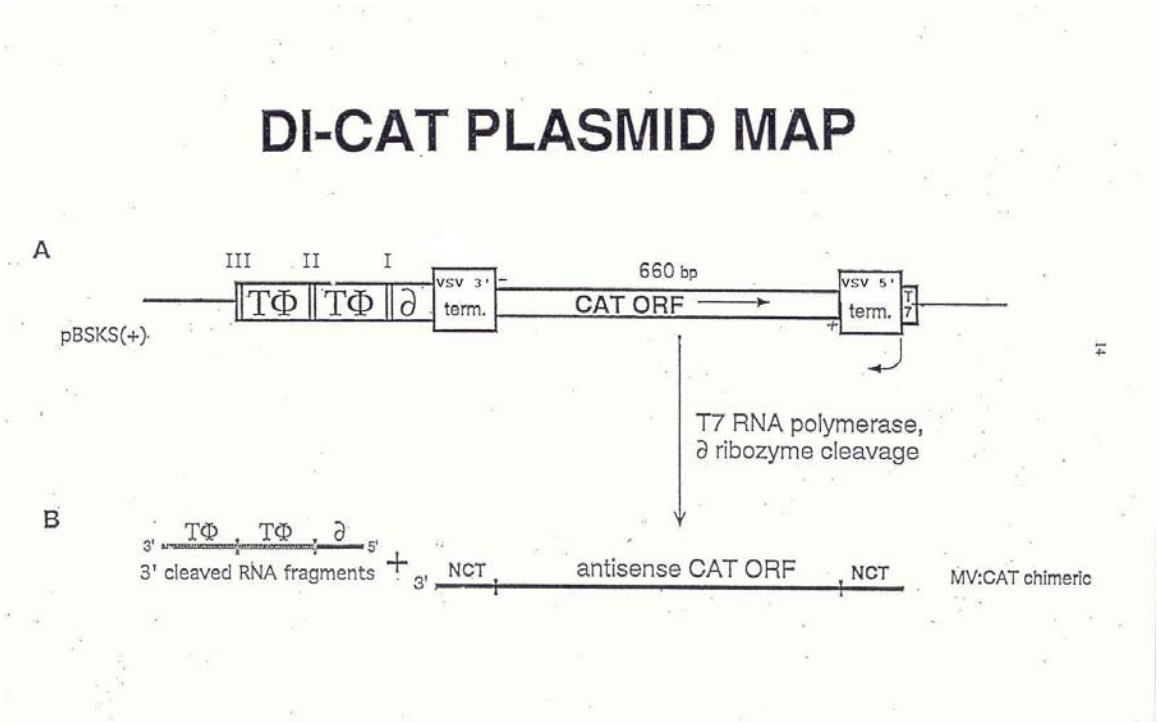

Figure 2-2. Representation of the DI-CAT plasmid used in the CAT assay to measure VSV RNA polymerase activity.

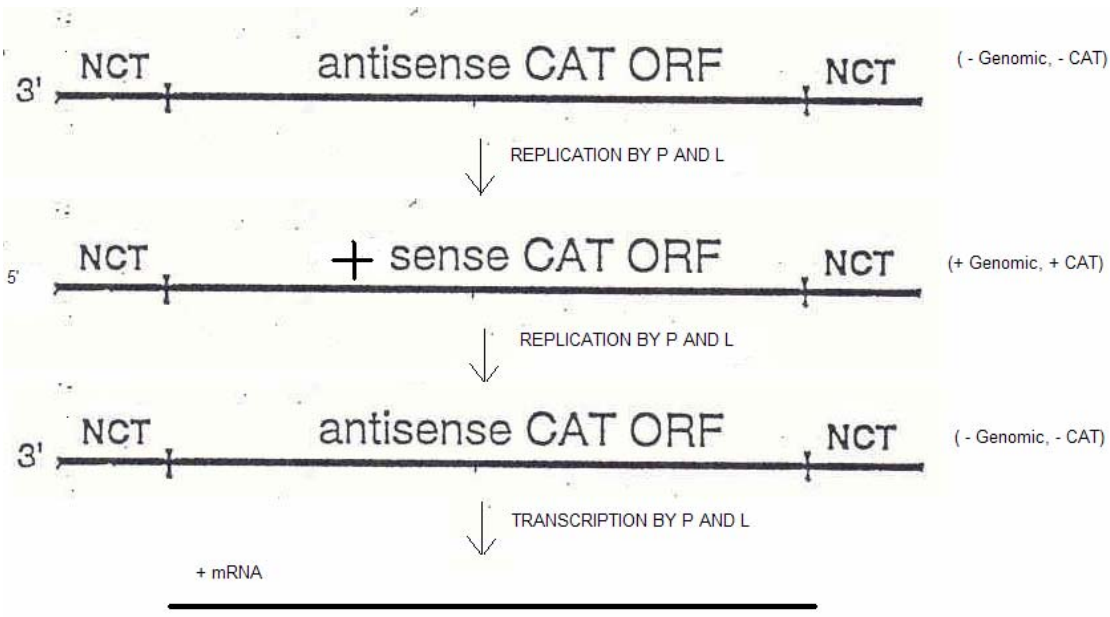

Figure 2-3. Representation of the transformation of the CAT ORF into a functional mRNA.

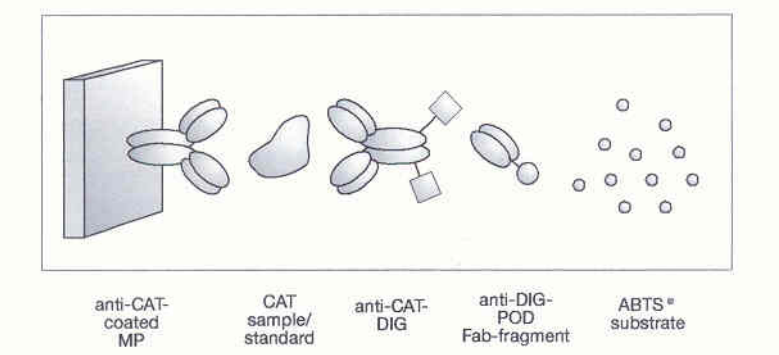

Figure 2-4. Representation of antibody binding in Roche CAT-ELISA kit.

CHAPTER 3
RESULTS: *HR*1

Clones

The original *hr*1 mutant contains two amino acid changes from WT L, ASP in place of ASN at position 505 and VAL in place of ASP at position 1671. In order to examine the effect that each amino acid change has on the function of the L protein, the mutations needed to be reproduced individually in the L protein. Therefore, each amino acid change was remade individually and the original *hr*1 mutant containing both mutations was also reconstructed.

*Hr*1-0 was constructed using the Stratagene QuikChange-XL Site-Directed Mutagenesis Kit described in the Material and Methods section. In summary, a PCR product was made where the WT or mutant L gene was used as the template and two complementary mutagenic primers created the desired mutation with a silent restriction site. If the silent restriction site, which was located near the desired mutation, was incorporated into the L gene the DNA was isolated by a Qiagen Midi-prep or Maxi-prep. Then, a TNT coupled transcription/translation (results below) was performed on the DNA to verify that it produced a full length L protein. Then a short region containing the mutation was sequenced to determine that the sequence was actually correct. That short region was subcloned back into the WT L gene via a double digestion and ligation to ensure that no other mutations were present. *Hr*1-1 had been made previously by Dr. Rich Hall in our laboratory. The combination *hr*1-0,1 was made strictly by subcloning the *hr*1-1 mutant region into *hr*1-0.

TNT Coupled Transcription/Translation Reaction Analysis of L Proteins

The amino acid changes that were introduced through the Stratagene QuikChange-XL Site-Directed Mutagenesis Kit were not meant to truncate the L protein, but only to exchange one amino acid for another. Therefore, *hr*1-0, which was produced via the Stratagene Kit, was tested by the TNT system described in Chapter 2 to verify that it produced full length L protein. Each DNA was mixed with the reagents and ^{3}H leucine and incubated at 37°C. The product was separated on an SDS-PAGE and detected by autoradiography. Although synthesis of the WT L protein in Lane 1 failed, Lane 5 in Figure 3-1 shows that *hr*1-0#22 is producing full length protein similar to *hr*8-0, another mutant L protein which was used as a marker. The other *hr*1-0 clone in Lane 4 had another mutation that truncated the protein. *Hr*1-0 was then sequenced by UF's DNA Sequencing Core to further confirm that only the correct amino acid had been incorporated into the L gene.

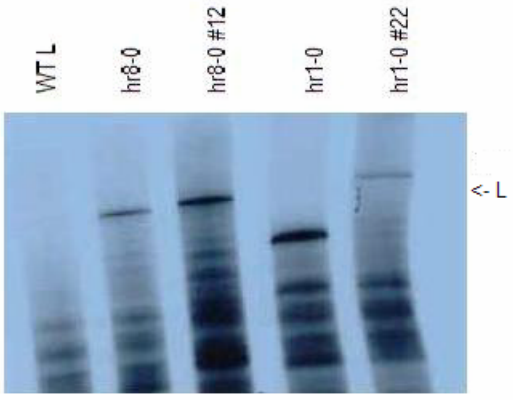

Figure 3-1. TNT of *hr*1-0 and other *hr* mutants. Analysis of the TNT product by SDS-PAGE shows that *hr*1-0 #22 is producing full length L-protein.

In vivo Expression of L Mutants

Not only is it important to ensure the L mutants produce full length L protein in a TNT reaction system, but it is also necessary for the L mutants to produce full length L protein *in vivo*. The *in vivo* expression system described in Chapter 2 was used to test the ability of all of the L mutant plasmids, whether produced via mutagenesis or subcloning, to produce full length L proteins in cells. Cells were infected with VVT7 and then transfected with VSV P and VSV L plasmids using lipofectamine. After four hours, the transfection media was aspirated and ^{35}S methionine overnight label was added. The following day, the cells were rinsed and scraped into a sterile microfuge tube. After the cells were spun down, and lyzed, the nuclei removed and the supernatant was analyzed on an SDS-PAGE. Figure 3-2 compares the synthesis of L protein produced by the mutants to the WT L protein. All of the *hr*1 mutants are producing full length L protein at about the same level as compared to WT L. P protein cannot be determined due to cell background labeling.

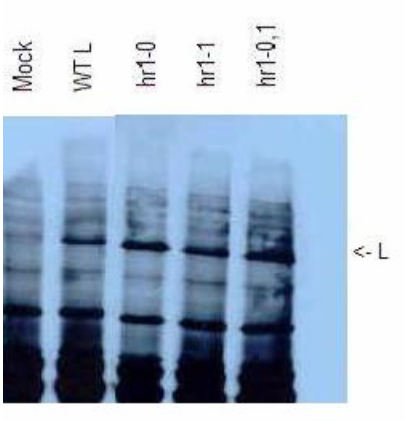

Figure 3-2. *In vivo* expression of *hr*1 L-mutants. This SDS-PAGE gel shows that the mutants all produce full length L-protein compared to WT in an *in vivo* system.

Analysis of the *hr*1 L Mutants for Transcription and Replication

The CAT-ELISA Kit (Roche) was used to test the abilities of the L mutants to direct replication and transcription from a CAT reporter minigenome. This was done by ELISA for the chloramphenicol acetyltransferase protein. As described in more detail in Chapter 2, the DI-CAT plasmid contains a gene encoding minus sense CAT downstream of a T7 promoter. Cells are infected with a modified vaccinia virus encoding a T7 RNA polymerase, which drives the synthesis of VSV N, P and L RNAs from plasmids as well as DI-CAT RNA. The N, P, and L mRNAs are then translated by the cellular machinery. DI-CAT is synthesized by the T7 polymerase to produce a minus sense CAT minigenome that is cleaved from the remaining RNA by a ribozyme contained in the DI-CAT transcript. This RNA is nonspecifically encapsidated by N proteins to create a template for the viral polymerase to replicate the RNA first to a positive sense and then to a negative sense minigenome. Then viral transcription of the amplified negative sense template occurs to produce a functional CAT mRNA. The mRNA is translated into chloramphenicol acetyltransferase protein by the cellular translation machinery. The Roche kit measures the amount of translated chloramphenicol acetyltransferase produced by the L mutants as compared to WT L as a measure of RNA synthesis. A CAT mRNA that is unmethylated during transcription will be inefficiently translated however, in this system vaccinia methyltransferases would methylate any unmethylated CAT mRNA. So methylation activity of L is not being measured. All CAT assays summarized below were performed twice on each mutant in triplicate.

The original *hr*1 mutant has host restriction as well as temperature sensitivity. Therefore, CAT assays were performed at three different temperatures and in three separate cell lines. The permissive and nonpermissive temperatures are 34°C, 40°C

respectively, while 37°C represents a central point between the two where virus is normally grown. BHK cells are permissive, while the HEp-2 and A549 cell lines are nonpermissive.

To test for temperature sensitivity in nonpermissive cells, CAT assays were performed for the L mutants on HEp-2 cells at all three temperatures. Tables 3-1, 3-2, and 3-3 show the percent of CAT protein translated by the L mutants relative to that of WT L at 34°C, 37°C and 40°C, respectively. In order to be considered temperature sensitive, the activity must drop by at least 80% at the higher temperature. At 34°C (Table 3-1), the three mutants are relatively WT except for *hr*1-1, which is 57% of WT L, indicating that 34°C does not significantly affect the activity of the L mutants. When the temperature is increased to 37°C, *hr*1-0 loses about 43% of its activity when compared to *hr*1-0 at the nonpermissive temperature. But, *hr*1-1 loses over 80% of its activity, suggesting that the *hr*1-1 mutation makes L temperature sensitive (Table 3-2). At 40°C (Table 3-3), the *hr*1-1 mutant still performs at only 20% of WT L thus further supporting the temperature sensitivity of this protein. Interestingly, the *hr*1-0,1 double mutant at 37°C and 40°C is not as reduced in viral RNA synthesis as *hr*1-1 alone. Perhaps the mutation at amino acid 505 stabilizes the protein. While the activity of the *hr*1-0 protein is decreased at 37°C compared to 34°C, it appears similar to WT L at 40°C. It is unclear what the cause of this result is. These experiments show that when the mutant proteins are made in these nonpermissive cells at 34°C significant activity is obtained, suggesting that the nonpermissive host cell is not a determinate of RNA synthesis. However, the *hr*1-1 protein is temperature sensitive due to the single mutation at amino acid 1671.

Table 3-1. CAT synthesis of *hr*1 mutants in HEp-2 cells at 34°C.

HEp-2 Cells @34°C	
Mutant	**Average***
WT	100
hr1-0	126
hr1-1	57
hr1-0,1	100.5

*VVT7 infected HEp-2 cells were transfected and incubated at 34°C. Six to nine CAT assay experiments were averaged and calculated against WT assuming 100% activity with less than 15% variation.

Table 3-2. CAT synthesis of *hr*1 mutants in HEp-2 cells at 37°C.

HEp-2 Cells @37°C	
Mutant	**Average***
WT	100
hr1-0	54
hr1-1	7.55
hr1-0,1	29.7

*VVT7 infected HEp-2 cells were transfected and incubated at 37°C. Six to nine CAT assay experiments were averaged and calculated against WT assuming 100% activity with less than 15% variation.

Table 3-3. CAT synthesis of *hr*1 mutants in HEp-2 cells at 40°C.

HEp-2 Cells @40°C	
Mutant	**Average***
WT	100
hr1-0	119.5
hr1-1	22.5
hr1-0,1	48

*VVT7 infected HEp-2 cells were transfected and incubated at 40°C. Six to nine CAT assay experiments were averaged and calculated against WT assuming 100% activity with less than 15% variation.

To further test the possible host range effects, CAT assays were also performed on A549 and BHK nonpermissive and permissive cell lines, respectively, at 37°C (Tables 3-4 and 3-5). Data fairly similar to that obtained in HEp-2 cells at 37°C was obtained. It appears that L mutants synthesized in A549 cells are slightly more active than in HEp-2

cells. *Hr*1-1 again appears temperature sensitive regardless of the cell line. The basis for the host range of the mutant virus does not appear to be a lack of RNA synthesis.

Table 3-4. CAT synthesis of *hr*1 mutants in A549 cells at 37°C.

A549 Cells @37°C	
Mutant	**Average***
WT	100
hr1-0	50
hr1-1	28
hr1-0,1	56.5

*VVT7 infected A549 cells were transfected and incubated at 37°C. Six to nine CAT assay experiments were averaged and calculated against WT assuming 100% activity with less than 15% variation.

Table 3-5. CAT synthesis of *hr*1 mutants in BHK cells at 37°C.

BHK Cells @37°C	
Mutant	**Average***
WT	100
hr1-0	ND
hr1-1	20.5
hr1-0,1	ND

*VVT7 infected BHK cells were transfected and incubated at 37°C. Six to nine CAT assay experiments were averaged and calculated against WT assuming 100% activity with less than 15% variation.

Dr. Valery Grdzelishvili et al. (in press) performed experiments on the *hr*1 L mutants that were inserted into recombinant vesicular stomatitis virus to look at cap methylation. His findings are clearer and more precisely support the result that both temperature sensitivity and host range phenotypes reside in the *hr*1-1 mutation. Table 3-6 compares both host range and temperature sensitivity by plaque assays of the virus under different conditions. The second to last column shows the ratio of virus titers at 34°C with respect to 40°C in BHK cells. The increased temperature had a significant inhibitory effect in *hr*1-1 virus and the original, as well as the reconstructed *hr*1-0,1,

viruses but not on *hr*1-0 virus as suggested by the CAT assays above. Additionally, the ratio of virus titers in BHK and HEp-2 cells is shown in the last column. Again, the *hr*1-1 virus and the original and reconstructed *hr*1 mutant viruses are clearly deficient for growth in HEp-2 cells, although *hr*1-0 is not. These results clearly show that both host range and temperature sensitivity lie in the same mutation, *hr*1-1 at amino acid 1671 in L.

Table 3-6. Comparative titers of recombinant VSV mutants in BHK and HEp-2 cell lines at 34°C and 40°C (Grdzelishvili et al., In Press).

Virus	**Virus titer (PFU/ml) in BHK cells**		**Virus titer (PFU/ml) in HEp-2 cells**		**Titer ratio 34ºC/40ºC in BHK**	**Titer ratio 34°C in BHK/HEp-2**
	34°C	**40°C**	**34°C**	**40°C**		
Wt	2.6×10^9	6.0×10^8	6.5×10^7	8.0×10^6	4.3	40.0
rHR1-0	2.5×10^9	1.0×10^9	4.4×10^7	8.5×10^6	2.5	56.8
rHR1-1	4.2×10^8	$< 10^3$	$< 10^3$	$< 10^3$	$> 10^5$	$> 10^5$
rHR1-0,1	1.1×10^9	$< 10^3$	$< 10^3$	$< 10^3$	$> 10^6$	$> 10^6$
hr1 (Orig.)	2.5×10^9	1.5×10^4	$< 10^3$	$< 10^3$	1.7×10^5	$>10^6$

CHAPTER 4
RESULTS: *HR*8

Clones

The original *hr*8 mutant contains five amino acid changes from WT L, ALA in place of THR at position 148 (*hr*8-0) , ILE in place of THR at position 1097 (*hr*8-1), ASP in place of ALA at position 1356 (*hr*8-2), SER in place of TYR at position 1375 (*hr*8-3), and ARG in place of GLY at position 1481 (*hr*8-4). In order to examine the effect that each amino acid change has on the function of the L protein, the mutations needed to be reproduced individually in the L protein. Therefore, each amino acid change was constructed individually or in combinations and the original *hr*8 mutant containing all five mutations was also reconstructed.

Two clones were made prior to the start of this project: *hr*8-1 and *hr*8-2. Eight more clones were successfully produced following the mutagenesis and subcloning methods described in Chapter 2. They are: *hr*8-0, *hr*8-3, *hr*8-4, *hr*8-1,2, *hr*8-1,2,3, *hr*8-1,2,3,4, and *hr*8-0,1,2,3,4. *Hr*8-0 and *hr*8-0,1,2,3,4 were later determined to be the incorrect sequence. Therefore, they as well as *hr*8-0,1,2,3 were remade by Sherin Smallwood in our laboratory. All subsequent tests proved that the three new *hr*8 mutants were correct (results not shown).

TNT Coupled Transcription/Translation Reaction Analysis of L Proteins

All the clones produced via the Stratagene QuikChange-XL Site-Directed Kit were tested for full length protein by the TNT system as the amino acid changes introduced were not meant to truncate the L protein. Those mutants are: *hr*8-0, *hr*8-3, *hr*8-4, *hr*8-1,2

*hr*8-1,2,3, *hr*8-0,1,2,3, *hr*8-1,2,3,4. Figures 4-1, 4-2, and 4-3 show the products of the TNTs analyzed by SDS-PAGE. Figure 4-1 indicates that each of the *hr*8-1,2 and *hr*8-3 clones are producing full length L protein relative to the WT L control. In Figure 4-2, the subclones of *hr*8-1,2 and *hr*8-1,2,3 are shown and they are also both producing full length L protein. Figure 4-3 shows a mutant that did not produce full length protein. *Hr*8-4#2 is truncated and that mutant was discarded however the other clone is correct. Both *hr*8-1,2,3,4 clones produced full length L proteins. Following the TNT reaction, clones that were producing full length L protein were then sequenced by UF's DNA Sequencing Core.

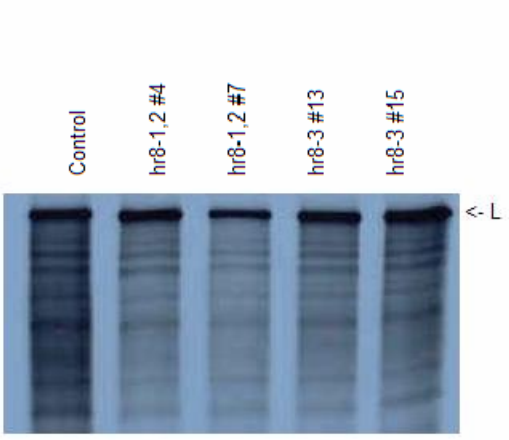

Figure 4-1. TNT of *hr*8-1,2 and *hr*8-3. Analysis of the TNT products by SDS-PAGE shows that *hr*8-1,2 and *hr*8-3 mutants are producing full length L-protein when compared with WT (control). Numbers 4 and 13 were used in future experiments.

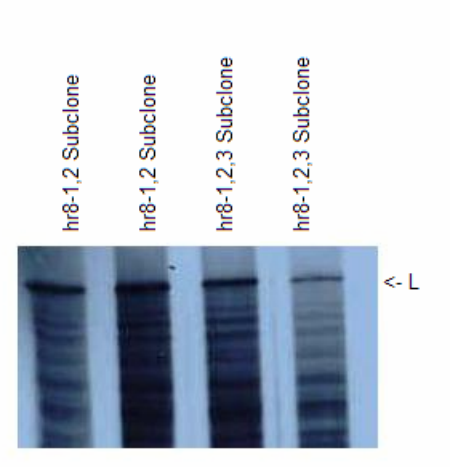

Figure 4-2. TNT of *hr*8-1,2 and *hr*8-1,2,3 subclones. Analysis of the TNT products by SDS-PAGE shows that the subclones of *hr*8-1,2 and *hr*8-1,2,3 mutants are producing full length L-protein.

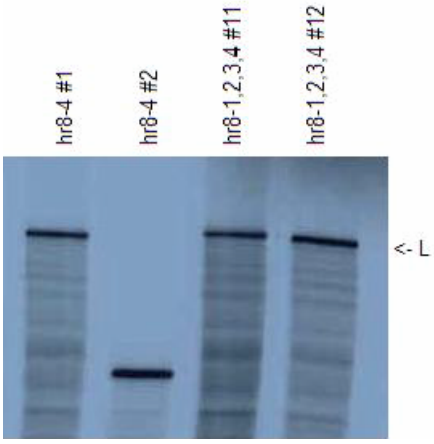

Figure 4-3. TNT of *hr*8-4 and *hr*8-1,2,3,4. Analysis of the TNT products by SDS-PAGE shows that *hr*8-4 #1 and *hr*8-1,2,3,4 are producing full length L-protein.

***In vivo* Expression of L Mutants**

All of the L mutants, whether produced via mutagenesis or subcloning must produce full length L protein *in vivo*. The *in vivo* expression system described in Chapter 2 was used to test protein synthesis of the L mutants by radiolabeling infected, transfected cells. Figure 4-4 compares the L proteins produced by the mutants to the WT L protein. All the *hr*8 mutants are producing full length L protein. In other experiments

(data not shown) reconstructed *hr*8-0, *hr*8-0,1,2,3 and *hr*8-0,1,2,3,4 also gave full length L proteins in cells.

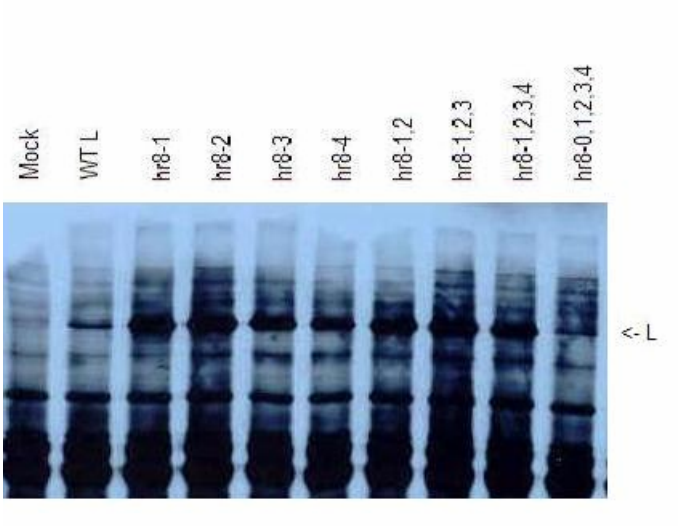

Figure 4-4. *In vivo* expression of *hr*8 L-mutants. This SDS-PAGE gel shows that the mutants all produce full length L-protein compared to WT in an *in vivo* system. Lanes from left to right: Mock, WT VSV, *hr*8-0, *hr*8-1, *hr*8-2, *hr*8-3, *hr*8-4, *hr*8-1,2, *hr*8-1,2,3, *hr*8-1,2,3,4, *hr*8-0,1,2,3,4. *Hr*8-0 and *hr*8-0,1,2,3,4 were later determined to be incorrect sequences.

Analysis of the *hr*8 L Mutants for Transcription and Replication

The CAT-ELISA Kit (Roche) was used to test the abilities of the L mutants to direct replication and transcription from a CAT reporter minigenome compared to WT VSV L. The CAT minigenome (described in more detail in Chapter 2) is constructed so that when it is transfected into cells along with N, P and L, chloramphenicol acetyltransferase protein is produced. The Roche kit measures the amount of translated CAT produced as a measure of viral RNA synthesis. All CAT assays summarized below were performed twice on each mutant in triplicate.

Like the original *hr*1 mutant virus, the original *hr*8 mutant virus showed host restriction as well as temperature sensitivity. Therefore, CAT assays were performed at three different temperatures and in three separate cell lines. The permissive and

nonpermissive temperatures are 34°C and 40°C, respectively, while 37°C represents a central point between the two where virus is normally grown. BHK cells are permissive, while the HEp-2 and A549 cell lines are non-permissive.

To test for temperature sensitivity, CAT assays were performed for the L mutants on HEp-2 cells at all three temperatures. Tables 4-1, 4-2, and 4-3 show the percent of CAT protein translated by the L mutants relative to that of WT L at 34°C, 37°C and 40°C respectively. In order to be considered temperature sensitive, the activity of the L mutants must drop by at least 80% at the higher temperatures. At the permissive temperature of 34°C (Table 4-1), most of the mutants are reduced somewhat but not below 50% of WT L. *Hr*8-4 and *hr*8-0,1,2,3,4 are more reduced to 43% and 34% respectively. Every mutant shows some effect on RNA synthesis at the permissive temperature. Surprisingly, as the temperature was increased to 37°C and 40°C (Tables 4-2 and 4-3), the mutants apparently become more heat resistant. Especially notable is *hr*8-1,2 which is greatly stimulated to 600% of WT L. The reason is unknown but it could be that WT L is actually less stable at the nonpermissive temperatures than the L mutants giving higher mutant values. In any case, the results do not point to a specific mutation as being responsible for the temperature sensitivity of *hr*8 virus.

Table 4-1. CAT synthesis of *hr*8 mutants in HEp-2 cells at 34°C.

HEp-2 Cells @34°C	
Mutant	**Average***
WT	100
hr8-0	61
hr8-1	71
hr8-2	85
hr8-3	50
hr8-4	43
hr8-1,2	ND
hr8-1,2,3	ND
hr8-1,2,3,4	69
hr8-0,1,2,3	52
hr8-0,1,2,3,4	34

*VVT7 infected HEp-2 cells were transfected and incubated at 34°C. Six to nine CAT assay experiments were averaged and calculated against WT assuming 100% activity with less than 15% variation. ND – Not Done.

Table 4-2. CAT synthesis of *hr*8 mutants in HEp-2 cells at 37°C.

HEp-2 Cells @37°C	
Mutant	**Average***
WT	100
hr8-0	ND
hr8-1	ND
hr8-2	ND
hr8-3	100.5
hr8-4	77.5
hr8-1,2	ND
hr8-1,2,3	79
hr8-1,2,3,4	66
hr8-0,1,2,3	147.5
hr8-0,1,2,3,4	ND

*VVT7 infected HEp-2 cells were transfected and incubated at 37°C. Six to nine CAT assay experiments were averaged and calculated against WT assuming 100% activity with less than 15% variation. ND – Not Done.

Table 4-3. CAT synthesis of *hr*8 mutants in HEp-2 cells at 40°C.

HEp-2 Cells @40°C	
Mutant	**Average***
WT	100
Hr8-0	63
Hr8-1	146
Hr8-2	280
Hr8-3	48.5
Hr8-4	130.5
Hr8-1,2	602.5
Hr8-1,2,3	73
Hr8-1,2,3,4	108.5
Hr8-0,1,2,3	135.5
Hr8-0,1,2,3,4	85

*VVT7 infected HEp-2 cells were transfected and incubated at 40°C. Six to nine CAT assay experiments were averaged and calculated against WT assuming 100% activity with less than 15% variation.

To test for host range effects, CAT assays were performed for the L mutants on A549 and BHK cells at 37°C (Tables 4-4 and 4-5). When experiments were conducted on A549 cells (Table 4-4), the results were largely similar to HEp-2 cells, however *hr*8-0,1,2,3 and *hr*8-0,1,2,3,4 are not included in these results as they were being remade. Because *hr*8-4 had somewhat decreased activity in Table 4-2, it was tested along with *hr*8-0,1,2,3,4 in the permissive cell line, BHK (Table 4-5). As expected, the mutants were also more active in the permissive cell line at 37°C like in HEp-2 cells at 37°C. Thus the cell line appeared to play no role in the activity of the L protein. Even at a permissive temperature each of the mutants seemed to have some effect on RNA synthesis and increased temperatures increased rather than decreased activity. As indicated in Chapter 3, the vaccinia virus T7 polymerase that infected the cells probably methylated the mRNAs allowing CAT to be more efficiently translated. Clearly the

minigenome assays with expression of mutant L proteins are not predictive of functions seen in virus.

Table 4-4. CAT synthesis of *hr*8 mutants in A549 cells at 37°C.

A549 Cells @37°C	
Mutant	**Average***
WT	100
hr8-0	115.5
hr8-1	171.5
hr8-2	107.3
hr8-3	81.5
hr8-4	131
hr8-1,2	153
hr8-1,2,3	74.5
hr8-1,2,3,4	38
hr8-0,1,2,3	ND
hr8-0,1,2,3,4	ND

*VVT7 infected A549 cells were transfected and incubated at 37°C. Six to nine CAT assay experiments were averaged and calculated against WT assuming 100% activity with less than 15% variation. ND – Not Done

Table 4-5. CAT synthesis of *hr*8 mutants in BHK cells at 37°C.

BHK Cells @37°C	
Mutant	**Average***
WT	100
hr8-4	100
hr8-0,1,2,3,4	41.5

*VVT7 infected BHK cells were transfected and incubated at 37°C. Six to nine CAT assay experiments were averaged and calculated against WT assuming 100% activity with less than 15% variation.

As with the *hr*1 mutants, Valery Grdzelishvili performed experiments on the *hr*8 L mutants that were inserted into recombinant vesicular stomatitis virus. His findings clearly indicate that both the temperature sensitivity and the host range phenotypes of the *hr*8 virus reside in the *hr*8-4 mutation. The second to last column of Table 4-6 shows that any recombinant virus containing the -4 mutation is significantly less active at 40°C than

at 34°C while all the other *hr*8 mutations are like WT at the nonpermissive temperature. Additionally, the ratio of titers for each virus in BHK and HEp-2 cells is shown in the last column. Again, *hr*8-4, *hr*8-1,2,3,4, *hr*8-0,1,2,3,4, and the original *hr*8 mutant are deficient in HEp-2 cells relative to BHK cells, while the other mutants are not. These results clearly show that both host range and temperature sensitivity phenotypes lie in the same mutation, *hr*8-4 with a change from GLY to ARG at amino acid 1481.

Table 4-6 . Comparative titers of recombinant VSV mutants in BHK and HEp-2 cell lines at 34°C and 40°C.

Virus	**Virus titer (PFU/ml) in BHK cells**		**Virus titer (PFU/ml) in HEp-2 cells**		**Titer ratio 34ºC/40ºC in BHK**	**Titer ratio 34°C in BHK/HEp-2**
	34°C	**40°C**	**34°C**	**40°C**		
Wt	2.6×10^9	6.0×10^8	6.5×10^7	8.0×10^6	4.3	40.0
rHR8-0	8.0×10^8	7.0×10^8	1.5×10^7	1.5×10^6	1.1	53.0
rHR8-1	6.0×10^9	6.0×10^9	1.1×10^8	4.5×10^6	1.0	54.5
rHR8-2	2.0×10^8	2.0×10^8	5.5×10^6	1.2×10^6	1.0	36.4
rHR8-3	3.5×10^9	2.0×10^9	1.1×10^8	8.0×10^6	1.8	31.8
rHR8-4	3.0×10^9	**$< 10^3$**	6.0×10^4	$< 10^3$	**$> 3.0 \times 10^6$**	**5.0×10^4**
rHR8-0,1,2,3	3.5×10^9	2.0×10^9	3.2×10^8	5.0×10^7	1.7	10.9
rHR8-1,2,3,4	3.2×10^8	$< 10^3$	3.0×10^3	$< 10^3$	**$> 10^5$**	**$> 10^5$**
rHR8-0,1,2,3,4	2.0×10^9	**2.0×10^4**	4.0×10^3	$< 10^3$	**2.0×10^5**	**5.0×10^5**
hr8 (Orig.)	4.0×10^8	$< 10^3$	$< 10^3$	$< 10^3$	**$> 4.0 \times 10^5$**	**$>4.0 \times 10^5$**

CHAPTER 5
DISCUSSION

The overall focus of my research was to determine which amino acid change or combination of changes in the *hr*1 and *hr*8 L mutant viruses were responsible for the host range and temperature sensitivity phenotypes. The minigenome CAT assays that were performed on the constructed L mutants which measured the effect of temperature and host cells on RNA synthesis by the expressed proteins were not completely predictive. When comparing the CAT assay results to other data obtained in our laboratory, it is clear that assaying the L protein in cell culture is not the same as assaying the protein in a virus infection.

The CAT assays suggested that the *hr*1 mutation responsible for temperature sensitivity is the replacement of VAL for ASP at position 1671 regardless of the cell line in which it was expressed. The results of Grdzelishvili et al (J. Virology, 2005, in press) do show that *hr*1-1 is, in fact, the mutation responsible for temperature sensitivity. His plaque assay experiments on recombinant VSV, which had the *hr*1 L mutations (Figure 3-6), show that the virus titer ratio at 34°C/40°C is five logs greater in recombinant *hr*1-1 and *hr*1-0,1 and the original *hr*1 mutant indicating that all L proteins containing the *hr*1-1 mutation are temperature sensitive.

While the CAT assay results were predictive of temperature sensitivity in *hr*1, it was unclear which amino acid change was affecting host restriction as all the *hr*1 mutants had fairly similar activities when compared to WT L regardless of the cell line in which they were expressed. In nonpermissive cells, such as HEp-2 and A549, the original L

mutant does not methylate mRNA, however, mRNA is methylated in permissive cells, like BHK (Horikami et al., 1984 and Horikami and Moyer, 1982). This is because viral transcription occurs in the cytoplasm of the cell. The nucleus of the cell contains methyltransferase enzymes that are leaked out into the cytoplasm in BHK cells, but not in nonpermissive cells. Therefore the *hr*1 mutation responsible for unmethylated mRNAs is rescued by the permissive cells eliminating the appearance of a defect while the non-permissive cells are unable to rescue methylation and the amount of translated CAT protein would be decreased. Because the CAT assay requires cells to be infected with VVT7, which encodes its own methyltransferase enzymes, it is likely that the recombinant vaccinia virus rescued the methylation in nonpermissive cells eliminating the appearance of a defect. Thus, this assay cannot measure methylation affects in the L protein. Grdzelishvili et al. conducted plaque assay experiments to test for host restriction (J. Virology, 2005, in press) and concluded that *hr*1-1 is once again the responsible mutation. The ratios of virus titers in BHK and HEp-2 cells are five logs greater in *hr*1-1, *hr*1-0,1, and the original *hr*1 mutant indicating that L proteins containing the *hr*1-1 mutation are host restricted as non-permissive cells are unable to rescue the methylation defect.

Additional experiments have been conducted by Grdzelishvili to prove that *hr*1-1 is in fact causing a methylation defect. He demonstrated in our laboratory that the *hr*1-1 mutation does not have a significant effect on mRNA synthesis in virus. Additionally, his results showed that this L mutation did not affect capping of viral mRNA during *in vitro* transcription. However, when he conducted *in vitro* transcription by detergent-activated purified viruses using a labeled methyl donor (AdoMet), it was clear that *hr*1-1,

*hr*1-0,1 and the original *hr*1 mutants were not being methylated at all (results not shown). Further, when the cap structures of WT and *hr*1-0 mutants were analyzed by digesting the [^{3}H]AdoMet labeled RNA with P_1 nuclease to release the cap, the digestion products were shown to be 7mGpppAm and GpppAm indicating that the non-mutant virus mRNAs (WT and *hr*1-0) were being methylated at the cap structure, confirming previous results with the original *hr*1 virus (Horikami et al., 1984).

The *hr*1 mutation at position 1671 is located in conserved Domain VI of the L protein. Figure 5-1 indicates the location of Domain VI and also shows the sequence of several L proteins of other negative stranded RNA viruses with similar conserved regions. Similar conserved regions in cellular and viral methyltransferases have been proven to be the S-adenosylmethionine binding site and mutations in this area are known to abolish the activities of two methylating enzymes, mRNA guanine-N7 cap methyltransferase and nucleoside-2'-O cap methyltransferase (Luongo et al., 1998, Mao and Shuman, 1996, Wang and Shuman, 1997). Based on this data, we propose that the mutation at amino acid 1671 abolishes AdoMet binding and therefore all cap methylation.

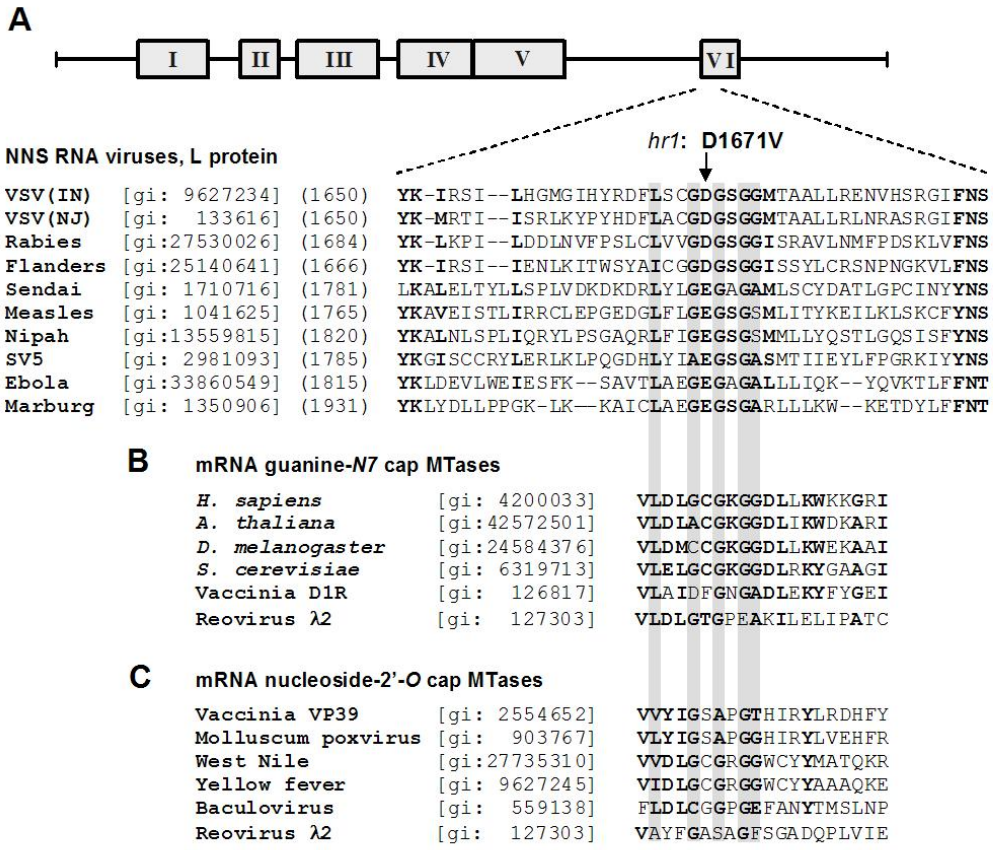

Figure 5-1. A) Conserved domains in VSV and the location of the *hr*1-1 mutation. B) The same conserved regions in known S-adenosylmethionine binding sites that are responsible for guanine-N7 cap methyltransferases. C) The same conserved regions in known S-adenosylmethionine binding sites that are responsible for nucleoside-2'O cap methyltransferases.

In the case of *hr*8, the data on the analysis of viral RNA synthesis were unclear and unexpected. When testing temperature sensitivity, none of the mutants were significantly decreased due to an increase in temperature. Surprisingly, *hr*8-2 and *hr*8-1,2 appear to be WT at the permissive temperature but were actually more stable relative to WT L at 40°C. The *hr*8-2 mutation is found in the nonconserved region between Domains V and VI at position 1356. Perhaps this mutation changes the overall conformation of the protein making it more stable. Host restriction results were equally inconclusive when analyzing the CAT assay results of *hr*8. Like *hr*1, the VVT7 required

for the CAT assays most likely rescued the methylation defect thus eliminating the appearance of a mutation.

It was impossible to predict which mutant was affected by temperature and the host cells by reviewing the CAT assay results. However, Grdzelishvili et al. (manuscript in preparation) have now shown by plaque assays that *hr*8-4 at position 1488 is the single mutation responsible for both temperature sensitivity and host restriction. The ratios of virus titers at 34°C/40°C and in BHK/HEp-2 are both five log greater for *hr*8-4, *hr*8-1,2,3,4, *hr*8-0,1,2,3,4 and the original *hr*8 mutant indicating that any mutant containing the *hr*8-4 mutation is both temperature sensitive and host restricted.

Similar experiments that were conducted on *hr*1 are in progress to further test for the methylation abilities of *hr*8-4. Grdzelishvili's initial results indicate that *hr*8-4 is not temperature sensitive for RNA synthesis but only for methylation. He has found that methylation is not abolished, as in *hr*1, but it is decreased by approximately 95%. It was originally thought that *hr*8 caused a defect specifically in the 7mG methyltransferase. Horikami and Moyer (1982) found that *hr*8 mRNA could be mono-methylated at the penultimate base or not methylated at all. However, some current results indicate that *hr*8 can be methylated at the 7mG position as well as at the 2'O penultimate base at very low levels.

The *hr*8-4 mutation at position 1488 is located in the nonconserved region between Domains V and VI of the L protein. This change causes a phenotype of limited methylation. Therefore, position 1488 is not part of the SAM binding site, as partial methylation can be achieved. It is possible that position 1488 is part of the catalytic site for one or both of the methylating enzymes. *Hr*8-4 could also change the overall

conformation of the protein thus decreasing its ability to methylate. Another option is that *hr*8-4 is part of a catalytic site for one enzyme and changes the overall conformation of the protein affecting the other methylation event. Grdzelishvili's results, when completed, will help to determine the function that amino acid 1488 plays in methylation. Additionally, site-directed mutagenesis will be conducted in the future on the area between amino acids 1400 and 1671 to further analyze the potential role of the nonconserved region between Domains V and VI on methylation.

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BIOGRAPHICAL SKETCH

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