MATRIX PROTEIN OF VESICULAR STOMATITIS VIRUS INTERACTS WITH HOST RAE1 IN MULTIPLE COMPLEXES TO INHIBIT HOST TRANSCRIPTION

By

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DEDICATION

This thesis is dedicated to my grandfather who’s enthusiasm for science has always been an inspiration to me.
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<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>$C_T$</td>
<td>critical threshold cycle</td>
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<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>EMCV</td>
<td>encephalomyocarditis virus</td>
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<tr>
<td>FG</td>
<td>phenylalanine–glycine</td>
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<td>G</td>
<td>VSV glycoprotein</td>
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<tr>
<td>GST</td>
<td>glutathione- S- Transferase</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<tr>
<td>HA-Rae1</td>
<td>HA epitope tagged Rae1</td>
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<tr>
<td>hnRNPs</td>
<td>heterogenous nuclear riboproteins</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>L</td>
<td>large polymerase protein of VSV</td>
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<tr>
<td>M</td>
<td>matrix</td>
</tr>
<tr>
<td>MLB</td>
<td>multi-vesicular bodies</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>rM51R-M</td>
<td>recombinant VSV containing the M51R mutant M protein</td>
</tr>
<tr>
<td>N</td>
<td>VSV nucleocapsid protein</td>
</tr>
<tr>
<td>NCM</td>
<td>nucleocapsid-M protein</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export sequence</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>NLS</td>
<td>nuclear localization sequence</td>
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<td>NPC</td>
<td>nuclear pore complex</td>
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<td>NT</td>
<td>non-targeting</td>
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<tr>
<td>Nup</td>
<td>nucleoporin</td>
</tr>
<tr>
<td>P protein</td>
<td>viral phosphoprotein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>phosphate buffered saline with 0.05% Tween-20</td>
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<tr>
<td>PKR</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>rwt</td>
<td>recombinant wild type</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA binding protein</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris buffered saline with 0.02% Tween-20</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>WD</td>
<td>tryptophan-aspartic acid</td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
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<tr>
<td>Wt</td>
<td>wild type</td>
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ABSTRACT

Rajani, Karishma
MATRIX PROTEIN OF VESICULAR STOMATITIS VIRUS INTERACTS WITH HOST RAE1 IN MULTIPLE COMPLEXES TO INHIBIT HOST TRANSCRIPTION
Dissertation under the guidance of Douglas Lyles, PhD., Professor and Chairman of Biochemistry

Vesicular stomatitis virus (VSV) suppresses antiviral responses in infected cells. This suppression is due to the activity of the matrix (M) protein, which inhibits host gene expression at multiple levels, including transcription, and nuclear-cytoplasmic transport. Previous studies have shown that M protein interacts with cellular proteins such as Rae1 and Nup98. Rae1 has been implicated in regulating nuclear-cytoplasmic transport and functions in mitotic spindle assembly and chromosome segregation and has multiple locations with the cell. Given its multiple functions and localization, I hypothesized that there may be multiple forms of Rae1 and that VSV M protein interacts with Rae1 to form multiple M protein-Rae1 complexes to inhibit host gene expression at multiple levels. Size exclusion chromatography determined that Rae1 exists in high, intermediate, and low molecular weight complexes. The high and intermediate molecular weight complexes containing Rae1 also have other proteins involved in nuclear-cytoplasmic transport, including Nup98, and Nup62.

To determine the importance of M protein-Rae1 complex to the ability of VSV to inhibit host gene expression, the role of Rae1 during VSV infection was determined by examining the effects of silencing the expression of Rae1 using small interfering RNA. Silencing the expression of Rae1 had little if any effect on nuclear accumulation of host mRNA in VSV-infected cells, or VSV’s ability to inhibit host translation. Instead, silencing Rae1 expression reduced the ability of VSV to inhibit host transcription. These results support the
idea that M protein-Rae1 complexes serves as a platform to promote the interaction of M protein with other host factors involved in regulation of transcription.

In the last section of this thesis, host proteins present in the different compartments within the nucleus were isolated and tested for their ability to interact with M protein. Rae1, Nup98, and Nup62 were present in fractions containing proteins associated with chromatin, nuclear envelope, and nucleoporins. Rae1 and Nup98 present in all the fractions were competent to interact with M protein. In contrast to these proteins, Nup62 present in fractions containing nuclear envelope and nucleoporins was competent to interact with M protein. These results indicated that M protein interacts with Rae1, Nup98, and Nup62 localized within the nucleus.
INTRODUCTION

The anti-viral responses mounted by virus-infected cells include potent mechanisms to evade virus replication. Thus, in order for viruses to effectively propagate, most viruses have developed mechanisms to inhibit or evade these host antiviral responses. Vesicular stomatitis virus (VSV) induces potent suppression of host antiviral responses. This suppression is accomplished by inhibiting host transcription, host mRNA transport, and host mRNA translation. The matrix (M) protein, a major structural protein that participates in virus assembly process, is involved in mediating the suppression of host gene expression. Studies have shown that M protein inhibits host mRNA transport by interacting with a cellular protein called Rae1. Rae1 has multiple localizations in a cell, and functions in mitotic checkpoint regulation. However, the role of Rae1 in regulating nuclear-cytoplasmic transport is itself debated even though it interacts with proteins regulating nuclear-cytoplasmic transport. I had the hypothesis that M protein interacts with Rae1 to form M protein-Rae1 complex(es). These would serve as a platform that would allow M protein to interact with other cellular proteins and form multiple inhibitory complexes and interfere with their function(s). These complexes would serve for M protein to inhibit host gene expression at multiple levels.

The goal of this project was to address the “platform hypothesis” and determine the role of Rae1 in VSV’s ability to inhibit host gene expression at multiple levels. The data presented in this thesis indicate that M protein interacts with Rae1 present in multiple complexes to inhibit host gene expression. In support of the “platform hypothesis” Rae1 was required for M protein to interact with other host proteins such as
Nup98 and Nup62. Furthermore, the results from experiments provide evidence that M protein-Rae1 complex primarily suppresses host transcription to inhibit host gene expression rather than mRNA transport as previously proposed.

**Structure and replication cycle of VSV**

VSV is a negative stranded RNA virus that is enveloped and belongs to the *Rhabdoviridae* family. Under electron microscopy, VSV appears bullet shaped (7) which is one of the characteristic features of viruses belonging to this family. The 11 kb RNA genome of VSV encodes genes of five proteins. The RNA genome cannot function as messenger RNA as it lacks a 5’ cap and a 3’ poly A tail. Instead the RNA genome serves as a template for primary transcription of viral genes. The nucleocapsid (N) protein encapsidates the viral RNA genome and associates with two proteins, the phosphoprotein (P) and the large polymerase protein (L). The L and the P proteins constitute the viral RNA dependent RNA polymerases that transcribe the RNA genome. The viral genome, along with the N, P, and L proteins comprises the ribonucleoprotein complex. The matrix (M) protein binds to the nucleocapsid, thereby, condensing it into a tightly coiled helix (85). In contrast to the tightly coiled helix, nucleocapsids devoid of M protein are loosely coiled and flexible (85). It is this activity of the M protein that gives the virus its bullet like shape. In addition to binding to the nucleocapsid, M protein also binds to the lipid bilayer of the virus envelope. Spanning the envelope of the virus is the glycoprotein (G) protein, an integral transmembrane protein, which functions during the process of virus attachment and penetration during virus infection.

The viral lifecycle is composed of the early steps which include attachment, penetration, and primary transcription. Later steps include secondary transcription and
virus assembly. During VSV infection, the G protein mediates attachment of VSV to the surface of cells. To date, no known receptor of VSV has been identified indicating that nonspecific electrostatic and hydrophobic interactions probably mediate attachment of VSV to cells. Penetration occurs via clathrin-dependent endocytosis (78) after which uncoating of the virus releases the nucleocapsid into the host cytoplasm. Primary transcription is the first synthetic step in the viral replication cycle, in which the viral RNA polymerase transcribes the 5 viral mRNAs and a leader RNA from the viral genome. This step can occur without further synthesis of any viral proteins. With the exception of the leader RNA that does not encode for a protein, all viral mRNA are capped and polyadenylated. These mRNAs are translated into viral proteins using host cell machinery. The accumulation of viral proteins triggers the viral RNA polymerase to switch from transcription into replication of parental genomes. Replication occurs in two steps. First the negative strand genome is copied into full length positively stranded RNA antigenome. The antigenome is then replicated into full length negatively stranded progeny genomes. These progeny genomes can also undergo secondary transcription to produce viral proteins or can be packaged into virions that eventually bud from the surface of the infected cell. The secondary transcription is the major amplification step of viral proteins. Both viral assembly and secondary transcription begin at 2 hours post infection and reach a maximum at 8 to 10 hours post infection and continues until 20 hours post infection [reviewed in reference (77)]. Both replication and transcription occur entirely in the cytoplasm as VSV can replicate efficiently in enucleated cells (42). A well characterized manifestation of VSV infection is the cytopathic effect of infected cells which results from activation of apoptosis (68). However, most of the replicative cycle of
VSV is complete before the cells die (66) and thus apoptosis does not affect the amount of progeny virions released from the infected cell.

**Role of M protein in viral lifecycle and in virus assembly**

The matrix (M) protein plays vital roles in the process of viral replication and in the pathogenesis of the virus. However, the role of M protein in virus induced cytopathic effects are genetically separable from its function in virus assembly (12, 73). The M protein along with the G protein and the nucleocapsid are major structural proteins of the virus. In the infected cell, M protein binds to the nucleocapsid, which exists as a random coil in the cytoplasm, to form nucleocapsid-M protein (NCM) complex. Binding of M protein causes the nucleocapsid to assume a helical structure (7, 28, 47, 73-75, 87). Through cryo-EM imaging of intact VSV particles, it was established that the helix is left handed and is bound by an outer layer of M protein that bridges the helix to the membrane (48). Although the M protein is present in high concentration in the cytoplasm, the plasma membrane is the only site where the M protein and the nucleocapsid interact (88). Moreover, only about 10-20% of the total M protein is associated with the plasma membrane (24, 64). Flood *et al* (41) have shown using purified M and NCM complexes, that cytosolic and membrane bound M protein can bind to nucleocapsids, whereas intracellular nucleocapsids are unable to bind to M protein. These results suggested a two-step mechanism for viral assembly where there is an initial addition of M protein to NCM, which is fundamentally different from M protein that is further recruited to the NCM complexes (74).

M protein in the absence of any viral components can bind plasma membrane (24) and form membrane vesicles that contain M protein (76). Using immunoelectron
microscopy, Swintek et al have shown that while M protein is not present in the same membrane microdomains as the N protein, both nucleocapsids and G protein are present in the same membrane microdomains. These experiments have led to the proposal of a new model for the budding process, in which the formation of microdomains containing N and G proteins are the precursors for the initiation of the budding process. This is followed by M protein binding to the nucleocapsids. However, the mechanism that initiates the process of binding of M protein to nucleocapsids is not known. Following the formation of NCM complexes, the release of budding virion follows. M protein also participates in this late budding function which is the final step in virus assembly. A proline rich region, PPPY motif within the M protein has been identified to play an important role in the budding process (61). It has been proposed that the process of budding may be mediated by the interaction of M protein with host factors such as Nedd 4, a ubiquitin ligase (55, 56). Ubiquitin ligases play regulatory roles in the formation of multi-vesicular bodies (MLB), which are generated as a result of maturation of the endosome (59). The process of virus budding at the plasma membrane and the budding of vesicles into the lumen of an endosome are similar and it is generally accepted that enveloped viruses use host cellular machinery regulating the formation of MLB as a platform to facilitate the release of the budding virion.

**Cytopathic effects of VSV infection**

Infection with VSV leads to severe cytopathic effects in vertebrate cells (120). These cytopathic effects are associated with the morphological changes associated with VSV infection which largely arise due to induction of apoptosis.

**Morphological changes in VSV infected cells.**
During VSV infection, the earliest morphological changes include cell rounding which results from sequential disassembly of cytoskeletal elements (103). In the absence of any other viral products, M protein can induce cell rounding (14). It was further demonstrated that the activity of M protein to induce cell rounding correlated with its ability to inhibit host gene expression and not with its role in virus assembly (73). Moreover, the induction of apoptotic pathways during VSV infection was required for M protein to induce cell rounding (66) further demonstrating that activation of apoptosis is the cause of the morphological changes associated with cell rounding.

**Induction of apoptosis**

During the course of VSV infection, following cell rounding are membrane blebbing, DNA fragmentation, cytoplasmic shrinkage and lysis. In the absence of any other viral components, induction of apoptosis and cell rounding have been attributed to the ability of M protein to inhibit host gene expression and is not associated with M protein’s ability to participate in virus assembly process (66, 67, 73). However, in context of viral infection, M protein as well as another unidentified viral component is needed for inducing apoptosis. This was shown using isogenic recombinant viruses, expressing either wild type M protein (rwt) or mutant M protein that has the amino acid methionine at position 51 substituted with arginine (rM51R-M). This mutation renders the virus defective in its ability to inhibit host gene expression (2, 4, 105, 115, 122) while still participating functionally in virus assembly process (12). HeLa cells infected with rwt virus entered apoptosis a lot faster than HeLa cells infected with rM51R-M virus. In contrast, BHK cells infected with wt virus entered apoptosis slower than BHK cells infected with rM51R-M virus (67). These results also suggested that the ability of M
protein to induce apoptosis depends on the cell type, in particular, the requirement of new protein synthesis for the cell to undergo apoptosis (67). Several studies using different cell types have demonstrated that VSV is a potent inducer of apoptosis and can activate both mitochondrial and the death receptor pathways to induce apoptosis (44, 45, 91).

**M protein plays a major role in inhibition of host gene expression**

The products of virus replication trigger the activation of inducers of host anti-viral response that are aimed at suppressing viral replication. Interferons (IFNs) are a group of cytokines that are secreted by the cell and are an early line of defense against viral infections [reviewed in reference (101, 108)]. Products of viral replication, such as double stranded (ds) RNA are recognized by membrane receptors such as toll like receptors (TLR) as well as intracellular receptors such as retinoic acid inducing gene (RIG-I) and melanoma differentiation associated gene (MDA-5). Recognition by these receptors results in activation of multiple signal transduction pathways, including kinases which phosphorylate IFN regulatory factors (IRF) such as IRF3 and nuclear factor kappa B (NF-κB). Phosphorylated IRF-3 and NF-κB serve as transcription regulators of IFN production. The secreted IFN binds to its receptors present on both infected and uninfected cells followed by the phosphorylation of IFN receptors by the Janus family of tyrosine kinases. Following this, STAT proteins are activated, and upon interacting with IRF-9 translocate into the nucleus. This complex acts as a transcription factor by associating with the IFN stimulated response element to activate the production of hundreds of IFN stimulated genes and induce an anti-viral state. Other kinases such as protein kinase R (PKR) also play a role in inducing an anti-viral response to viral infection. Although PKR is expressed constitutively at basal levels, its expression is
upregulated upon IFN production. PKR is activated upon binding to dsRNA and activated PKR phosphorylates the translation initiating factor, eIF2α which leads to the suppression of host translation machinery.

Nearly all viruses have mechanisms to inhibit or evade these host anti-viral responses in order for the virus to propagate efficiently and induce pathogenesis. In some viruses, viral proteins directly target specific host factors that participate in induction of anti-viral responses. For example the phosphoprotein in rabies virus prevents the induction of IFN by inhibiting the phosphorylation of IRF-3, therefore preventing IRF-3 to function as a transcription activator for the production of IFN-β (19). In the case of paramyxovirus simian virus 5, the V protein doesn’t prevent phosphorylation of IRF-3, but prevents its translocation into the nucleus (57). Moreover, the V protein also targets STAT1 for degradation thereby preventing production of IFN (35).

In contrast to these viruses that employ specific mechanisms to evade anti-viral response, VSV has a more general mechanism to counteract the anti-viral response that comprises a rapid and potent inhibition of all host gene expression. This suppression is mediated by the viral M protein, which inhibits multiple steps in the expression of host genes (2, 4, 11, 27, 58, 110) including expression of genes that code for production of antiviral cytokines such as interferons (4, 39, 105). The rapid inhibition of host gene expression occurs at the level of transcription, nuclear -cytoplasmic transport, and mRNA translation.

Earlier studies have suggested that the Leader RNA in VSV is responsible for inhibition of host gene expression (29). Leader RNA is a small positive sense RNA encoded at the 3’ end of the VSV genome. The leader RNA does not encode for any
protein and does not have a 5’cap and 3’ poly A tail [reviewed in reference (77)].

However, several lines of experiments now suggest that instead of the leader RNA, it is
M protein that likely plays a role in the inhibition of host gene expression.

Naturally occurring viruses that have mutations in the M gene are defective in
their ability to inhibit host gene expression (2, 4, 12, 105, 115). However, these mutations
do not affect M protein’s functions in virus assembly as these viruses are able to
assemble into virions (12). Conversely, M protein mutants that have truncations in the N
terminus that affect M protein’s ability to participate in virus assembly can still largely
induce inhibition of host gene expression (12). Furthermore, M protein, in the absence of
other viral components, inhibits the expression of host transcription and of cotransfected
genes (3, 11) further demonstrating the role of M protein in suppression of host gene
expression.

One of the intriguing questions is, how does a relatively small protein with no
enzymatic activity accomplish inhibition of host gene expression? One of the most
widely accepted notions is that the M protein interacts with host proteins directly or
indirectly to inhibit host gene expression at multiple levels. Purified M protein is very
sticky and it has been difficult to identify cellular proteins with which M protein might
interact specifically in order to inhibit host gene expression because of the large number
of nonspecific interactions. However, over the last decade there have been a few host
proteins identified with which the M protein interacts specifically. These proteins are
primarily proteins such as Rae1 (38), Nup98 (110), and heterogenous nuclear riboprotein
U, (23). This thesis concentrates on the molecular mechanisms of the interaction of M
protein with different host proteins and the implications of these interactions to VSV’s ability to inhibit multiple steps in host gene expression.

**Inhibition of host transcription**

M protein, in the absence of any other viral products was shown to inhibit transcription of target genes using nuclear runoff assays (2, 11). In contrast to the potent inhibitory activity of M protein, other viral proteins, such as the N, P, G proteins do not inhibit host gene expression (39). In transfected cells, M protein inhibits transcription by all three RNA polymerases (2). Using recombinant viruses with wt M protein or mutant M protein, it was further demonstrated that the ability of VSV to inhibit host transcription was due to the activity of M protein (4). Furthermore, the lack of promoter specificity in the inhibitory effects of M protein suggested that M protein targets proteins in host transcriptional apparatus common to all polymerases. Using nuclear extracts isolated from VSV infected cells, it was identified that the TATA binding protein (TBP) subunit in the basal transcription factor TFIID was inactivated (122). However, M protein does not interact with TFIID and the phosphorylation of TBP and assembly of TBP were not affected in VSV infected cells (121). Thus, M protein inhibits host transcription by indirectly inactivating the activity of TFIID and is possibly mediated by M protein’s interaction with other host factors.

**Inhibition of host protein synthesis**

M protein in the absence of other viral components is unable to inhibit host mRNA translation (10) and works in concert with other viral factors to inhibit host translation. The cytoplasm of VSV- infected cells has both host and viral messages. Viral and host messages appear structurally similar i.e. the 5' ends have 2'-O- methylated
adenosine capped by 7-methly guanosine (1, 106) and the 3’ end have poly (A) tail (60). Despite the structural similarity between host and viral mRNAs, inhibition of host protein synthesis is generally completed by four hours post infection (71). In contrast to host messages, at similar time point viral messages are in abundance (71). This preferential translation of only viral messages is not due to degradation of host mRNA (71, 115) or through the use of cis acting sequences on viral messages (116). Recent work has shed light into how viral messages are preferentially translated during infection. During VSV infection, the translation initiation complex, eIF4F complex, is targeted by dephosphorylating the eIF4E subunit (27). Whitlow et al (115) have shown using in vitro synthesized mRNA that translation of mRNA transfected an hour after infection was not inhibited whereas the translation of mRNA transfected prior to infection was inhibited. Thus viral messages which are constantly generated during infection escape translation inhibition whereas host messages that have a slower rate of turnover are subjected to translation inhibition. Moreover, host mRNA translation would be further prevented from being synthesized by suppressing preceding steps in host gene expression, probably via inactivating host transcription apparatus, transport machinery, incorporation of mRNA into inactive messenger ribonucleoproteins (116), and inactivating host translational machinery.

**Inhibition of host nuclear-cytoplasmic transport.**

The effect of M protein on nuclear accumulation of RNA depends on the cell type and target being analyzed. Nuclear accumulation of RNA resulting from the inhibition of transport is most obvious in cells in which M protein has little if any effect on transcription, such as *Xenopus* oocytes (58, 92, 110). Her et al (58) were the first to
demonstrate that M protein, in the absence of any other viral components can inhibit host nuclear-cytoplasmic transport in *Xenopus* oocytes. However, in most mammalian cells, there is relatively little net accumulation of host RNAs in the nucleus, because their synthesis as well as their transport is inhibited by M protein. This was originally demonstrated in the pulse-chase experiments of Weck and Wagner (112).

Nuclear cytoplasmic transport is a complex and well orchestrated process and occurs via the nuclear pore complex. The nuclear envelope, which lines the nucleus, is a double membrane and separates the genetic material enclosed within the nucleus and the nucleoplasm from the cytoplasm. This double nuclear membrane is not continuous and has perforations which are occupied by the nuclear pore complexes (NPC). The NPC are large protein assemblies, their mass is estimated to be approximately 90-120MDa, and they have an eight fold symmetry (37, 65, 83, 107). The structure of the NPC can be divided into three components, the central plug and the nuclear and cytoplasmic filaments. The central plug is surrounded by eight spokes and embedded in the nuclear bilayer. Emanating from this central tube and into both the cytoplasm and the nucleus are filaments. The filamentous structures on the nuclear side of the NPC converge to form a nuclear basket. Nuclear pore proteins or nucleoporins (Nups) are components of NPC and have diverse functions. Some of the Nups constitute the bulk of the central plug and thus contribute to the structural integrity of the NPC while a subset of Nups mediate transport [reviewed in reference (37)]. A characteristic feature of transport Nups is the presence of repeats of short sequences ending in phenylalanine-glycine (FG) repeats (107). Multiple FG repeats can be present in each FG Nup, thus allowing multiple binding sites for cargo for each Nup.
The NPC serves as a highly selective gateway between the nucleus and the cytoplasm which macromolecules must traverse in order to translocate between the two compartments of the cell. Water, ions, and molecules less than 40kDa in mass can freely diffuse through the NPC, however, transport of molecules (larger than 40kDa) is mediated by karyopherins that recognize either nuclear or cytoplasmic transport signals located on the molecules. For import, nuclear localization signal (NLS) and for export nuclear export signal (NES) are recognized by karyopherins. The transport of karyopherins bound macromolecules is in turn regulated by binding of the small GTPase Ran and its regulatory factors. In the nucleus, Ran exists primarily in a GTP-bound state and in the GDP-bound state within the cytoplasm. These states of Ran are in turn regulated by guanine-nucleotide exchange factors present within the nucleus that replace the RanGDP form with GTP form and by GTPase activating protein present in the cytoplasm that favors the GDP form. Import and export of most RNA and proteins occurs through karyopherins. In the case of import, karyopherins recognize and bind to NLS containing cargo, and upon translocating to the nucleus, karyopherins bind to RanGTP thereby triggering the release of the cargo. For export, however the reverse process is favored. Karyopherins recognize and bind to cargo containing NES in the presence of RanGTP and upon translocating to the cytoplasm, hydrolysis of RanGTP promotes the dissociation of karyopherins from the cargo. The transport of macromolecules occurring via the NPC is bidirectional, i.e. protein and RNA can be imported and exported simultaneously (36). In the case of mRNA which are transported as mRNP complexes, the translocation is facilitated by the receptors that are not related to karyopherins. In mammalian cells, unlike export of RNA which is dependent on Ran gradient, the
dependence of mRNA transport on Ran gradient is highly debated (65, 83). Only mRNA lacking introns are transported into the cytoplasm with the 5’ end of the mRNA leading first through the NPC (83). Moreover, additional soluble factors that act in trans such as shuttling heterogenous nuclear riboproteins (hnRNPs) (94, 95) and TAP (8, 17, 83) also mediate mRNA transport. Although the precise molecular details of how translocation occurs via the fine meshwork of Nups is debated, it is widely accepted that during translocation, cargo bound receptors interact with the FG repeat Nups (107). FG repeat regions are natively disordered which allow them to freely writhe and form many transient, low affinity interactions with the cargo-receptors facilitating rapid transport.

M protein inhibits transport of small nuclear RNAs and mRNA, however it does not affect transport of t-RNA (58), intron lariat bearing the constitutive transport element (110) and complexes containing hnRNP-A1 and other hnRNPs (93). It was demonstrated further that M protein also affected the localization of transport karyopherin importin alpha while the localization of Ran and its associated factors remained largely unaffected (58). The exact mechanism of how M protein affects transport is still not known. This is further impeded due to the inherent complexity in understanding multiple pathways engaged in nuclear cytoplasmic transport.

M protein was shown to inhibit mRNA transport by interacting with host proteins such as Nup98 (110) and later Rae1 (38), and that this interaction is genetically correlated with the ability of M protein to inhibit nuclear-cytoplasmic RNA transport. Using oligo-dT in situ hybridization assays, it was demonstrated that M protein inhibited mRNA transport and this inhibition could be reverted upon overexpressing Rae1 (38). Further, the M protein-Rae1 interaction was shown to be independent of Nup98 (38). Thus it is
conceivable that disrupting the interactions between cargo-receptor and Nups would be one of the mechanism by which M protein in VSV would disrupt nucleoplasmic transport.

**Nup98**

Nup98 is one of the widely studied FG repeat containing Nup. Nup98 is encoded by two alternatively spliced mRNA transcripts that are auto proteolytically processed. This processing is required for the maturation of Nup98 and for its proper localization in the NPCs (43), and for the interaction with other Nups (51). Using immunoelectron microscopy it has been demonstrated that Nup98 is present in the cytoplasmic and the nuclear side of the NPC (51). This is further supported by fluorescence recovery after photobleaching analysis which indicated that Nup98 is a dynamic protein and shuttles between the nucleus and cytoplasm (50). Nup98 has been implicated in regulating nuclear cytoplasmic transport of proteins and RNA (13). Several studies highlight the pertinence of this Nup not only in regulating transport but also in other steps in gene expression such as transcription (21) and cell cycle regulation (63). In embryonic cells obtained from Nup98 null mice, the stoichiometry of cytoplasmic oriented Nups such as Nup358, Nup62, Nup214, and Nup88 were altered and import of proteins with NLS or M9 signal was impaired, highlighting the important role of Nup98 in transport (118). Furthermore, silencing the expression of Nup98 in *Xenopus* oocytes impairs export of ribosomal and mRNA (98). Recently it was discovered that Nup98 interacts with the export factor CRM1 (89) for proteins carrying leucine rich NES.

**Rae1**
In contrast to Nup98, the function of Rae1 in mRNA transport in higher eukaryotes remains unclear. This is largely due to discrepancies reported for the requirement of Rae1 to regulate transport, especially in higher eukaryotes, even though it can interact with other transport proteins such as Nup98 (99) and TAP (13) and can be cross linked to RNA (99). Rae1 was first identified in yeast where deletion of the gene inhibited polyadenylated RNA transport (9, 18, 81, 114) which was shown by oligo-dT in situ hybridization assays. However, deleting Rae1 gene in mice and analyzing cultured blastocytes from mouse embryos did not show evidence of mRNA transport inhibition. Interestingly, depletion of Rae1 leads to chromosome missegregation and spindle assembly (5, 15, 104, 117), indicating a role for Rae1 in mitotic spindle assembly.

Rae1 and Nup98 can form a complex during mitosis as well as interphase (79). Splenocytes from mice that are haploinsufficient for both Rae1 and Nup98 exhibit severe aneuploidy. It was further demonstrated that Rae1 in complex with Nup98 prevented premature degradation of the regulators of sister chromatids separation (62), indicating that these proteins may play a role in proper chromosome segregation. Recently a role for M protein in regulating mitosis was proposed. It was demonstrated using recombinant viruses that express wt M protein and mutant M(D) protein, that M could inhibit mitotic progression of synchronized HeLa cells. Furthermore, wt M protein was also shown to interact with Rae1 and Nup98 in HeLa cells synchronized at interphase and at mitosis (23). Therefore, M protein could possibly inhibit mitotic progression through its interaction with Rae1 in complex with Nup98.

Given the observation that Rae1 is not essential for nuclear-cytoplasmic RNA transport, it is unlikely that the VSV M protein inhibits host gene expression simply by
interfering with Rae1 function. Furthermore, it is not clear whether the interaction of M protein with Rae1 is responsible for inhibition of other steps in host gene expression besides nuclear-cytoplasmic RNA transport, such as host transcription and mRNA translation. In higher eukaryotes, Rae1 is localized in the cytoplasm, nucleoplasm, and the nuclear rim (9, 69, 99, 104). However, upon over expressing Rae1, it is localized primarily in the nucleus (99). Given its multiple sites of localization and the uncertainty about its function, I hypothesized that there may be multiple forms of Rae1 and that VSV M protein interacts with Rae1 to form multiple M protein-Rae1 complexes. These complexes may serve as platforms for M protein to interact with other cellular protein(s) in order to globally inhibit host gene expression (figure 1).

The data presented here support this hypothesis by demonstrating that Rae1 is required for Nup98, a previously characterized binding partner of Rae1 (99), as well as Nup62 to interact with M protein. Furthermore, Rae1 exists in high, intermediate, and low molecular weight complexes. Of these, the intermediate and low molecular weight complexes containing Rae1 are competent to interact with M protein. The high and intermediate molecular weight complexes also contain Nup98, as well as other Nups including Nup62, Nup214, and most likely Nup358. These experiments also showed that not all of the cellular Rae1 can interact with M protein, but instead there is a sub-population of cellular Rae1 that is competent to bind to M protein. While the properties that distinguish the Rae1 that is competent to interact with M protein from the remaining cellular Rae1 are not known, I was able to rule out many of the common forms of post-translational modifications.
To further test the role of Rae1 in VSV’s ability to inhibit host gene expression at multiple levels, the expression of Rae1 was silenced using small interfering RNA (siRNA) and the inhibition of host transcription, nuclear-cytoplasmic transport, and translation were measured in VSV-infected cells. Silencing the expression of Rae1 does not affect VSV’s ability to inhibit translation of host mRNA or the nuclear accumulation of host mRNA. However, silencing the expression of Rae1 impedes the ability of VSV to inhibit host transcription in infected cells. These results demonstrate that M protein-Rae1 complexes are involved in the inhibition of host RNA synthesis in VSV-infected cells.

Rae1, Nup98, and Nup62 have multiple localizations within cells. These proteins present in the different compartments within the nucleus were isolated and tested for their ability to interact with M protein. Rae1, Nup98, and Nup62 were present in fractions containing proteins associated with chromatin, nuclear envelope and Nups. Rae1 and Nup98 present in all the fractions were competent to interact with M protein. In contrast to these proteins, only Nup62 present in fractions containing nuclear envelope and Nups was competent to interact with M protein. These results indicated that M protein interacts with Rae1, Nup98, and Nup62 present in the nucleus and suggest that perhaps, M protein may interact with these proteins present in the nucleus to inhibit host gene expression.
Figure 1. Model for inhibition of host gene expression by M protein-Rae1 complexes:

“The platform hypothesis” M protein interacts with Rae1 to form M protein-Rae1 complexes that serve as a platform for M protein to interact with other host proteins to interfere with their function(s) to inhibit host gene expression at multiple levels. M protein-Rae1 complexes can interact with Nup98 to inhibit RNA transport or can inhibit host transcription by interacting with unknown target protein X or can inhibit host translation by interacting with target protein Y.
Figure 1: Model for inhibition of host gene expression by M protein-Rae1 complexes
**EXPERIMENTAL PROCEDURES**

*Construction of Glutathione –S- transferase (GST) tagged M protein(s).* To construct a wild type (wt) M protein with BamHI and NotI restriction site, the wt M gene in pET21d vector (28) served as a template. A BamHI restriction site was added using the primer 5’GCGGCCGGATCCATGGCTTCCTAA 3’, and a NotI restriction site was added using reverse primer 5’ GCCCGCGCGGCCGCTACTCGAGTTTG 3’. The resulting PCR fragment was cleaved and ligated into the vector pGEX-6P-1 (GE Healthcare). The resulting M protein had an N-terminal glutathione-S-transferase tag [GST-M]. Mutations within the M gene were made using the Quick change™ mutagenesis kit (Stratagene). Sequences of all clones were verified through DNA sequencing. The plasmids were transformed into BL21(DE3)pLysS *E.coli* cells.

*Purification of GST-M protein.* Day cultures were grown until the cells reached an optical density of $A_{600nm} \sim 0.7$ and induced with 200 µM isopropyl-β-D-thiogalactopyranoside for 3 hours at 37°C. Cells were centrifuged at 4000 x g for 20 minutes at 4°C, and pellets were frozen at -20°C until use. Recombinant wt and mutant M proteins with the GST tag were prepared using the protocol described in (92) with a few modifications which are as follows. The cells were lysed in phosphate buffered saline (PBS) with 1% TritonX100 with 1 mM phenyl methylsulfonyl fluoride. Following sonication and centrifugation, the protein was immediately loaded onto glutathione-Sepharose beads (GE Healthcare) and incubated for 1 hour at 4°C. Beads were washed with PBS and used in experiments.

*Infections and transfections of HEK 293 cells* - HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 2mM glutamine. To
prepare lysates, the cells were washed with PBS and incubated for 10 minutes on ice in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 15 mM MgCl₂, and 0.5% NP40) with an EDTA-free protease inhibitor mixture (Roche). Lysates were centrifuged at high speed for 15 minutes at 4°C. The resulting supernatant was collected and frozen at -80°C or used immediately in experiments. For infecting cells, recombinant wild type (rwt) or recombinant VSV containing the M51R mutant M protein (rM51R-M) virus stocks were prepared in BHK cells as described (67). Twenty four hours prior to infection, 1X10⁶ HEK 293 cells were seeded in 100mm dish. Cells were infected with either rwt or rM51R-M virus at a multiplicity of infection (MOI) of 10 plaque forming units/cell for 6 hours. Following infection, cells were harvested in lysis buffer as described above. For transfections, 1X10⁶ cells were seeded in 100mm dishes 24 hours prior to transfection. Cells were transfected with 9 µg of plasmid encoding hemagglutinin (HA) epitope tagged Rae1 [a gift from B. Fontoura used in (38)] using the calcium phosphate method. The plasmid encoding HA epitope tagged Rae1 (HA-Rae1) was modified to encode the entire sequence of the HA epitope tag to enhance antibody binding (This was done by Elizabeth Kneller, PhD). Twenty four hours post-transfection, the cells were washed with PBS and cell lysates were prepared as described above.

**Binding of cellular proteins to GST-M protein.** Wt or mutant GST-M proteins on glutathione beads were incubated in binding buffer (20 mM HEPES pH 7.4, 110 mM potassium acetate, 2 mM MgCl₂, and 0.1% Tween 20) for 1 hour at 4°C. Lysates from transfected or untransfected cells (250 µl) were incubated with 25 µl packed volume of GST-M proteins on glutathione beads (500 ng of GST-M protein) suspended in 250 µl of binding buffer at 4°C. With the exception of the experiment to monitor time of
interaction, and unless otherwise noted, where the incubation time was varied, the proteins were incubated for 1 hour. Bound and unbound fractions were separated by spinning the samples at 4000 x g for 2 minutes at 4°C. The bound fraction was washed several times with the binding buffer and analyzed along with the unbound fraction by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

**Immunoblotting.** Proteins were resolved by SDS-PAGE using either 10% Bis-Tris NuPAGE gel (Invitrogen) or 10% Tris-HCl polyacrylamide gels. Following electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and blocked in Tris buffered saline with 0.02% Tween-20 (TBS-T) with 5% milk (Difco) or in PBS with 0.05% Tween-20 (PBS-T) and 3% milk. The membranes were probed using primary antibodies to HA (Sigma), Nup98 (Sigma), Nup62 or FG repeat proteins (mAb414: Covance, 610497: BD Biosciences) prepared in TBS-T with 2.5% milk. Antibody against Rae1 (R2905: Sigma) was prepared in PBS-T with 1% milk. After several washes in either TBS-T or PBS-T, the blots were incubated with respective secondary antibodies linked to horseradish peroxidase (Amersham) used at 1:10000 in TBS-T with 2.5% milk or PBS-T with 1% milk. The blots were washed in either TBS-T or PBS-T, and proteins were detected using enhanced chemiluminescence substrate (Thermo Scientific). The intensities of the bands were quantified by scanning and analysis using Quantity One software (Bio-Rad).

**M protein(s) competition experiment:** About 80 ng of GST-M protein (prepared as described above) on glutathione beads and either 270 ng, 800 ng or 2500 ng of purified his-tagged M protein was added to the binding buffer with high salt (20 mM HEPES pH
7.4, 110 mM potassium acetate, 2 mM MgCl2, and 0.1% Tween 20 + 230 mM NaCl) and incubated for 40 minutes at 4°C. Lysates containing HA-Rae1 were then added and allowed to incubate for 1 hour at 4°C. The bound and unbound fractions were separated by spinning at 6000 rpm at 4°C. The bound fractions were washed in binding buffer with high salt and analyzed along with the unbound fraction by SDS-PAGE and immunoblotting.

Two-dimensional (2D) gel electrophoresis. Bound and unbound fractions of cell lysates containing endogenous Rae1 or HA-Rae1 after incubation with GST-M protein on glutathione beads were prepared as described above. The bound fraction was washed and the beads were incubated with fresh lysates twice more to increase the amount of bound protein for analysis. The bound fraction was washed after each incubation. The bound fraction was re-suspended in rehydration buffer (8 M urea, 2% chaps, 50 mM dithiothreitol and 0.2% ampholytes (Bio-Rad). The unbound fraction was precipitated using 1:1 ethanol: ether solution and then re-suspended in rehydration buffer. The bound and unbound fractions were incubated for 16 hours at room temperature in an IPG strip pH 3-10, 11 cm (Bio-Rad). The strips were focused in a Protean IEF cell. Following focusing, the strips were run in the second dimension using an 8-16% Tris–HCl gel (Bio-Rad), transferred onto PVDF and probed for Rae1.

Wheat germ agglutinin binding. Wheat germ agglutinin (WGA)-binding proteins were isolated using the glycoprotein isolation kit (89805: Thermo Scientific). Lysates from cells containing endogenous Rae1 or HA-Rae1 were diluted 4:1 with 5X binding buffer provided in the kit. Flow through and eluate fractions were prepared according to the manufacturer’s protocol. The diluted lysate, flow-through and eluate fractions were
incubated with GST-M protein on glutathione beads as described above for 14 hours at 4°C to obtain bound and unbound fractions. The fractions were analyzed by SDS-PAGE and immunoblotting.

*Gel filtration chromatography.* Lysates from cells containing endogenous Rae1 or HA-Rae1 and lysates from virus-infected or mock-infected cells were prepared as described above. Lysates from mock-infected cells were concentrated using Ultracel-10K (Millipore) before chromatography on size exclusion chromatography. Lysates were chromatographed on a Superdex 200 column (length = 30 cms, diameter = 1 cm) in cell lysis buffer using an FPLC apparatus (Bio-Rad), and thirty 1 ml fractions were collected. Equal volumes of fractions were analyzed for the presence of proteins by SDS-PAGE followed by immunoblotting. The standards used to calibrate the column were bovine serum albumin (BSA), ovalbumin, and aldolase prepared in cell lysis buffer without NP40. Gel filtration fractions obtained were incubated with GST-M protein on glutathione beads for 1 hour to obtain bound and unbound fractions as described above.

*Sucrose gradient centrifugation.* Lysates containing endogenous Rae1 or lysates containing HA-Rae1 were overlaid on 5-20% sucrose gradients in cell lysis buffer. Gradients were centrifuged at 35,000 rpm for 18.3 hours (for lysates containing endogenous Rae1) or at 40,000 rpm for 14 hours (for lysates containing HA-Rae1) at 4°C in a SW41.0 rotor (Beckman Instruments). Twenty fractions of equal volumes were collected from the top of the gradient and analyzed by SDS-PAGE and immunoblotting. The gradient was calibrated with standard proteins of known sedimentation coefficient, BSA (4.4S) and phosphorylase b (8.8S). Sucrose gradient fractions of lysates containing
endogenous Rae1 obtained were incubated with GST-M protein on glutathione beads for 14 hours at 4°C to obtain bound and unbound fractions as described above.

**siRNA transfections, lysate preparations, and infections.** Rae1 siRNA (D-011482-02, Dharmacon) was either used at final concentration of 5 µM or 10 µM with similar results. Nup98 siRNA (D013078-01, Dharmacon) was used at a final concentration of 5 µM and Nup62 siRNA was used (D012468-04, Dharmacon) at a final concentration of 20 µM. The nontargeting (NT) siRNA whose sequence is scrambled and does not match any sequence on the human genome used was as control (D-001210-01, Dharmacon). All transient siRNA transfections were carried out in HeLa cells using Hiperfect transfection reagent (Qiagen Corporation) according to the manufacturer’s instructions and as described in paper (93). For binding experiments, lysates from cells transfected with siRNAs were prepared after 48 hours post transfection in the buffer as described above and incubated on GST-M protein or GST for 2 hours. Silencing of each protein was confirmed by immunoblotting.

**RNA synthesis.** HeLa cells were transfected with Rae1, Nup98 or NT siRNA, and at 48 hours post transfection, cells were re-seeded at a density of approximately 1X10^6 cells in 35-mm culture dishes. After 24 hours cells were mock-infected or infected with rwt virus in the presence or absence of actinomycin D at MOI= 30 as described in paper (3). At 6 hours postinfection, cells were labeled with [³H]-uridine (100 µCi/ml) for 30 minutes, washed, and harvested in PBS. RNA was precipitated with tri-chloro acetic acid, and radioactivity was determined by scintillation counting. For cells transfected with Nup62 siRNA, the experiment was performed at 48 hours post transfection, without re-seeding the cells.
Cytoplasmic and nuclear RNA isolation. All solutions used for RNA purification were prepared in diethyl pyrocarbonate-treated water. At 72 hours post transfections with Rae1 siRNA or NT siRNA, cells were mock-infected or infected with rwt virus at MOI=10 for 6 hours. RNA was isolated from the nucleus and cytoplasm as described in (49) with a few modifications which were as follows. After scraping the cells in cold PBS, the pellet was resuspended in lysis buffer (10 mM NaCl, 10 mM Tris-Cl [pH 7.4], 3 mM MgCl2) containing 20 mM vanadyl-ribonucleoside complex (Sigma). An equal volume of the same lysis buffer with 10% (vol/vol) deoxycholate and 20% Tween-40 was added to the cells on ice with gentle mixing. Nuclei and cytoplasmic fractions were separated using by centrifugation over a sucrose cushion. To normalize the data for RNA recovery samples were spiked with 3 µg of E.coli total mRNA (Ambion) before isolation RNA using TRIZOL reagent.

Real time reverse transcription-polymerase chain reaction (RT-PCR).

Oligonucleotide primers and probes were designed and purchased from Sigma-Genosys. Primers for uidA gene (beta-glucuronidase) in E.coli were (forward) 5’-AGGTGCACGGGAATATTTCG-3’ and (reverse) 5’-ACGCGTCGGGTCGAGTT-3. The probe for E.coli uidA was CCACTGGCGGAAGCAACGCG which was labeled at the 5’ end with the reporter dye carboxyfluorescein and at the 3’ with the quencher tetramethylrhodamine. The primers and probe sequences for β-actin were as described in (22). Real time RT-PCR analysis was performed with a TaqMan One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems) as described by the manufacturer using a 25-µl sample volume and 0.25 ng of sample RNA. For actin, 5 µM concentrations of primers, and 2.5 µM concentration of probes were used, and for E.coli uidA, 10 µM concentration of
primers and 5 µM concentration of probe were used. TaqMan PCR assays were performed using an ABI 7700 instrument (Applied Biosystems, Foster City, CA) as described in (22). All samples were tested in triplicate. The critical threshold cycle (C_T) is defined as the cycle at which the fluorescence becomes detectable above background and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer-probe set with C_T values obtained from amplification of known quantities of plasmid DNA coding for either β-actin or of total E.coli mRNA. The standard curves were used to transform C_T values of the experimental samples to the relative number of DNA molecules.

**Determination of rates of protein synthesis.** At 72 hours post transfection with Rae1, Nup98, or NT siRNA cells were mock-infected or infected with rwt virus at MOI= 30 and then labeled with [35S] methionine for 10 minutes at varying times after infection, as described (27). Lysates were assayed for protein content and equal amounts of protein were resolved on SDS-PAGE gel. Gels were stained with Coomassie blue and were analyzed by phosphorescence imaging (Amersham Biosciences). The intensities of corresponding host and viral protein bands were quantified using ImageQuant™ software (Molecular Dynamics). For cells transfected with Nup62 siRNA, the experiment was performed at 48 hours post transfection, without re-seeding the cells.

**Isolation of Nups in the nucleus.** The nuclei from spinner HeLa’s were fractionated according to the protocol described in (80). All the solutions had protease inhibitors added to them right before use. Briefly, 1.4 X10^3 cells were pelleted and resuspended in ice-cold RSB buffer (10 mM Tris-Cl [pH 7.4], 0.5 mM MgCl₂, 10 mM KCl). Cell membranes were disrupted in a Dounce homogenizer by 35 strokes of Teflon coated pestle. The integrity of
the nuclei membranes were generally intact as monitored by light microscopy. The lysates were overlaid on buffer B (2.3 M sucrose, 50 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM MgCl₂, 2 mM DTT) and spun at 2000 rpm to pellet the nuclei. The pellets were resuspended in 100 μl buffer A (0.25 sucrose, 50 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM MgCl₂, 2 mM DTT) and the nuclei were counted and frozen at -80 °C until use. 1X10⁷ nuclei were thawed by placing in 30°C water bath and centrifuged at 2500 rpm for 1 minute. The pellet was resuspended by adding 300 μl of lysis buffer (0.1 mM MgCl₂, 1 mM DTT, 5 μg/ml of DNaseI and 5 μg/ml of RNaseI) dropwise, and vortexing. Following resuspension, 1.3 ml of extraction buffer (10% sucrose, 20 mM triethanolamine [pH 7.5], 0.1 mM MgCl₂, 1 mM DTT) was added dropwise and the pellet was incubated for 15 minutes at room temperature. The resuspended nuclei were underlaid with 500 μl of 30% sucrose cushion (30% sucrose, 20 mM triethanolamine [pH 7.5], 0.1 mM MgCl₂, 1 mM DTT) and centrifuged by slowly increasing the speed up to 4000 rpm for 10 minutes. The supernatant and pellet fractions were separated and the pellet was resuspended in 300 μl of extraction buffer [pH 7.5] dropwise followed by 170 μl of extraction buffer [pH 7.5] containing 0.3mg/ml of heparin. The resuspended nuclei were underlaid on 30% sucrose cushion and centrifuged as before. The process was repeated, with the pellet resuspended in 170 μl of extraction buffer [pH 7.5] containing 3% TX-100, and 0.075% SDS. The pellet obtained after centrifugation was resuspended in 300 μl of extraction buffer [pH 7.5] containing 0.3% of Empigen and incubated on ice for 10 minutes. The samples were centrifuged and the pellet fraction was solubilized in 300 μl of extraction buffer [pH 7.5] containing 0.3% of Empigen. The isolated fractions were incubated with GST-M protein or GST for 14 hours suspended in cell lysis buffer and analyzed by SDS-PAGE and immunoblotting.
RESULTS

Specificity of interaction of GST-M protein with Rae1: VSV containing wild type (wt) M protein inhibits host transcription (2, 4, 11, 122), nucleo-cytoplasmic transport (38, 105, 110), and translation (27, 115). Viruses containing mutations in the M gene, such as rM51R-M virus, which has amino acid substitution M51R, are fully functional in virus assembly (12), but are defective in inhibiting host transcription (4, 122), translation (115), and nuclear-cytoplasmic RNA transport (105). Wt M protein has been shown to interact with HA-Rae1 expressed in transfected cells and with endogenous Rae1 (38). In contrast, a mutant M protein, M(D), which has three amino acid substitutions (D52A, T53A, and Y54A), has been shown to be defective in interacting with HA-Rae1 and endogenous Rae1 (38). This mutant is defective in inhibiting host mRNA transport (38, 110), but has not been tested in its ability to inhibit host transcription and translation. The purpose of the experiment in figure 2A was to compare the abilities of wt, M(D), and M51R mutant M proteins to interact with Rae1. Recombinant wt or M51R mutant M proteins were expressed in bacteria as GST fusion proteins and purified on glutathione beads. HEK 293 cells were transfected with a plasmid encoding HA-Rae1. Lysates from transfected cells were incubated with recombinant GST-M proteins bound to glutathione beads, and bound (shown in duplicate) and unbound fractions were analyzed by SDS-PAGE and immunoblotting using antibody against the HA tag. Negative controls included beads containing the M(D) mutant M protein or GST not fused to M protein. Forty percent of the total bound fraction was analyzed and for the unbound fraction, 2% of the total was analyzed. As shown in figure 2A (upper panel), wt M protein interacted
with HA-Rae1, while no interaction was detected with M51R mutant M protein, similar to the negative controls.

Similar experiments were performed using lysates from untransfected cells to determine if M proteins could interact with endogenous Rae1. Cell lysates were incubated with wt, M51R, or M(D) M proteins or GST on glutathione beads, and bound and unbound fractions were analyzed by SDS-PAGE and immunoblotting using an antibody against Rae1. Similar to the results with HA-Rae1, wt M protein interacted with endogenous Rae1 while the two mutant M proteins were unable to interact with Rae1 (figure 2A, lower panel). Rae1 in the unbound fractions in this experiment was very faint, but could be seen in longer exposures (data not shown) and upon loading a larger percentage of the unbound fraction (shown in figure 2B). Thus, these experiments are in agreement with the published report that wt M protein interacts with Rae1 (38) and correlated the interaction between M protein and Rae1 with the ability of recombinant viruses containing wt versus mutant M protein to inhibit host transcription and translation as well as mRNA export.

In figure 2A, 40% of the total bound fraction was analyzed, and for the unbound fraction, 2% of the total was analyzed. Thus, it was observed that most of Rae1 remained in the unbound fraction. In order to more accurately estimate the amount of Rae1 that interacted with GST-M protein, amounts of bound and unbound fractions of Rae1 protein were analyzed by SDS-PAGE to give approximately equal intensities in immunoblots (figure 2B). Quantification of the bound and unbound fractions in multiple experiments indicated that approximately 10% of HA-Rae1 and 4% of endogenous Rae1 interacted with GST-M protein in a 1 hour incubation. In this exposure, there was another band with
slower electrophoretic mobility that was immunoreactive with the antibody against endogenous Rae1. Transfecting cells with Rae1 siRNA silenced Rae1 expression but had little if any effect on expression of the slower migrating band (unpublished results), indicating that this band was not derived from Rae1. Despite the low amount of Rae1 that interacted with M protein compared to the total cellular Rae1, the results with the mutant M proteins suggests that the interaction of M protein with this sub-population of Rae1 is largely responsible for inhibiting host gene expression.

Further evidence for specificity of the interaction between M protein and Rae1 was obtained in a competition assay. Purified soluble M protein was tested for its ability to compete with GST-M protein on glutathione beads for interaction with HA-Rae1. In the experiment shown in figure 2C, increasing amounts of soluble M protein was added to GST-M protein on glutathione beads. Lysates containing HA-Rae1 were added to the mixture of M proteins. Following an hour of incubation, bound and unbound fractions were analyzed by SDS-PAGE and immunoblotting using an antibody against the HA-tag. As shown in the figure 2C, there was a reduction in the amount of HA-Rae1 interacting with GST-M protein with increasing amounts of purified soluble M protein. Thus, purified soluble M protein was able to compete for interaction with HA-Rae1.
Figure 2: Specificity of M protein interaction with Rae1. (A) Wild-type (wt) or mutant GST-M proteins or GST alone on glutathione beads were incubated for 1 hour with cell lysates containing HA-Rae1 or lysates from untransfected cells. Bound (40% of the total fraction) and unbound fractions (2% of the total fraction) were analyzed by immunoblotting and probed for HA (top panel) or Rae1 (lower panel). Bound fractions were analyzed in duplicate. (B) Lysates containing HA-Rae1 or lysates from untransfected cells were incubated with wt GST-M protein on glutathione beads for 1 hour at 4°C. Bound and unbound fractions were probed for HA (upper panel) and Rae1 (lower panel). 40% of the total bound and 4% of the total unbound fraction of HARae1 were analyzed. For endogenous Rae1, 40% of the total bound and 6% of total unbound fraction were analyzed. (C) wt GST-M protein (0.08µg of M protein) or GST alone on glutathione beads was incubated with the indicated amount of soluble wt M protein. Cell lysates containing HA-Rae1 was added to the mixture of M proteins and incubated for 1 hour at 4°C. Bound fractions were analyzed by immunoblots using antibody against HA.
Figure 2: Specificity of M protein interaction with Rae1
The time course of Rae1 interaction with GST-M protein on glutathione beads was determined as shown in figure 3A and B. The amount of HA-Rae1 in the bound fraction at each time point was quantified and normalized to the amount bound at one hour. From these data, the time required to achieve 50% of maximal interaction of HA-Rae1 with M protein was approximately 30 minutes, and was virtually complete by 1 hour (figure 3A). Compared to HA-Rae1, endogenous Rae1 appeared to interact more slowly with M protein. The time course was extended and the data were normalized to the amount bound at 14 hours (figure 3B). The time required for 50% of maximal interaction of endogenous Rae1 was approximately 3 hours. The difference in the time course between HA-Rae1 and endogenous Rae1 is likely due to differences in their distribution among different types of complexes (addressed in figures 6 and 7).

To determine if the amount of GST-M protein on glutathione beads was limiting, and accounted for the large percentage of HA-Rae1 in the unbound fraction, the unbound HA-Rae1 obtained after incubation with GST-M protein was further incubated with a second aliquot of GST-M protein on glutathione beads. The bound and unbound fractions were analyzed by SDS-PAGE and immunoblotting (figure 3C). The bound fraction of the first incubation is designated B and the bound and unbound fractions of HA-Rae1 after second incubation are labeled 2B and 2U respectively. The amount of HA-Rae1 interacting with GST-M protein after the second incubation was substantially less than the amount of HA-Rae1 bound after the first incubation. Thus most of the HA-Rae1 remained unbound despite the excess of GST-M protein. Thus, the observation that 10% of HA-Rae1 interacting with GST-M protein was not due to limiting amounts of M protein.
It is also possible that the small percentage of HA-Rae1 in the bound fraction could be due to dissociation of the complex during the washing steps. In order to address the stability of the M protein–Rae1 complex, the bound fractions containing HA-Rae1 were washed with binding buffer and incubated for varying times in the binding buffer to allow the complex to dissociate. Following incubation, the bound and unbound fractions were analyzed by SDS-PAGE and immunoblotting (figure 3D). If the interaction between the proteins is unstable then the amount of HA-Rae1 in the bound fraction should decrease and most of HA-Rae1 should be in the unbound fraction over the course of the dissociation time. The amount of HA-Rae1 in the bound fractions did not decrease over the course of a 1 hour incubation and was similar to the amount of HA-Rae1 bound in the control. Thus the interaction between M protein and HA-Rae1 was stable over the time course of these experiments.
Figure 3: M protein interacts with a sub-population of Rae1. (A) Lysates from cells containing HA-Rae1 were incubated with wt GST-M protein on glutathione beads for the indicated times. Bound fractions were analyzed in duplicate for HA-Rae1 respectively. The graph represents quantification of the percentage of Rae1 bound normalized to the 1 hour sample. The curves were drawn to fit to exponential rise to max equation in Sigma Plot. The data shown are the means ± standard deviation of three separate experiments. (B) Lysates from HEK 293 cells were incubated with wt GST-M protein on glutathione beads for the indicated times. Bound fractions were analyzed in duplicate for Rae1 respectively. The graph represents quantification of the percentage of Rae1 bound normalized to the 14 hour sample. The curves were drawn to fit to exponential rise to max equation in Sigma Plot. The data shown are the means ± standard deviation of three separate experiments. (C) Unbound fraction of HA-Rae1 after the first round of incubation with GST-M protein was incubated with fresh GST-M protein on glutathione beads for an additional hour at 4°C. Bound and unbound fractions were probed for HA. B represents the bound fraction obtained after first round of incubation, while 2B and 2U represent the bound and unbound fractions obtained after second round of incubation respectively. (D) Bound fractions of HA-Rae1 after incubation with GST-M protein on glutathione beads were allowed to dissociate in binding buffer for the indicated times. Bound and unbound fractions were probed for HA.
Figure 3: Specificity of M protein interaction with Rae1
M protein interacts with major forms of post-translationally modified forms of Rae1: Another possible explanation for the observation that only a sub-population of Rae1 interacts with M protein is that Rae1 has different forms of post-translational modification and that M protein interacts with only one or a few forms of post-translationally modified Rae1. To determine whether Rae1 undergoes post-translational modification that changes its isoelectric point (such as phosphorylation, acetylation), endogenous Rae1 and HA-Rae1 were analyzed by 2D gel electrophoresis. Lysates from untransfected cells or lysates containing HA-Rae1 or were incubated with GST-M protein on glutathione beads. Bound and unbound fractions were separated in the first dimension by isoelectric focusing and in the second dimension by SDS-PAGE. The electrophoretically separated proteins were transferred and immunoblotted using antibodies against the Rae1 or HA tag. As shown in figure 4A, both endogenous Rae1 and HA-Rae1 were primarily detected as four spots which may be due to different degrees of phosphorylation or other modifications that affect the net charge of the protein. The four spots for HA-Rae1 have lower isoelectric points than endogenous Rae1 because of the acidic nature of the HA tag. The distribution of the four spots between bound and unbound fractions were similar for both endogenous Rae1 and HA-Rae1. Thus, there was no difference in the isoelectric points of most of the endogenous Rae1 or HA- Rae1 in the bound and unbound fractions. There was one spot of endogenous Rae1 with a high isoelectric point (>8.5) that was primarily in the unbound fraction but could also be seen in lower amounts in the bound fraction. At this point the modification that results in the spot with high isoelectric point is not clear. With the exception of this spot,
the results indicate that M protein interacts equally well with all post-translationally modified forms of Rae1 that have a difference in isoelectric point.

*Rae1 is not glycosylated:* Many Nups are glycosylated (110). To determine if Rae1 is glycosylated and if GST-M protein interacts with a glycosylated form of Rae1, cell lysates were incubated with WGA beads, which have affinity for N-acetyl glucosaminyi residues commonly found on nuclear proteins. Lysates containing HA-Rae1 or endogenous Rae1 were incubated on WGA beads and the flow-through and eluate fractions were obtained. These fractions were incubated with GST-M protein on glutathione beads. Following incubations, the bound and unbound fractions were analyzed by SDS-PAGE and immunoblotting using antibody against Rae1 or the HA-tag. As shown in the figure 4B, GST-M protein interacted with Rae1 which was present only in the flow-through fractions and not in the eluate fractions. Thus, Rae1 is not glycosylated and GST-M protein interacts with non-glycosylated Rae1. Similar results were seen with HA-Rae1 indicating that HA-Rae1 is not glycosylated and that GST-M protein interacts with non-glycosylated form of HA-Rae1.
Figure 4: Analysis of post-translational modification of Rae1. (A) 2D gel electrophoresis of bound and unbound fractions of endogenous Rae1 (upper panel) or HA-Rae1 (lower panel) after incubation with wt GST-M protein on glutathione beads. The fractions were probed for Rae1 and HA. The arrows depict the isoelectric points of known standards subjected to the same conditions. (B) Flow and eluate fractions of cell lysates either containing endogenous or HA-Rae1 after incubation with wheat germ agglutinin beads (WGA) were incubated with wt GST-M protein on glutathione beads for 14 hours. Bound and unbound fractions were probed for Rae1 and HA.
Figure 4: Analysis of post-translational modification of Rae1
Specificity of interaction of GST-M protein with other Nups. In addition to Rae1, M protein also interacts with the nucleoporin Nup98 (38, 110). To determine if other Nups were competent to interact with M protein, cell lysates were incubated with either GST-M protein or GST alone on glutathione beads, and bound fractions were probed with mAb414 that recognizes several FG repeat-containing Nups (33, 52). M protein interacted specifically with Nups whose electrophoretic mobility is consistent with Nup62, Nup214 and Nup358 (figure 5A). Nup153 also interacted with GST-M protein, but this interaction was non-specific as Nup153 also bound to the negative control GST. To further confirm the specificity of the interaction of M protein with Nup62 and Nup98, lysates from HEK 293 cells were incubated with recombinant wt and mutant GST-M proteins bound to glutathione beads, and bound fractions (shown in duplicate) were analyzed by SDS-PAGE and immunoblotting using antibody against Nup62 and Nup98 (figure 5B). Wild type M protein interacted with Nup98 and Nup62 but the M protein mutants were not able to interact with these proteins. These experiments confirm the specificity of the interaction between M protein and Nup62, and are also in agreement with the published reports that wt M protein interacts with Rae1 (38) and Nup98 (38, 110). These experiments further correlated the interaction between M protein and these host proteins with the ability of recombinant viruses containing wt versus mutant M protein to inhibit host transcription and translation as well as mRNA export.
Figure 5: M protein interacts with other Nups. (A) Wild-type (wt) or mutant GST-M proteins or GST alone on glutathione beads were incubated for 1 hour with lysates from HEK 293 cells for 1 hour at 4°C. Bound fractions were probed with mAb414. (B) Cell lysates were incubated with wt or mutant GST-M proteins or GST alone on glutathione beads for 1 hour at 4°C. Bound fractions were probed for Nup98 and Nup62.
Figure 5: M protein interacts with other Nups
Interaction of M protein with Rae1 in multiple complexes. Rae1 interacts with multiple proteins involved in regulating mRNA transport (13, 15, 99), and in mitotic spindle (117) and checkpoint regulation (111). To determine whether M protein interacts with Rae1 in different complexes, size exclusion chromatography was used to first separate complexes containing Rae1 and Rae1 in these column fractions was tested for its ability to interact with M protein. In the experiment shown in figure 6A, cell lysates were chromatographed on a Superdex 200 column and fractions were analyzed using SDS-PAGE and immunoblotting with antibody against Rae1. Rae1 was present in fractions 10-13 and in fractions 16-18. These data indicate that Rae1 exists in multiple forms, as high molecular weight complexes (corresponding to fractions 10-13) and as low molecular weight complexes (corresponding to fractions 16-18). Approximately 30% of Rae1 was present in a low molecular weight form. The low molecular weight form of Rae1 eluted in later fractions than would be expected compared to the ovalbumin standard which has a similar monomeric molecular weight to Rae1. This is most likely due to Rae1 adsorbing non-specifically to the chromatography matrix.

To determine if both high and low molecular weight forms of Rae1 are competent to interact with M protein, the fractions containing Rae1 10-13 and 16-18 were tested for interaction with GST-M protein on glutathione beads. Shown in figure 6B are the bound fractions obtained after incubation with GST-M protein or GST alone. Rae1 in fractions 11-13 was competent to interact with GST-M protein. However, the amount of Rae1 in fraction 10 that interacted with GST-M protein was much less than that in fractions 11-13, indicating that there were at least two complexes in the high molecular weight fractions, a high molecular weight complex (corresponding to fraction 10), which was relatively
ineffective in interacting with M protein, and an intermediate molecular weight complex (corresponding to fractions 12-13), which did interact with M protein. Fraction 11 probably contained a mixture of the high and intermediate molecular weight complexes. Rae1 in fractions 16-18 was also competent to interact with GST-M protein, which was observed on longer exposures (unpublished results).

Cell lysates were subjected to rate zonal centrifugation using sucrose gradients to confirm the existence of the low molecular weight form of Rae1 and to estimate its size in the absence of adsorption to a column matrix. After centrifugation twenty fractions were collected and analyzed by SDS-PAGE and immunoblotting. Under the conditions of sedimentation, the high molecular weight forms of Rae1 would be found in later fractions. Shown in figure 6C are fractions 1-11 collected from the top of the gradient. Rae1 was present in fractions 7-8, which corresponds to an $s_{20,w}$ value of 5.0 ± 1S. This is the estimated $s_{20,w}$ of either a homo-dimer of Rae1 or a complex of Rae1 with another similarly sized component(s), depending on their shape and buoyant density. Fractions 4-8 containing Rae1 were tested for interaction with GST-M protein on glutathione beads (figure 6D). Rae1 in these fractions was competent to interact with GST-M protein, similar to the results from the gel filtration experiments.

To determine whether the high molecular weight fractions containing Rae1 include the Rae1-Nup98 protein complex and potentially complexes of other Nups which are implicated in mRNA transport, fractions from gel filtration of cell lysates were analyzed by immunoblotting for the presence of Nup98 and Nup62 using specific antibodies. Nup98 was present primarily in fractions 11-12 obtained from size exclusion chromatography and Nup62 was present in fractions 9-12 (figure 6E). The fractions were
also probed with antibody mAb414 that was used to determine the interaction of Nup214, and Nup358 with M protein. Nups whose electrophoretic mobility is consistent with Nup153, Nup214 and Nup358 were also detected in the high and intermediate molecular weight fractions 9-12 (not shown). These results indicate that fractions corresponding to the intermediate molecular weight complexes containing Rae1 that is competent to bind M protein (fractions 11 and 12 in figure 6B) also had Nups Nup98 and Nup62. However, Nup62 was also present in the high molecular weight fractions (9 and 10) containing Rae1 that did not interact efficiently with GST-M protein.
Figure 6: Analysis of complexes containing Rae1. (A) Cell lysates were chromatographed on Superdex 200. Fractions of equal volume were collected and probed for Rae1. Arrows represent the fractions where standards of the indicated molecular weight eluted under the same conditions. The graph represents quantification of % Rae1 in each fraction normalized to total Rae1 eluting in all fractions. Vo indicates the void volume. (B) Superdex 200 fractions from (A) were incubated with wt GST-M protein (M) or GST (G) on glutathione beads for 1 hour at 4°C. Bound fractions were probed for Rae1. (C) Cell lysates were subjected to sucrose gradient centrifugation. Fractions were collected from the top and probed for Rae1. Arrows represent fractions containing standards with the indicated s_{20,w} value subjected to the same conditions. (D) Sucrose gradient fractions from (C) were incubated with wt GST-M protein (M) or GST (G) on glutathione beads for 14 hours at 4°C. Bound fractions were probed for Rae1. (E) Cell lysates were incubated with wt or mutant GST-M proteins or GST alone on glutathione beads for 1 hour at 4°C. Bound fractions were probed for Nup98 and Nup62.
Figure 6: Analysis of complexes containing Rae1
Interaction of M protein with HA-Rae1 in multiple complexes: The distribution of overexpressed HA-Rae1 was analyzed by gel filtration to determine which complexes were formed by overexpressed Rae1. HA-Rae1 was present in fractions 10-12 and in fractions 16-18 (figure 7A). These data indicate that HA-Rae1 exists in multiple forms, as high molecular weight complexes (corresponding to fractions 10-11), intermediate (corresponding to fractions 11-13) and as low molecular weight complexes (corresponding to fractions 16-18) similar to endogenous Rae1. Quantification of six of such experiments indicated that approximately 80% of HA-Rae1 was in the low molecular weight complex. This distribution is in contrast to endogenous Rae1 which was present mostly in the high and intermediate molecular weight complexes (compare figures 6A and 7A).

The ability of the different molecular weight complex(es) of HA-Rae1 to interact with M protein was similar to that of the endogenous Rae1. The low molecular weight complex of HA-Rae1 in fractions 16-18 and the intermediate molecular weight complex in fractions 11-13 interacted with GST-M protein, while HA-Rae1 in fraction 10 interacted less effectively.

Also similar to endogenous Rae1, the low molecular weight complex of HA-Rae1 eluted in later fractions than expected based on its monomer molecular weight, likely due to HA-Rae1 adsorbing non-specifically to the chromatography matrix. When analyzed by rate zonal centrifugation using sucrose gradients, HA-Rae1 was present in fractions 3-7 (figure 7C), which corresponded to an $s_{20,w}$ value of $4.8 \pm 1S$, similar to the estimated $s_{20,w}$ of endogenous Rae1. HA-Rae1 in fractions 3-7 was competent to interact with GST-M protein on glutathione beads (Figure 7D). Based on these results, HA-Rae1 exists in
similar complexes as the endogenous Rae1 and the interaction of M protein with HA-Rae1 in the different complexes is similar to endogenous Rae1. However, the net distribution of the HA-Rae1 differs from the endogenous Rae1 with the excess HA-Rae1 primarily in the low molecular weight complex.
Figure 7: M protein interacts with HA-Rae1 present in different complexes. (A) Lysates from cells containing HA-Rae1 were chromatographed on Superdex 200. Fractions of equal volume were collected and probed for HA. Arrows represent the fractions where standards of the indicated molecular weight eluted under the same conditions. The graph represents quantification of % HA-Rae1 in each fraction normalized to total HA-Rae1 eluting in all fractions. Vo indicates the void volume. (B) Superdex 200 fractions from (A) were incubated with wt GST-M protein (M) or GST (G) on glutathione beads for 1 hour at 4ºC. Bound fractions were probed for HA. (C) Lysates from cells containing HA-Rae1 were subjected to sucrose gradient centrifugation. Fractions were collected from the top and probed for HA. Arrows represent fractions containing standards with the indicated $s_{20,w}$ value subjected to the same conditions. (D) Sucrose gradient fractions from (C) were incubated with wt GST-M protein (M) or GST (G) on glutathione beads for 1 hour at 4ºC. Bound fractions were probed for HA.
Figure 7: M protein interacts with HA-Rae1 present in different complexes
**Complexes containing Rae1 and Nup98 are altered during infection.** The ability of M protein to interact with complexes containing Rae1, Nup98 and Nup62 raises the question of whether the presence of M protein in VSV-infected cells alters the distribution of these complexes compared to mock-infected cells. The elution profile of Rae1, Nup98, and Nup62 was determined by size exclusion chromatography of lysates from cells infected with recombinant virus containing a wt M protein (rwt virus). As controls, lysates were analyzed from mock infected cells and cells infected with rM51R-M virus, which is defective in suppression of host gene expression (4, 105, 115, 122). As shown in figure 8A, both intermediate and low molecular weight complexes of Rae1 shift to higher molecular weight fractions in cells infected with rwt virus but not in cells infected with the rM51R-M virus. In the case of the intermediate molecular weight complexes, this can be most easily seen by the greater amount of Rae1 in fraction 10 and the lesser amounts in fractions 12 and 13. Similar changes occurred in complexes containing Nup98 with greater amounts of Nup98 in fractions 9 and 10 in lysates from rwt virus-infected cells (figure 8B). The Superdex 200 column used for these experiments has a high level of resolution and reproducibility, so that the shifts in the elution profiles by one or two fractions were very reproducible. In contrast to Rae1 and Nup98, there were no detectable changes in the complexes containing Nup62 in lysates from rwt virus-infected cells (figure 8C). These data are consistent with the results that Rae1, in both the low molecular weight complexes and intermediate molecular weight complexes with Nup98 is competent to interact with wt M protein, and this interaction shifts these complexes to higher molecular weights. In the case of Nup62, it is likely that most of the
Nup62 is in the high molecular weight complexes that cannot interact with M protein, so that any shift in a minor population would not be apparent.
Figure 8: Analysis of complexes containing Rae1, Nup98, and Nup62 during infection with VSV containing wt or mutant M protein. Lysates from mock, rM51R-M virus, and rwt virus-infected cells were chromatographed on Superdex 200. The collected fractions were probed for (A) Rae1, (B) Nup98, and (C) Nup62.
Figure 8: Analysis of complexes containing Rae1, Nup98, and Nup62 during infection with VSV containing wt or mutant M protein
Rae1 is required for the interaction of Nup98 and Nup62 with M protein.

Mutagenesis experiments have indicated that the interaction of Rae1 with M protein is independent of Nup98 binding (38). However, it has not been determined whether the presence of Rae1 or Nup98 is required for M protein interaction with Nup62, or other Nups. To address the interdependence of these proteins for the interaction with M protein, the expression of each protein was silenced and the ability of the proteins to interact with M protein was tested. As reported previously (15), silencing the expression of Rae1 was not lethal to the cells, and silencing the expression of Nup98 also had little if any effect on cell viability in the time period of these experiments (48-72 hours post-transfection). In the case of Nup62, whose expression has been reported to be important for cell viability (16), the duration of these experiments was shortened to 48 hours post-transfection so that silencing did not significantly affect cell viability.

In the experiments shown in figure 9A, HeLa cells were transfected with Rae1 siRNA or with a non-targeting (NT) siRNA. Cell extracts were prepared 48 hours post-transfection and were incubated with GST-M protein or GST alone on glutathione beads. Cell extracts and the bound fractions were analyzed by immunoblotting with antibodies against Rae1, Nup62, and Nup98. Silencing the expression of Rae1 using siRNA resulted in Rae1 expression levels less than 2% protein levels of those in NT siRNA controls. In lysates from Rae1 siRNA cells, the amount of Nup98 and Nup62 that interacted with M protein was considerably less than that in lysates from NT siRNA cells. Quantification of immunoblots from multiple experiments indicated that the amounts of Nup98 and Nup62 that interacted with M protein in lysates from Rae1 siRNA cells were <10% of those in lysates from NT siRNA cells. Analysis of cell lysates indicated that silencing the
expression of Rae1 did not affect the overall levels of expression of Nup98 and Nup62. These results demonstrate that Rae1 is required for the interaction of M protein with both Nup62 and Nup98.

Similar experiments were performed with cells transfected with Nup98 and Nup62 siRNAs. Silencing the expression of Nup98 resulted in approximately 10% of Nup98 protein levels compared to NT siRNA controls. In lysates from Nup98 siRNA cells, the amount of Rae1 that interacted with M protein was reduced slightly (figure 9B), but this could be attributed to a slightly lower level of expression of Rae1 in lysates from Nup98 siRNA cells (70±9% of NT siRNA cells, as determined by densitometry of 5 separate experiments). The amount of Nup62 that interacted with M protein in lysates from Nup98 siRNA cells was reduced dramatically compared to lysates from NT siRNA cells (17±9% of NT siRNA cells). However, this could not be accounted for by an effect on the expression of Nup62 in Nup98 siRNA cells, which was 93% of that in NT siRNA cells, indicating that Nup98 is required for the interaction between Nup62 and M protein.

In lysates from Nup62 siRNA cells, the silencing of Nup62 expression was not as effective as the silencing of Rae1 and Nup98 by their respective siRNAs (31% of NT siRNA cells). The amount of Nup98 in lysates from Nup62 siRNA cells that interacted with M protein was reduced (32% of that of NT siRNA cells), and this could be attributed primarily to the reduced amount of Nup98 expressed in lysates from these cells (58% compared to NT siRNA cells). Silencing the expression of Nup62 did not affect the amount of Rae1 that interacted with M protein even though the expression of Rae1 in these cells was slightly less (78%) compared to the amount expressed in lysates from cells transfected with NT siRNA. These results suggest that Nup62 is not required for
Nup98 or Rae1 to interact with M protein, although it is also possible that residual Nup62 expression in Nup62 siRNA cells could be involved in the interaction.
Figure 9: Effects of silencing the expression of Rae1, Nup98, and Nup62 on interaction with M protein. (A) Lysates from Rae1 siRNA cells or from NT siRNA cells were incubated with wt GST-M protein (M) or GST (G) for 2 hours at 4 C. Bound fractions were probed for Nup62, Nup98 and Rae1. On the right are immunoblots of lysates from Rae1 siRNA cells or NT siRNA cells probed for Nup62, Nup98, Rae1, and actin. (B) Lysates from Nup98 siRNA cells or NT siRNA cells were incubated with wt GST-M protein (M) or GST (G). Bound fractions and lysates (on the right) were probed for the indicated proteins. (C) Lysates from Nup62 siRNA cells or NT siRNA cells were incubated with wt GST-M protein (M) or GST (G). Bound fractions and lysates (on the right) were probed for the indicated proteins.
Figure 9: Effects of silencing the expression of Rae1, Nup98, and Nup62 on interaction with M protein.
Rael is required for VSV to inhibit host transcription. The effect of silencing Rael on VSV’s ability to inhibit host transcription, mRNA transport, and host mRNA translation was tested. The inhibition of host transcription was quantified by the incorporation of [\(^3\)H] uridine into host RNA. This approach measures RNA synthesis by all three host RNA polymerases, with RNA polymerase I making the largest contribution. RNA polymerases I, II, and III have similar sensitivities to the inhibitory activity of M protein (2, 3, 112, 113). HeLa cells were transfected with siRNA against Rael or with NT siRNA. At 72 hours post-transfection, cells were mock infected or infected with rwt virus in the presence or absence of actinomycin D, an inhibitor of host transcription, which does not affect transcription by the viral RNA-dependent RNA polymerase. At 6 hours postinfection, cells were pulse labeled with [\(^3\)H] uridine and cell lysates were precipitated with trichloroacetic acid to measure acid precipitable radioactivity. This experiment was done by Margie McKenzie in our lab. In cells infected with rwt virus, RNA synthesis in the absence of actinomycin D represents the synthesis of both host RNA and viral RNA. Synthesis in the presence of actinomycin D represents viral RNA synthesis. Thus host RNA synthesis was determined by subtracting the amount of host RNA synthesized in the presence of actinomycin D from the amount of RNA synthesized in the absence of actinomycin D. Both host RNA synthesis (actinomycin D sensitive) and viral RNA synthesis (actinomycin D resistant) were expressed as a percentage of total RNA synthesis in mock-infected controls, in order to normalize for differences in cell number between Rael siRNA cells and NT siRNA cells. This is because Rael siRNA cells divide slightly more slowly than NT siRNA cells during the time course of the experiment. In mock-infected cells, >99% of the RNA synthesis was actinomycin D-
sensitive, and there was no significant difference in total RNA synthesis between Rae1 siRNA cells, NT siRNA cells, and non-transfected control cells that could not be accounted for by differences in cell number.
Figure 10: Effects of silencing the expression of Rae1, Nup98, and Nup62 on the rate of host and viral transcription (A) Untransfected or cells transfected with Rae1 siRNA or NT siRNA were either mock or infected with rwt virus at MOI= 30 for 6 hours in the presence of absence of actinomycin D (ActD). Cells were labeled with $[^3]H$uridine for 30 minutes. Cells were lysed and RNA was precipitated using trichloroacetic acid and acid precipitable counts were measured. The graph represents host (ActD sensitive) and viral (ActD insensitive) RNA synthesis expressed as a percentage of mock infected cells. The data shown are means ± standard deviation from three independent experiments. Similar experiments were done to determine effects of silencing the expression of (B) Nup98 and (C) Nup62 on the rate of host and viral transcription.
Figure 10: Effects of silencing the expression of Rae1, Nup98, and Nup62 on the rate of host and viral transcription
As shown in figure 10A, host RNA synthesis in NT siRNA cells or non-transfected control cells infected with rwt virus was reduced to approximately 10% of that in mock-infected cells. However, host RNA synthesis in Rae1 siRNA cells infected with rwt virus continued at approximately 40% (p value = 0.003) of that in mock-infected cells. In contrast to host RNA transcription, viral RNA transcription was similar in all three cell types. These results indicate that cells transfected with siRNA against Rae1 are more resistant to host transcription inhibition by VSV and that the expression of Rae1 is important for the ability of VSV to inhibit host transcription. Similar experiments tested the effects of silencing Nup98 and Nup62 on the ability of VSV to inhibit host transcription and in contrast to Rae1, silencing the expression of these Nups had little if any effect on host transcription inhibition by VSV (figure 10B, and 10C respectively). In the experiments pertaining to Nup62, the synthesis of viral RNA in both Nup62 siRNA and in NT siRNA cells were lower compared to cells that were not transfected. These differences could not be accounted for by differences in cell number among the different cell types. Despite the low level of viral RNA synthesis, it does not affect synthesis of VSV proteins and VSV’s ability to inhibit host translation (see Figure 14).

*Silencing Rae1 expression has little effect on nuclear accumulation of actin mRNA.* Rae1’s role as an important player in the mRNA transport is debated (5, 15, 104). To determine the effect of silencing Rae1 expression on the accumulation of host mRNA in the nucleus, the amount of actin mRNA transcripts in the nucleus was measured by real time RT-PCR in cells transfected with Rae1 siRNA or with NT siRNA. At 72 hours post-transfection, cells were mock infected or infected with rwt virus. At 6 hours postinfection, cells were lysed and separated into nuclear and cytoplasmic fractions, and
RNA was isolated from each fraction. The nuclear fractions were largely free of cytoplasmic contamination, since 28S and 18S rRNAs were undetectable by gel electrophoresis and ethidium bromide staining. As a control to monitor the efficiency of RNA isolation in the nuclear and cytoplasmic fractions, a known quantity of *E.coli* mRNA was added to the cytoplasmic and the nuclear fractions before harvesting RNA. The amount of actin mRNA measured by real time RT-PCR in the cytoplasm and the nucleus was normalized to the amount of *E.coli* uidA transcript measured in the same samples and the percentage of actin mRNA in the nucleus was calculated (figure 11). The amounts of nuclear actin mRNA in mock-infected Rae1 siRNA cells and in NT siRNA cells were similar. This result is consistent with the idea that Rae1 is not required for transport of actin mRNA. There was no significant difference in the levels of actin mRNA in the nuclei of rwt virus-infected cells compared to mock-infected cells for either Rae1 siRNA or NT siRNA cells. This result is consistent with earlier data indicating that there is little if any net accumulation of host RNA in the nuclei of VSV-infected cells, due to the inhibition of host transcription (112, 113). Collectively these results indicate that silencing Rae1 expression has little if any effect on the nuclear accumulation of actin mRNA.
Figure 11. Effects of silencing the expression of Rae1 on nuclear-cytoplasmic transport.

(A) Cells were transfected with Rae1 siRNA or NT siRNA and were either mock infected or infected with rwt virus for 6 hours. Cells were lysed and separated into nuclear and cytoplasmic fractions. *E. coli* mRNA was added to each fraction to quantify recovery of RNA. Actin transcripts were measured in each fraction using real time RT PCR and normalized to the amount of *E. coli* uidA transcripts measured. The graph represents the amount of nuclear actin expressed as a percentage of total actin in NT siRNA cells (hatched bars) or Rae1 siRNA cells (black bars). The data shown are means ± standard deviation from three independent experiments.
Figure 11: Effects of silencing the expression of Rae1 on nuclear-cytoplasmic transport.
Silencing expression of Rae1 and other Nups does not affect VSV’s ability to inhibit host translation. VSV inhibits translation of host mRNA (4, 27, 115). To determine the effects of silencing the expression of Rae1 on the ability of VSV to inhibit host translation, cells were transfected with Rae1 siRNA or with NT siRNA for 72 hours, and were mock infected or infected with rwt virus for various times. Cells were pulse-labeled with $[^{35}\text{S}]$ methionine, and lysates were analyzed by SDS-PAGE and phosphorescence imaging. Figure 12A shows a phosphoimage that compares the inhibition of host protein synthesis in Rae1 siRNA cells versus NT siRNA cells that were mock infected or infected with rwt virus for 2, 4, and 6 hours. The ladder of bands in mock infected cells represents synthesis of host proteins. During infection with rwt virus, the synthesis of host proteins was inhibited in both Rae1 siRNA and NT siRNA cells, and was almost completely inhibited at 6 hours postinfection. In rwt virus-infected cells, the synthesis of viral proteins L, G, N, P and M can be observed at 2 hours postinfection and by 6 hours the synthesis has reached its maximum (27). Host protein synthesis was quantified from regions of the gel devoid of viral proteins in three separate experiments and is shown in figure 12B expressed as a percentage of that in mock-infected cells. Viral protein synthesis was quantified from radioactivity in all the viral bands and is expressed as a percent of synthesis at 6 hours postinfection (figure 12C). There was no significant difference between Rae1 siRNA cells and NT siRNA cells in either the inhibition of host protein synthesis or the levels of viral protein synthesis. These data indicate that the expression of Rae1 does not affect the ability of VSV to inhibit host translation.
Figure 12. Effects of silencing the expression of Rae1 on host and viral protein synthesis. (A) HeLa cells were transfected with Rae1 siRNA or with NT siRNA. 72 hours post transfections, cells were mock infected or infected with rwt virus for the indicated times and labeled with $[^{35}\text{S}]$ methionine for 10 min. Lysates from Rae1 siRNA cells or NT siRNA cells were analyzed by SDS-PAGE and phosphoimaging. Shown is a phosphoimage with the viral proteins indicated on the right. (B) The graph represents quantification of host proteins synthesis at each time point post infection in NT siRNA cells (open circles) or Rae1 siRNA cells (closed circles) and expressed as a percentage of mock infected cells. The host proteins were quantified by measuring the amount of radioactivity present in between each viral band. The data shown are the means ± standard deviation of three separate experiments. (C) The graph represents quantification of viral protein synthesis at each time following infection in NT siRNA cells (open circles) or Rae1 siRNA cells (closed circles) and expressed as a percentage of synthesis at six hours post infection. Viral proteins were quantified by measuring the amount of radioactivity in all the viral proteins. The data shown are the means ± standard deviation of three separate experiments.
Figure 12: Effects of silencing the expression of Rae1 on host and viral protein synthesis.
Similarly, the rate of translation inhibition was measured in cells transfected with Nup98 siRNA, and similar to the results in figure 12, silencing expression of Nup98 did not affect the ability of VSV to inhibit host protein synthesis and affect viral protein synthesis (figure 13). For cells transfected with Nup62 siRNA, there appears to be a slight delay in the viral protein synthesis at 2 hours post infection (figure 14) compared to cells transfected with NT siRNA. However this difference in the delay of viral protein synthesis dissipates at subsequent hours post infection. Silencing the expression of Nup62 does not affect inhibition of host protein synthesis by VSV or affect viral protein levels.
Figure 13. Effects of silencing the expression of Nup98 on host and viral protein synthesis. (A) HeLa cells were transfected with Nup98 siRNA or with NT siRNA. 72 hours post transfections, cells were mock infected or infected with rwt virus for the indicated times and labeled with $[^{35}S]$ methionine for 10 min. Lysates from Nup98 siRNA cells or NT siRNA cells were analyzed by SDS-PAGE and phosphoimaging. Shown is a phosphoimage with the viral proteins indicated on the right. (B) The graph represents quantification of host proteins synthesis at each time point post infection in NT siRNA cells (open circles) or Nup98 siRNA cells (closed circles) and expressed as a percentage of mock infected cells. The host proteins were quantified by measuring the amount of radioactivity present in between each viral band. The data shown are the means ± standard deviation of three separate experiments. (C) The graph represents quantification of viral protein synthesis at each time following infection in NT siRNA cells (open circles) or Nup98 siRNA cells (closed circles) and expressed as a percentage of synthesis at six hours post infection. Viral proteins were quantified by measuring the amount of radioactivity in all the viral proteins. The data shown are the means ± standard deviation of three separate experiments.
Figure 13: Effects of silencing the expression of Nup98 on host and viral protein synthesis.
Figure 14. Effects of silencing the expression of Nup62 on host and viral protein synthesis. (A) HeLa cells were transfected with Nup62 siRNA or with NT siRNA. 72 hours post transfections, cells were mock infected or infected with rwt virus for the indicated times and labeled with $[^{35}S]$ methionine for 10 min. Lysates from Nup62 siRNA cells or NT siRNA cells were analyzed by SDS-PAGE and phosphoimaging. Shown is a phosphoimage with the viral proteins indicated on the right. (B) The graph represents quantification of host proteins synthesis at each time point post infection in NT siRNA cells (open circles) or Nup62 siRNA cells (closed circles) and expressed as a percentage of mock infected cells. The host proteins were quantified by measuring the amount of radioactivity present in between each viral band. The data shown are the means ± standard deviation of three separate experiments. (C) The graph represents quantification of viral protein synthesis at each time following infection in NT siRNA cells (open circles) or Nup62 siRNA cells (closed circles) and expressed as a percentage of synthesis at six hours post infection. Viral proteins were quantified by measuring the amount of radioactivity in all the viral proteins. The data shown are the means ± standard deviation of three separate experiments.
Figure 14: Effects of silencing the expression of Nup62 on host and viral protein synthesis.
M protein interacts with Rae1, Nup62, and Nup98 present in the nucleus: The method of preparation of cell lysates described by Faria et al (38) and used for all of the experiments above preferentially extracts cytoplasmic proteins. To determine if Rae1, Nup98 and Nup62 present in the nucleus was competent to interact with M protein, nuclei were harvested from spinner HeLa cells and fractionated as described in (80). These fractions were probed for the presence of Rae1, Nup98, and Nup62 and the ability of M protein to interact with these proteins was tested. The isolation procedure (shown in figure 15) fractionates nuclear envelope and the associated proteins by utilizing reagents that affect permeability of the nuclear envelope. After each treatment, the fractions were centrifuged to pellet the nuclear envelope and lamina fractions. First the nuclei were treated with DNaseI/RNase followed by heparin, a negatively charged polyanion, to solubilize chromatin. The supernatant fractions after treatment with DNaseI and RNaseI and heparin represents chromatin and any proteins interacting with chromatin and separates these from the pellet fraction, which largely represents crude nuclear envelope. This was followed by treatment with buffer containing the detergent Triton X-100 and a small amount of SDS which solubilized the membrane and left the isolated NPC and lamina in the pellet. Finally, the nups were solubilized and separated from the lamina using the detergent Empigen.
Figure 15. A flow chart depicting the procedure for isolating Nups. Nuclei were purified from spinner HeLa cells. The 1X10^7 nuclei were treated with DNaseI/RNase and centrifuged at 4000 rpm for 10 minutes. The supernatant (S) fraction and the pellet (P) fractions were separated. The pellet fraction was treated with heparin to solubilize chromatin from the nuclear envelope. The resulting pellet fraction was treated with SDS and Triton X 100 (TX100) to solubilize the membrane from the nuclear pore complex and lamina found in the pellet. The pellet fraction was treated with Empigen to solubilize the Nups from the lamina fraction.
Figure 15: A flow chart depicting the procedure for isolating Nups.
The pellet and supernatant fractions obtained after each treatment were analyzed by SDS-PAGE and immunoblotting with antibodies against Rae1, Nup98 and Nup62. As controls, to monitor the quality of the purification at each step, the fractions were also probed for lamin A and the transcription factor TBP. Shown in figure 16A are cytoplasmic fractions and the supernatant and pellet fraction after each treatment of the nuclei. Rae1 was present in the cytoplasm, and was also present in the pellet fractions after each treatment, indicating that it is associated with the nuclear envelope. However, Rae1 was also detected in the supernatant fraction after treatment with DNaseI/RNase that contains proteins associated with chromatin. Similarly, Rae1 was also present in supernatant and pellet fraction after Empigen treatment indicating that it is present in fraction containing the nuclear pore complex and in fraction containing lamina respectively. Similar results were also observed with Nup98 and Nup62 indicating that these proteins are associated with fractions containing chromatin bound proteins, nuclear pore complex and lamina. TBP was detected largely in the supernatant fractions after DNaseI/RNase treatment confirming that it is associated with chromatin. TBP was not detected in any other fractions indicating that the fractionation procedure isolated intact chromatin and nuclear envelope. Lamin A was largely detected in the pellet fractions after each treatment further indicating the fractionation procedure isolated purified lamina.
Figure 16: Localization of Rae1, Nup98, and Nup62 within the nucleus. Cytoplasmic and nuclear fractions were isolated from spinner HeLa cells. The nuclear fractions were treated with DNaseI/RNaseI, followed by Heparin, SDS and TritonX100 (TX100), and Empigen. The supernatant and pellet fractions after each treatment were analyzed by SDS-PAGE and probed for Rae1, Nup98, and Nup62. The fractions were also probed for TBP and lamin as controls for proper fractionation.
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Figure 16: Localization of Rae1, Nup98, and Nup62 within the nucleus.
To determine if these proteins found in different sub-compartments within the nucleus were competent to interact with M protein, supernatant fractions obtained after each treatment were incubated on GST-M protein or GST for 14 hours. The bound fractions were analyzed by SDS-PAGE and immunoblotting with antibodies against Rae1, Nup98, and Nup62. Rae1 present in the cytoplasm interacted with M protein, further confirming our results. Rae1 present in fractions associated the chromatin, nuclear envelope and nuclear pore complex was also competent to interact with M protein (figure 17A). Similar results were obtained for Nup98. In the case of Nup62, no interaction with M protein was detected with Nup62 present in the cytoplasm. Similarly no interaction of Nup62 present in the supernatant fraction obtained after DNaseI/RNaseI treatment was detected even though Nup62 was clearly detected in these fractions. However, Nup62 present in fractions comprising nuclear envelope and nuclear pore complexes was competent to interact with M protein. These results indicate that M protein can interact with Rae1, Nup98 and Nup62 present within the nucleus.
Figure 17. M protein interacts with Rae1, Nup98, and Nup62 present in the different sub-compartments within the nucleus. Cytoplasmic and nuclear fractions were isolated from spinner HeLa cells. The nuclear fractions were treated with the indicated reagents. 250 μl of the supernatant after each treatment were incubated on either GST-M (M) or GST (G) for 14 hours at 4°C. The bound fractions were analyzed by SDS-PAGE and probed for Rae1, Nup98, and Nup62.
Figure 17: M protein interacts with Rae1, Nup98, and Nup62 present in the different sub-compartments within the nucleus.
DISCUSSION

One of the remarkable aspects of VSV pathogenesis is the ability of M protein, a relatively small protein of 26kDa, to induce its pleiotropic effects in infected cells. M protein plays multiple roles in both virus assembly and in the inhibition of host gene expression (77). M protein inhibits transcription by all three host RNA polymerases (2, 4, 11, 122), inhibits nucleo-cytoplasmic RNA transport (58, 92, 110), and inhibits translation of host mRNA (4, 27, 115). One of the mechanisms by which the M protein may serve these diverse functions is through interaction with host proteins, such as Rae1, that also serve multiple functions in a cell. My hypothesis was that the M protein-Rae1 complex serves as a scaffold for M protein to interact with other cellular proteins to suppress host gene expression. The data presented here support this idea by showing that M protein interacts with multiple complexes that contain Rae1. These include low molecular weight complexes and intermediate molecular weight complexes that include the previously described complex of Rae1 with Nup98 (13, 38, 62, 79, 99). My data indicate that the high and intermediate molecular weight complexes also contain complexes of Rae1 with other Nups, including Nup62, Nup214, and Nup358. However, in contrast to the low and intermediate molecular weight complexes, the high molecular weight complexes containing Rae1 do not interact effectively with M protein.

Based on my data from size exclusion chromatography, it was demonstrated that complexes containing Nup98 and Rae1 got larger after infection with virus containing wt M protein (figure 7). At this point, the molecular mechanisms governing the alterations of complexes containing Nup98, and Rae1 during infection with VSV were not pursued further. However, several hypotheses, (that are not mutually exclusive) to address how
the complexes may be modified could be proposed. For instance, it is possible that during VSV infection, the M protein-Rae1 complex by interacting with Nup98 would induce a response within the cell such that Nup98 could undergo post-translation modifications. Nup98 has FG repeat sequences (98, 110) that are known to be intrinsically disordered (34). It is possible that during VSV infection, Nup98 by interacting with its binding partner Rae1, in complex with M protein, would undergo a conformation change making interaction with other proteins or the formation of oligomers more favorable. This would allow Nup98 to interact with other cellular proteins and form larger complexes or these complexes might result from Nup98 oligomerizing with itself. It is possible similar changes would occur without any post-translation modification of Nup98. In either case, these alterations would serve as inhibitory complexes during VSV infection for suppression of host nuclear–cytoplasmic transport. These proposed explanations for addressing the alteration of Nup98 complexes during VSV infection may also account for the changes associated with Rae1 containing complexes during VSV infection. M protein, by interacting with Rae1, would alter interactions of proteins associated with Rae1 and perturb their function. These changes in the complexes containing Rae1 would serve as inhibitory complexes during VSV to primarily induce suppression of host transcription as well as transport.

Besides M protein interacting with Rae1, another mechanism by which M protein may suppress host gene expression is by inducing post-translation modification of host proteins. To date, it is largely unknown if any post-translation modifications of nups are induced in VSV-infected cells. Although studies comparing post-translation modifications of Rae1, Nup98, and Nup62 in VSV-infected and mock infected cells were
not pursued, it is quite possible that that VSV infection induces post-translation modification(s) of these proteins.

From my binding assays, Nup62 was also shown to interact with M protein. This protein was originally identified to be one of the major proteins isolated from rat NPC (32) and is present both at the cytoplasmic and nuclear side of the NPC (32, 102). Nup62 is glycosylated (32) and has the N terminal domain containing five FG repeats (102). Nup62 along with other Nups namely, Nup62, Nup58, Nup54, and Nup45 forms a very stable complex, called Nup62 complex. Depleting of Nup62, Nup54, and Nup58 from Nup62 complex using antibodies directed against them in *Xenopus* oocytes, resulted in nuclei with impaired import capabilities (40). This demonstrates a role for Nup62 in regulating import. Nup62, has been shown to interact *in vitro* with importin alpha, an import receptor that interacts with importin beta to form a heterodimer complex that imports cargo containing classical NLS (82). Moreover, Nup62 can be crosslinked to mRNA (31), indicating that Nup62 may play a role in regulating export as well. The C terminal domain of Nup62 interacts with Sp1(54), a transcriptional factor which suggests that this Nup may participate in regulating transcription. The importance of Nup62 for cellular gene expression was recently demonstrated by silencing the expression of Nup62, which affected cell viability as reported by Boettcher *et al* (16), and this was confirmed with my observation of Nup62 siRNA cells.

The most unexpected result in our experiments was that only a sub-population of cellular Rae1 was competent to interact with M protein. I was not able to determine the properties that distinguish the Rae1 that is competent to interact with M protein from the remaining cellular Rae1, but I was able to rule out the most common forms of post-
translational modifications. I also considered the possibilities that M protein-Rae1 complex was unstable, with most of Rae1 disassociating, or that the amount of M protein used was not in excess. However, based on results from my experiments, I was able to rule out each of these possibilities. It is also possible that the process of preparation of cell lysates may inherently account for only a small fraction of Rae1 interacting with M protein.

The results from sedimentation velocity experiments indicate that Rae1 in the low molecular weight fractions exists as an oligomer. Thus, I also considered the possibility that the interaction of Rae1 with other protein(s) might interfere with association of M protein with Rae1. These proteins may compete with the M protein for the same binding sites on Rae1, thus preventing the association of M protein with Rae1. In the case of the high molecular weight complexes, Rae1 in fraction 10 interacted poorly with M protein compared to Rae1 in other fractions (figure 5). However, the intermediate molecular weight fractions 11-12 and low molecular weight fractions 16-18 also had only a small percentage of Rae1 that interacted with M protein (estimated from the amount remaining in unbound fractions, data not shown) even though Rae1 present in these fractions has a better ability to interact with M protein. Thus it is possible that fraction 10 has a large proportion of protein(s) that may interfere with interaction of Rae1 with M protein than in the intermediate molecular weight fractions 11-12 and the low molecular weight fractions 16-18.

Another possible explanation for the sub-population of Rae1 that is competent to interact with M protein is that Rae1 exists in multiple conformations, and M protein preferentially interacts with one of the conformations. There are several observations that
support this hypothesis. Rae1 is a member of the family of tryptophan-aspartic acid (WD) repeat proteins (9, 69, 99) that typically have a conserved core of amino acids which are bordered by glycine-histidine dipeptide on the N terminus and (WD) dipeptide sequence on the C terminus (46, 70, 84). WD repeat proteins, like Rae1 (9, 69, 99), are primarily found in eukaryotes, and functional forms of these proteins have been difficult to express in *E. coli* (46, 84). For example, Rae1 expressed in bacteria is sensitive to proteolytic degradation, indicating that Rae1 is largely misfolded (99) and does not assume its native protease resistant form (46, 99). Moreover, Rae1 expressed in *E. coli* is unable to interact with Nup98 (99). In contrast to bacterial expression, *in vitro* translation of Rae1 using rabbit reticulocyte lysates produces Rae1 in its protease-resistant conformation (99). HA-Rae1 transcribed and translated in rabbit reticulocyte lysates by Faria *et al*, assumed a proper fold that allowed it to interact with M protein (38). However, in my hands, HA-Rae1 translated in rabbit reticulocyte lysates did not interact with M protein (unpublished results). It is possible that the conditions of *in vitro* translation used by Faria *et al* led to proper folding of Rae1 needed for interaction with M protein. It is quite likely that possibilities discussed above could account for a sub-population of Rae1 interacting with M protein, however these were not pursued further.

M protein does not inhibit host gene expression by simply interfering with Rae1 function, since Rae1 is not essential for host gene expression (5, 104). This raises the question of how interaction of M protein with a sub-population of a protein that is not essential for gene expression can have a global effect on host gene expression at multiple levels. To address this paradox, I have proposed a model where M protein interacts with Rae1 in complexes that serve as a platform for M protein to interact with other essential
host proteins, thereby interfering with their function (figure 1). WD repeat proteins, such as Rae1 (9, 69, 99) are known to adopt beta propeller folds (46, 70, 84) that have large surface areas suitable for multiple protein interactions. Human Rae1, which has seven WD repeats in its sequence (9, 99) has been shown to form seven bladed β propellers with extensive surface loops, provides large surface areas (100). These surfaces would serve as large interacting regions for M protein to disrupt function of other proteins associated with Rae1.

As an alternative to the “platform hypothesis”, M protein could also suppress host gene expression by inhibition the function of Rae1 itself. This hypothesis suggests that inhibitory effects of M protein are due to its ability to form a complex with Rae1, thereby perturbing Rae1 function. This hypothesis is supported by experiments published in Faria et al (38) in which M-protein-mediated inhibition of host gene expression was reversed upon overexpression of Rae1 with an epitope tag (HA-Rae1) and this reversal was due to the recovery in the export of mRNA transport (38). This was demonstrated using two different assays.

Using luciferase reporter gene assay as a measure of gene expression, cells cotransfected with a plasmids encoding for M protein and HA-Rae1, had a higher amount of luciferase activity than cells transfected with plasmid encoding for M protein alone. To measure mRNA transport in VSV–infected and in cells transfected with plasmid encoding for M protein, in situ hybridization with oligo-dT was used. In the absence of any exogenous Rae1, in both VSV-infected and cells transfected with plasmid encoding for M protein, poly A+ RNA was largely detected in the nucleus, demonstrating a block in mRNA transport. However, upon overexpression of Rae1 there was an apparent
increase in polyA+ RNA in the cytoplasm demonstrating that overexpressing Rae1 reversed M protein mediated suppression of host gene expression, largely by reversing the block in mRNA transport. The alternative hypothesis based on M protein inhibition of Rae1 function (which addresses M protein’s suppression of host gene expression), predicts that Rae1 siRNA cells should be more sensitive than control cells to the effects of M protein. This is because of the lower level of Rae1 expression. In contrast the “platform hypothesis” predicts that Rae1 siRNA cells should be less sensitive to the effects of M protein than controls, since there is less Rae1 to mediate the interaction of M protein with other cellular targets. The results from the experiments presented in this thesis support the “platform hypothesis” by showing that Rae1 siRNA cells are relatively resistant to the inhibitory effects of M protein.

A major focus of this thesis was to determine the pertinence of the expression of Rae1 to VSV’s ability to inhibit host gene at the three steps of host gene regulation. The assumption by Faria et al (38) that the M protein-Rae1 complex suppresses host gene expression primarily by interfering with nuclear-cytoplasmic transport, was not supported by my data. Instead, my data indicates that M protein by interacting with Rae1 mainly suppresses host transcription (figure 9A). This data not only supports M protein’s role as a potent suppressor of transcription but also suggests a plausible mechanism by which M protein may inhibit host transcription. For instance, experiments by Weck and Wagner (112) have shown that transcription activity of all three host RNA polymerases was sensitive to suppression by VSV. Moreover, M protein in the absence of any viral components was able to inhibit host transcription by all three host RNA polymerases (2).
The ability of M protein to inhibit host transcription by all three polymerases suggests that a factor that interacts with all three polymerases is targeted by the M protein.

VSV inhibits the activity of polymerase I, II and III, however the transcription activity of polymerase II was the most sensitive to the effects of VSV infection (2, 112) and this has been attributed to the potent transcriptional suppression activity of the M protein (2, 11). The polymerase II transcriptional machinery is responsible for transcribing genes encoding for mRNA and small nuclear RNAs (86). Transcription initiation by polymerase II requires the formation of preinitiation complex, which is composed of transcription factors and polymerase II. TFIID is the nucleation factor that begins the formation of preinitiation complex by binding to the DNA promotor element through its TBP subunit. Subsequently polymerase II and other transcription factors are recruited to the pre-initiation site to initiate transcription [reviewed in refs (20, 86, 123)].

However no cellular protein has been identified with which M protein interacts directly to inactivate the transcriptional apparatus. TBP, a subunit in the basal transcription factor TFIID was inactivated in VSV-infected cells (122). However the exact mechanism of inactivation of TBP is largely unknown. This is because M protein does not interact with TFIID or affect the phosphorylation status of TBP (121). Thus, M protein inhibits host transcription by indirectly inactivating the activity of TFIID and is possibly mediated by M protein’s interaction with other host factors, possibly by interacting with Rae1. It is therefore conceivable that M protein-Rae1 complex may inactivate the TBP subunit of TFIID through direct or indirect mechanisms. The mechanism of modulating the host transcriptional apparatus by VSV is largely different from other RNA viruses such as poliovirus. During poliovirus infection, the transcriptional factor TBP (25), and
transcriptional activators such as Oct-1 (119) are cleaved by the viral encoded protease 3C to inactive host polymerase II mediated transcription.

My data strongly suggests that M protein-Rae1 complex inhibits host gene expression primarily by inhibiting host transcription (figure 6). Both Rae1 and Nup98 are present in the nucleoplasm, as well as the nuclear envelope and cytoplasm (9, 50, 51, 69, 99, 104, 110). Furthermore, the localization of Rae1 at the nuclear envelope is affected by inhibitors of RNA polymerase I and II activity (99). This suggests that the localization of Rae1 is dependent on ongoing transcription. Similarly, the mobility of Nup98 in the nucleus is also dependent on ongoing transcription (50). Furthermore, Rae1 can be cross-linked to poly A-containing mRNA (69), and Rae1 interacts with other mRNA binding proteins (15) which includes Nup98, suggesting that Rae1 and Nup98 may be a part of larger ribonucleoprotein complex. Recent evidence suggests that the steps involved in gene transcription, nascent mRNA processing, and transport may be coupled (30, 109). Rae1 and Nup98 may form mRNP complexes which include other proteins during transcription and transport of nascent mRNA. M protein, by interacting with Rae1 and Nup98, would target these complexes to inhibit both transcription and transport of nascent mRNA.

Lysates from siRae1 RNA cells were separated using size exclusion chromatography to determine which complexes containing Rae1 were silenced. Preliminary results indicate that the intermediate and the low molecular weight complexes containing Rae1 were silenced (unpublished data). This suggests that M protein interacts with Rae1 present in these molecular weight complexes to form inhibitory complexes that suppress host transcription. These results combined with the results from transcription
measured in VSV infected Rae1 siRNA cells provide evidence in support of the platform hypothesis.

Experiments were conducted in our laboratory by Elizabeth Kneller, PhD. in order to reproduce the results by Faria et al (38), measuring the effects of overexpressing Rae1 on host gene expression in the presence of M protein. In these experiments, a luciferase reporter assay was used to measure gene expression in transfected cells. Results indicated that cells overexpressing Rae1 were resistant to the inhibition of host gene expression by M protein, however, Rae1 was not able to completely attenuate the inhibitory effects of M protein (Drs. Elizabeth Kneller and Douglas Lyles, unpublished results).

Similarly, luciferase activity was measured in Rae1 siRNA cells transfected with M mRNA. Cells silenced for the expression of Rae1 are resistant to inhibition of gene expression by M protein, however, it does not completely reverse the inhibitory effects of M protein (Drs. Elizabeth Kneller and Douglas Lyles, unpublished results). These results are consistent with the results from VSV-infected cells, demonstrating that silencing Rae1 expression makes cells more resistant to the suppression of host gene expression by VSV. The observations that both silencing of Rae1 expression and as well as overexpression of Rae1 lead to resistance of M protein suggest that there maybe an optimal level of expression of Rae1 required for both, M protein to suppress host gene expression and for Rae1 to attenuate the inhibitory effects of M protein. Therefore, the two models of M protein-Rae1 complex inhibiting host gene expression are in apparent disagreement with each other with data supporting each of two hypotheses. It is important
to point out that neither of results are conclusive, and either model could be refuted by additional experiments.

I also compared the properties of overexpressed HA-Rae1 that was competent to interact with GST-M protein to endogenous Rae1. Similar to endogenous Rae1, HA-Rae1 exhibited similar post-translationally modified forms that result in a difference in charge and was present in multiple complexes that were competent to bind to M protein. However, in contrast to endogenous Rae1, HA-Rae1 was present primarily in the low molecular weight complex and interacted more rapidly with GST-M protein. These results suggest that the low molecular weight complex of Rae1 may be the form that reverses the inhibition of host gene expression by M protein, possibly by competing for M protein interacting with higher molecular weight complexes of Rae1.

The effect of M protein on nuclear accumulation of RNA depends on the cell type and the target being analyzed. Nuclear accumulation of RNA resulting from the inhibition of transport is most obvious in cells in which M protein has little if any effect on transcription, such as Xenopus oocytes (58, 92, 110). In this system, M protein inhibited export of mRNA, 5S ribosomal RNA and export of small nuclear ribonucleoprotein particles U1 and U2. However, M protein did not affect transport of t-RNA, demonstrating that not all nuclear-cytoplasmic transport is inhibited by M protein. For example, transport of tRNA (58, 110) and intron lariat bearing the constitutive transport element (110) are resistant to the inhibition. Similarly, even in mammalian cells, not all nuclear-cytoplasmic transport is inhibited by M protein. For example, export of complexes containing hnRNP-A1 and other hnRNPs were not inhibited during VSV infection but rather their export was enhanced (93).
In most mammalian cells, M protein inhibits host transcription and therefore there is relatively little net accumulation of host RNAs in the nucleus, because their synthesis as well as their transport is inhibited by M protein. This was originally demonstrated in the pulse-chase experiments of Weck and Wagner (112), and is confirmed by our analysis of the distribution of actin mRNA using real time RT-PCR (figure 10).

Experiments measuring mRNA transcripts using real time RT-PCR as an assay for transport were done by Stojdl et al. However, as opposed to actin mRNA transcripts, the distribution of IFN-β mRNA transcripts during VSV infection was monitored in their assays. In their analysis, there was an increase in the amount of IFN-β in the nucleus compared to the amount in cytoplasm in VSV infected cells (105). The authors attributed this increase in IFN-β in the nucleus to the ability of the virus to inhibit export. However, this conclusion is further complicated by the fact that this apparent increase in IFN-β transcript is due to the cells anti-viral response to VSV infection. Furthermore, no controls to monitor the efficiency of RNA extraction from the nucleus and the cytoplasm were used. Experiments using in situ hybridization with oligo-dT have shown an apparent accumulation of total mRNA in the nucleus of VSV-infected cells (38). However, this result has not been confirmed by an independent approach and may be subject to artifacts such as masking of poly A-containing mRNAs in the cytoplasm of VSV-infected cells as a result of their accumulation in nontranslating ribonucleoprotein particles (116).

Therefore, it maybe that M protein is able to inhibit host gene expression by inhibiting transcription and nuclear-cytoplasmic transport. However, the dominant mode of inhibition may vary such that in systems in which M protein has little effect on
transcription inhibition (such as *Xenopus* oocytes), it primarily inhibits host gene expression by suppressing host nuclear-cytoplasmic transport.

Silencing Rae1 expression inhibits export of hnRNP-A1 in VSV-infected cells (93), but has little if any effect on nuclear accumulation of actin mRNA (figure 10). The level of actin mRNA in the nucleus reflects a balance of its transcription, transport, and turnover. Thus it is possible that silencing Rae1 expression may have an effect on mRNA transport in VSV-infected cells that is balanced by changes in transcription or turnover. All classes of RNAs such as mRNA, rRNA, tRNA that are synthesized in the nucleus are transported into the cytoplasm. These molecules that need to be transported are recognized by multiple adapter proteins and transport factors. The pathways and the associated proteins that regulate transport of RNA are different from those associated with transport of mRNA. Transport of mRNA is accomplished by the interactions of hnRNPs with nascent pre-mRNA transcripts. There are 20 known hnRNPs and the stoichiometry of hnRNPs associating with each pre-mRNA transcript differs. Apart from hnRNPs there are other export factors that also mediate transport. Moreover these mRNA-hnRNP protein complexes must exit via the nucleus via the NPC by interacting with Nups [reviewed in refs (82, 83)]. Therefore, it is conceivable that although there maybe some common factors needed for mRNA transport, there maybe certain factors unique to transporting certain mRNAs. It is possible that during VSV-infection, M protein may not significantly perturb the pathway(s) or any of the protein(s) associated with export of actin.

Other RNA viruses also interfere with nuclear cytoplasmic transport although, through fundamentally different mechanisms. For instance, using an *in vitro* import
assay, it was shown that rhinovirus type 14 inhibits import of multiple pathways, and
docking of nuclear import receptor cargo complexes. Moreover this inhibition correlated
with the ability of the virus to degrade Nups Nup153 and Nup62 (53). Similarly Nup153
and Nup62 are both proteolytically cleaved during infection with poliovirus and this
correlated with the ability of the virus to inhibit classical import pathway (52). In contrast
to these proteins, cleavage of Nup98 by the 2A protease during infection with poliovirus
(90) did not correlate inhibition of mRNA transport (52, 90). These results indicate that
poliovirus targets different Nups to accomplish different functions and not all transport
pathways are altered during infection with poliovirus which is similar to results in VSV-
infected cells. These results also suggest that viruses may alter cellular transport
machinery differentially to inhibit host gene expression in order to replicate efficiently in
infected cells. It is possible that RNA viruses alter import pathways to prevent certain
host machinery from entering the nucleus in order to reserve the cytosolic supplies of
such factors and be utilized by RNA viruses for the replication process. Alternatively,
this impaired import pathways might serve as stress signals for cells to globally shut
down gene regulation and induce cell death.

Unlike enteroviruses that have proteases, such as 2A protease, to cleave Nups to
interfere with nuclear-transport trafficking, cardioviruses such as encephalomyocarditis
virus (EMCV) that lack a 2A protease like polypeptide, and instead express leader
protein. Like VSV M protein, the EMCV leader protein lacks any enzymatic activity and
is relatively a small protein of sixty seven amino acids in length. Leader protein interacts
with small GTPase, Ran (96) and also induces hyper-phosphorylation of Nup62,
Nup153, and Nup214 to inhibit import (97).
Unlike picornaviruses, VSV infection does not degrade Nup98 (110) and based on my data does not degrade Rae1 and Nup62 (unpublished data). Nup98 was not necessary for VSV induced inhibition of at least host transcription and translation. It has been demonstrated that the expression of Nup98 is important for M protein to localize to the nuclear rim. This was shown using mouse embryonic cells from Nup98 knockout cells (110). These cells were transfected with a plasmid encoding for M protein and the localization and the ability of M protein to inhibit host gene expression were measured. In these cells, M protein was unable to both associate around the nuclear rim and inhibit host gene expression. Although the protein levels of Rae1 in these cells were not determined, these results suggest that Nup98 may play a role in M protein’s ability to inhibit nuclear-cytoplasmic transport. However, the effects of silencing the expression of Nup98 on the ability of VSV to inhibit nuclear-cytoplasmic transport has never been tested directly. Similar to Nup98, Nup62 did not appear to play a role in the ability of VSV to inhibit host transcription and translation. Although the effects of silencing the expression of Nup62 on the ability of VSV to inhibit nuclear-cytoplasmic transport were not tested, it is possible that Nup62 may play a pertinent role in the ability of M protein to inhibit nuclear-cytoplasmic transport.

VSV infection also results in robust inhibition of host mRNA translation which can be observed as early as four hours post-infection (26, 27, 116). Contrary to transcription and nuclear-cytoplasmic transport, M protein in the absence of any viral component is unable to suppress host translation. In fact, wild type M protein stimulates translation of cotransfected mRNAs in the absence of any viral components (10). However, rM51R-M virus is unable to inhibit host translation as efficiently as the wild
type virus (27) which supports M protein’s role in inhibiting host translation. However, it also suggests that other viral components, besides M protein, are needed for this step in host gene inhibition. The inhibition of host translation in VSV-infected cells is not due to depletion of host mRNAs from the cytoplasm as a result of the inhibition of host transcription and nuclear-cytoplasmic transport (72, 115, 116). Instead, the translational apparatus is altered in VSV-infected cells such that only newly appearing mRNAs are translated (115), namely those produced by the viral RNA-dependent RNA polymerase. This alteration of the translation apparatus is correlated with the dephosphorylation of the cap-binding translation factor eIF4E (26, 27). The results presented here indicate that silencing Rae1 expression has little if any effect on this process (figure 11), suggesting that other molecular targets, besides Rae1 are involved in the suppression of translation.

The method of preparation of cell lysates described by Faria et al (38) and used here preferentially extracts cytoplasmic proteins. The results from size exclusion chromatography indicate that Rae1 and Nup98 are present in a complex in the cytoplasm which may include other Nups, such as Nup62, as well. It is difficult to ascertain if these complexes are inherently present in the cytoplasm or represent the complexes formed in the nucleus that are extracted during cell lysis. Moreover, it is possible that the sub-population of Rae1 that interacts with M protein originates in the nucleus and is exported into the cytoplasm. Preliminary work included in this thesis to ascertain Rae1-containing complexes in the nucleus indicate that Rae1, Nup98 and Nup62 are present in different sub compartments within the nucleus. These proteins were present in fractions containing proteins interacting with chromatin, lamina, and in the NPC embedded in the nuclear envelope. Moreover, M protein interacts with these proteins present in the nucleus.
Further experiments to monitor changes in the Rae1-containing complexes within the nucleus during infection with VSV will address the implications of M protein interacting with Rae1, Nup98, and Nup62 localized within the nucleus to suppress host gene expression.

I have addressed mechanisms by which M protein inhibits host gene expression at all three levels by interacting with Rae1. Based on my data, I provide evidence in support of the “platform hypothesis” in which Rae1 serves as a platform for interaction of M protein with other molecular targets. However, in my binding assays using Nup98 siRNA cells, a smaller amount of Nup62 interacted with M protein compared to control cells, suggesting that Nup98 may also be required for the interaction. Although this assay does not directly address the molecular mechanisms of binding of these proteins to M protein, the data however suggest, to an extent, the order of binding. For example Rae1 and Nup98 are known binding partners (13, 38, 62, 79, 99) and the interaction of M protein with Rae1 is independent of Nup98. Data from my silencing experiments indicate the both Rae1 and Nup98 are required for Nup62 to interact with M protein. Therefore, it is conceivable that that Rae1 interacts with M protein first, followed by the interaction Nup98, and Nup62. It is noted that although this hypothesis is not conclusive and further experiments will be needed to address the composition of these complexes.

My findings also lead me to propose a new function for Rae1 in regulating transcription, and provide new insights into the mechanism of VSV-mediated inhibition of host gene expression. In either case, I propose that the M protein-Rae1 complex in the nucleus may primarily suppress transcription, possibly by interacting with transcriptional machinery.
Future Directions

The results included in this work strongly suggest that M protein interacts with Rae1, present in multiple complexes, to inhibit host transcription. Through this work, a novel function of the host protein, Rae1 in regulating transcription has been identified. However, the exact mechanism by which Rae1 regulates transcription and importantly the mechanism by which M protein-Rae1 complex inhibits host transcription are not known.

Recently, Nups have been shown to interact with chromatin and with genes that are actively transcribing (21, 63). From my preliminary work in isolating and understanding the role of Rae1 in the nucleus suggested that Rae1 is associated with proteins associated with chromatin (Figure 16). Although the basal transcriptional factor was inactivated in VSV-infected cells, M protein does not directly interact with TFIID (121, 122). Therefore, it is possible that Rae1 may interact with the transcriptional apparatus of polymerase II, possible with TFIID and play a role in regulating transcription. Besides transcription factors, transcription by polymerase II also requires additional components such as coactivators and adapter proteins [reviewed in refs (20, 86, 123)]. Rae1 may also play a role in regulating transcription by either functioning as an adapter protein or could potentially interact with other adapter proteins.

Rae1 can be crosslinked to polyA +RNA (69). It would be interesting to expand studies to determine whether M protein-Rae1 complex also plays a role in modulating transcription by polymerase I and III. This would be analogous to poliovirus infection, in which the 3C protease inactivates SL1 factor by cleaving one of its subunits (6). SL1 is
the basal transcription factor that nucleates the process of transcription initiation by polymerase I and participates in promoter recognizing process [reviewed in ref (123)].

VSV’s replication cycle is short compared to other viruses, and in order to replicate efficiently, the virus has evolved mechanisms to evade host immune response by inducing a robust suppression of host gene expression. One of the mechanisms of suppression would be to abrogate the functions of host proteins that play multiple roles within the cells. Therefore, additional studies are necessary to identify other host proteins that are targeted by the virus, specifically by the M protein to address the mechanism of virus induced suppression of host gene expression.
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ABSTRACTS


