Health Science Campus

FINAL APPROVAL OF MASTER THESIS
Master of Science in Biomedical Sciences

Characterization of Vesicular Stomatitis Virus Strains with Adaptability Differences

Submitted by:
John Presloid

In partial fulfillment of the requirements for the degree of
Master of Science in Biomedical Sciences

Examination Committee

Major Advisor: Isabel Novella, Ph.D. _____

Academic Advisory Committee: Dorothea Sawicki, Ph.D. _____

James Trempe, Ph.D. _____

Senior Associate Dean
College of Graduate Studies
Michael S. Bisesi, Ph.D. _____

Date of Defense: July 18, 2008

Characterization of Vesicular Stomatitis Virus Strains with Adaptability Differences

John B. Presloid

University of Toledo, Health Science Campus

2008

ACKNOWLEDGEMENTS

- Isabel S. Novella, P.I. and major advisor, for overall guidance
- Arthur K. Chan, for technical assistance and help in fitness determinations
- Bonnie E. Ebendick-Corpus, for technical assistance
- Ranendra N. Dutta, Sarah D. Smith, and Siming Yang, for contributions to the analysis of evolved strains
- The above-mentioned along with Kim L. Lust for discussion of ideas and information
- Committee Members: Dr. Dorothea Sawicki, Dr. James Trempe, and Dr. Robert Blumenthal.

TABLE OF CONTENTS

INTRODUCTION

RNA viruses are a major source of infection and disease in humans, livestock and crops. Many pathogens are RNA viruses, including human immunodeficiency virus (HIV), hepatitis viruses, and influenza virus. RNA viruses have high mutation rates, which can lead to rapid adaptation to changes in the environment, such as immune pressure or drug treatment. Vesicular stomatitis virus (VSV) is an often-used model for RNA virus evolution. In this study, I used VSV to gain insight into the dynamics of RNA virus evolution by studying its potential for adaptation. Previous studies indicated that certain VSV strains had adaptation deficiencies, and I explored this by studying differences in phenotype of such strains. For this project I confirmed the adaptability defect in two strains and estimated the extent of the defect. In addition, I hypothesized that the impaired adaptability could be the result of a lower beneficial mutation rate. I tested this hypothesis through the examination of phenotypic distributions of their viral quasispecies. I used two methods to study the composition of clones within each population: determination of plaque size and determination of fitness within each population. Fitness analyses led to the identification of overall defects in robustness, with higher deleterious mutation rates and lower beneficial mutation rates in one of the populations. These studies could be useful to help combat clinically relevant RNA viruses, such as those listed above.

LITERATURE REVIEW

RNA Viruses: Evolution, Disease, and the Red Queen Hypothesis

RNA viruses are a broad class of infectious agents that can cause infection and serious disease in humans. Examples of RNA viruses include the influenza viruses, human immunodeficiency virus (HIV), poliovirus, Ebola virus, hepatitis C virus and coronaviruses, among many others. Millions of humans become infected with RNA viruses every year, and in the United States alone hospitals average nearly 500,000 cases per year of influenza or influenza-like diseases (Thompson, Shay et al. 2004). A major obstacle in the treatment of RNA viruses is their high mutation rates. One consequence of these high mutation rates is that RNA viruses are often able to generate mutants which can overcome changes in the environment such as the production of antibodies and treatment with antiviral drugs (Domingo, Menendez-Arias et al. 1997), leading to rapid adaptation.

Adaptation exhibited by RNA viruses that leads to immune evasion is often explained under the Red Queen Hypothesis, also known as an "arms race". This principle was named after a character in Lewis Carroll's novel “Through the Looking-Glass”, in which the Red Queen says “It takes all the running you can do, to keep in the same place”. In terms of evolution, the Red Queen Hypothesis states that competing systems must constantly adapt in order to stay in equilibrium with each other (Van Valen 1973). There are two scenarios where the Red Queen applies. The first scenario is in systems where two populations of the same species compete in a single niche; the second is in predator-prey systems. In the first case, a competitive

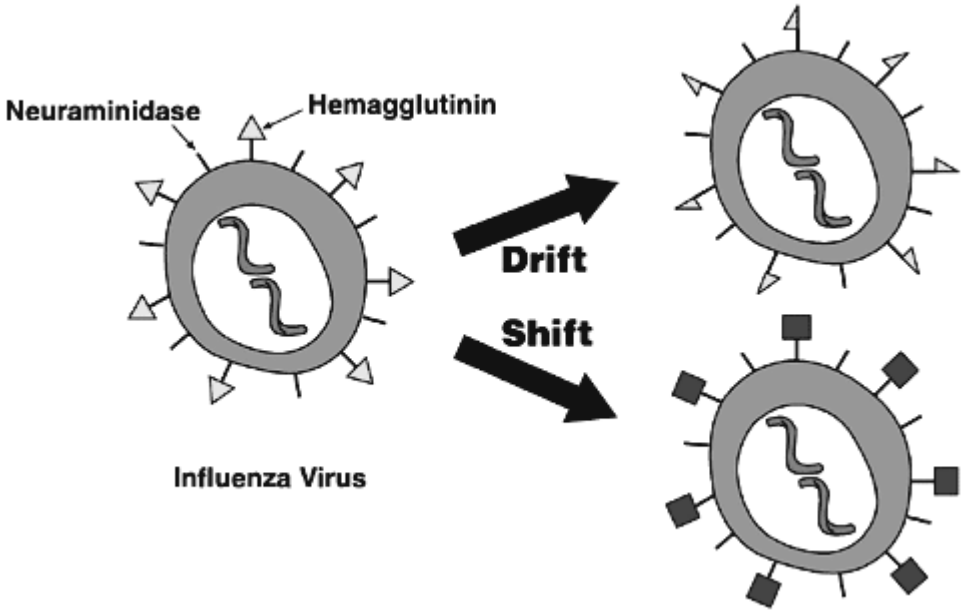
coexistence is allowed as long as both populations gain in fitness to a similar extent during the competition (Clarke, Duarte et al. 1994; Sole, Ferrer et al. 1999). Infecting virus and the immune system can be considered a predatory-prey system where both undergo constant adaptation and where each new change is designed to beat the latest mutation of the opponent. Viral infection is followed by the development of antibodies, to which the virus responds with the acquisition of mutations that allow it to evade these new antibodies. The immune system will then develop new antibodies and cytotoxic T-lymphocytes (CTLs) to attack the newly adapted virus and its infection. The virus will then acquire further mutations to adapt to and evade the new antibodies and CTLs. This process will continue until one of the populations becomes extinct, so either the virus overcomes the immune system (and likely kills the host) or the host clears the infection. So in this manner, a constant "running in place" of mutations is needed for both the viral and immune system populations to keep up with each other. The Red Queen may operate at an individual host level (e.g. hepatitis C virus, HIV-1) or at a host population level (e.g. influenza).

Viral Escape from Immune Responses

RNA viruses escape immune responses through the modification of antigenic sites. The best-characterized example of immune escape is influenza virus (Smith, Lapedes et al. 2004; Nelson and Holmes 2007). The main targets of antibody recognition are found in the external proteins, and particularly in the hemagglutinin (HA) protein. Two different genetic processes result in two different mechanisms of evolution: antigenic drift and antigenic shift. Antigenic drift is characterized by the

more gradual accumulation of point mutations in antigenic sites, while antigenic shift occurs when an entirely new NA gene is acquired. The differences between the two processes are illustrated in figure 1:

Figure 1: Antigen drift versus antigen shift. In antigen drift, epitope sites are mutated, modifying the antigenic sites slightly to evade immune defenses. In antigen shift, antigens are replaced with a new set of antigens, resulting in more rapid modifications.



Five epitopes in the HA protein of influenza virus have been identified (Plotkin and Dushoff 2003). These epitopes are directly involved in antibody recognition, and thus are a direct target of the host's immune defenses. Also in the 2003 Plotkin and Dushoff study, mutations found in the influenza genome among many samples were analyzed. The researchers found that codons in the HA epitopes

had higher rates of substitution mutations than other areas of the HA gene, as well as higher rates than found in other genes (Plotkin and Dushoff 2003). This is important because it shows that selection can operate at the nucleotide level and favor sites that would likely produce a protein change when mutated. By mutating frequently at such sites, the viral population has a better chance of generating antibody-resistant mutants. These results were confirmed by another study, which sampled different influenza virus strains from 37 years (Shih, Hsiao et al. 2007). This study found there were 63 substitution sites in the HA gene in all samples combined. Of these 63 sites, 57 were identified to be immune system targets. Again, these epitope mutations are beneficial to the virus, allowing it to escape immune defenses. In addition to escaping antibodies, influenza virus can also escape recognition by CTLs, except that CTL epitopes are not necessarily external components of the virion, and can thus theoretically map to any part of the viral genome. For example, nucleocapsid mutants were found which abrogated CTL recognition, in a study which also found that CTLs from one infection did not recognize mutant viruses from a later infection (Rimmelzwaan, Boon et al. 2004).

Antigen shift is the second mechanism that affects viral interaction with host defenses, but in a different manner than is seen in antigen drift. Rather than evading immune defenses by mutating specific epitopes as seen in antigen drift, in antigen shift viruses evade immune defenses by completely replacing their antigenic sites through reassortment (Figure 1). This can be accomplished in influenza virus because of its segmented genome, which means that different genes exist as entirely

separate strands of RNA inside the virion. When multiple viruses infect the same cell, the progeny genomes are free to associate with products from other viruses of the same type. In this manner, a virus can pick up an entirely new gene from a different influenza virus, possibly representing a completely different set of epitopes and antigenic sites.

Antigen shift plays a major role in the current concern over avian flu, which represents a major epidemiological fear. Influenza virus has caused several major pandemics in the human population, including the Spanish Flu of 1918 (Potter 2006). Influenza has two main surface proteins: HA and neuraminidase (NA). These proteins mediate cell entry, and thus host range. There are 16 HA variants and 9 NA variants, with each influenza virus having one type of HA and one type of NA. Influenza strains are often identified by these proteins numerically, such as H1N1. Until recently only H1, H2 and H3 influenza strains were found in human infections. However, all known HA and NA variants exist endemically in avian populations, and both human and avian strains can also infect swine (Webster, Bean et al. 1992). Coinfection of swine and birds with human and avian viruses allows the exchange of genetic segments to produce new combinations of HA and NA proteins (as well as other viral proteins). All the pandemics from the 20th century were caused by viruses that evolved by antigenic shift. Currently, H5N1 is primarily an avian virus, but it has been known to infect humans who have extensive contact with infected avian populations (Hatta, Gao et al. 2001; Hatta and Kawaoka 2002). Currently, human-to-human transmission of the virus is believed to be rare (Ungchusak,

Auewarakul et al. 2005). However, if allowed to replicate in humans, it is possible that the strain can mutate to allow for better transmission between humans. Evidence suggests that this has happened previously throughout history, most notably for the H1 strain which caused the Spanish Flu of 1918, which killed nearly 20-40 million people around the world (Reid, Fanning et al. 1999; Suzuki 2006). The anticipated mutation of the H5N1 avian flu virus enabling it to efficiently spread via human-to-human contact is expected to cause major suffering in humans (Banks, Speidel et al. 1998; Hatta and Kawaoka 2002). Thus, the recombination and mutation of influenza genes represent an important source of disease outbreak and virulence in humans.

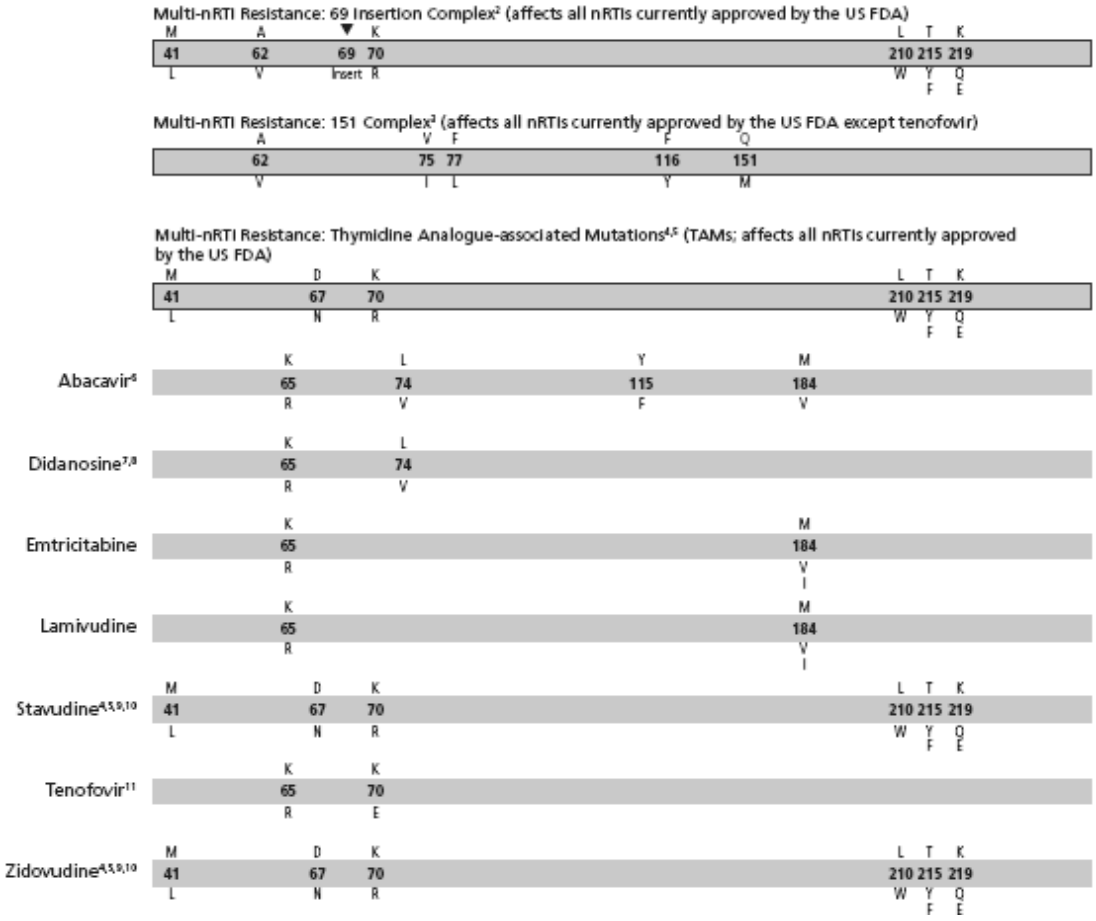
While the term "antigenic drift" is usually applied only to influenza viruses, many other RNA viruses avoid detection by the immune system through the accumulation of mutations in antigenic sites. One such example is HIV. HIV is a major human pathogen, with estimates of over 40 million people infected worldwide (Piot, Bartos et al. 2001). HIV attacks CD4 helper cells of the immune system, eventually crippling the ability of the immune system to fight off infections. There is extensive evidence of HIV mutants which escape recognition of circulating antibodies (Tomaras, Shen et al. 2006). Evidence of mutants capable of escaping CTL recognition has also been reported for HIV infections (Leslie, Pfafferott et al. 2004). Similar findings have been reported for hepatitis C virus (HCV), a nonsegmented positive-stranded RNA virus. In populations from infected human patients, HCV strains with high degrees of mutational diversity were more able to evade the effects

of the immune system (Kurosaki, Enomoto et al. 1994; Manzin, Solforosi et al. 1998; Pawlotsky, Germanidis et al. 1999).

Escape from Antiviral Drugs

Similarly, RNA viruses can produce mutants which escape the effects of antiviral drugs. One such example is HIV, which has many documented mutations conferring resistance to different antiviral drugs (Najera, Holguin et al. 1995; Johnson, Brun-Vezinet et al. 2007; Shafer, Rhee et al. 2007). These mutations cover a wide range of drug targets and affects. Nucleoside analog reverse transcriptase inhibitors (NRTIs) affect the virus by mimicking nucleosides, as their name suggests. However, the analogs lack a hydroxyl group necessary for chain extension, and thus when an analog is incorporated into the RNA, synthesis is terminated, resulting in shortened, defective RNA particles. Examples of NRTIs used to treat HIV infections include azidothymidine (AZT), lamivudine, and abacavir (ABC). Multiple mutations have been found in the reverse transcriptase of HIV conferring resistance to these drugs, and in some cases mutations have been found which confer resistance to all currently known NRTIs, such as an insertion at amino acid 69. These mutations are summarized in figure 2:

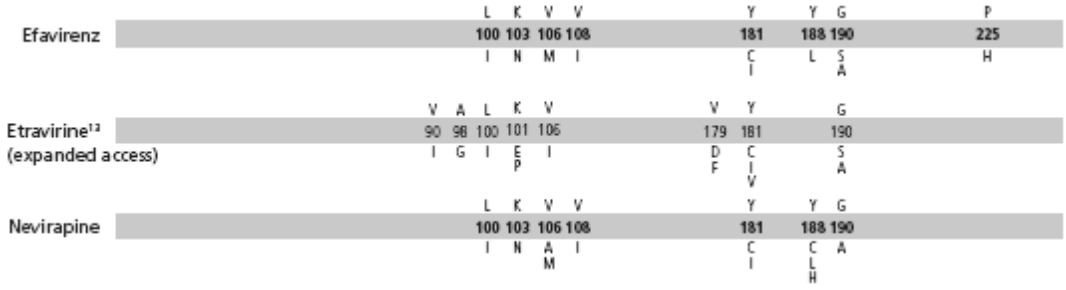
Figure 2: Summary of mutations of the reverse transcriptase of HIV conferring resistance to various nucleoside analog reverse transcriptase inhibitor (NRTI) antiviral drugs (Johnson, Brun-Vezinet et al. 2007).



Another class of drugs attacks the reverse transcriptase itself, and is termed non-nucleoside analog reverse transcriptase inhibitors (NNRTIs). One such example is nevirapine, which binds in a pocket of the reverse transcriptase, inactivating it (Beach 1998). Again, many mutations have been found conferring resistance to all

known NNRTIs, although the set of mutations is different than those found in populations resistant to NRTIs. These mutations are summarized in figure 3:

Figure 3: Summary of mutations of the reverse transcriptase of HIV conferring resistance to various non-nucleoside analog reverse transcriptase inhibitor (NNRTI) antiviral drugs (Johnson, Brun-Vezinet et al. 2007).



Other drugs have been developed to attack unique viral proteins. Two examples of this are protease and integrase. Protease is a cleavage protein, used to cut longer proteins into the proper proteins needed for virus assembly and function. Integrase functions in incorporating retroviral genetic material into the host DNA. Additionally, antivirals have been developed such as enfuvirtide which inhibit viral entry into the cell by disrupting virus fusion (Lalezari, Henry et al. 2003). Again, drugs of all these types have produced mutations in corresponding HIV domains (the protease gene, integrase gene, and envelope gene, respectively) conferring resistance to these antiviral drugs (Johnson, Brun-Vezinet et al. 2007). Thus, these mutations complicate the treatment of HIV. Currently, the recommended regimen includes the administration of multiple drugs simultaneously, to make it more difficult to acquire

multiple resistances. This therapy is known as highly active antiretroviral therapy (HAART). While HAART can help delay the appearance of resistant mutants, it is not completely effective as resistance can occur eventually (Daniel, Schneider et al. 2003).

Similar results have been found for influenza virus, another RNA virus. Two main targets are used by antivirals for influenza: neuraminidase (NA) and M2 proteins. NA protein is needed for viral budding, while the M2 protein is necessary for viral uncoating prior to transcription (Lamb and Krug 1996; Stouffer, Acharya et al. 2008). Examples of NA inhibitors include oseltamivir and zanamivir, while examples of M2 inhibitors include amantadine and rimantadine. NA inhibitors like oseltamivir function by blocking the NA protein, preventing the budding of virus from an infected cell. M2 inhibitors function by blocking M2 channels, preventing uncoating of infecting viruses and thus preventing transcription and replication. As is the case with HIV antivirals, influenza antiviral drugs have been shown to select for resistant mutations in influenza virus. M2 inhibitor-resistant mutants seem to occur in amino acids 26-34 of the M2 protein, most commonly at amino acid 31 (Shiraishi, Mitamura et al. 2003). Importantly, resistance to M2 inhibitors is thought to be increasing (Bright, Shay et al. 2006). Resistance to NA inhibitors seems to be selected for less-frequently than HA mutants (Gubareva, Kaiser et al. 2000), but it does occur, with mutants found resistant to both oseltamivir (Kiso, Mitamura et al. 2004) and zanamivir (Gubareva, Matrosovich et al. 1998). It is thought that through the use of antivirals in poultry farming, H5N1, the avian flu virus, has developed

resistance to amantadine, an M2 inhibitor (Cheung, Rayner et al. 2006; Smith, Fan et al. 2006; Hurt, Selleck et al. 2007). Additionally, recent reports have found H5N1 isolates resistant to NA inhibitors such as olsetamivir (de Jong, Tran et al. 2005; Le, Kiso et al. 2005). Thus, drug resistance via mutation remains an important concern for the treatment of many viral diseases.

Quasispecies.

One consequence of the high mutation rate of RNA viruses is that viruses produced during an infection are likely to include at least one mutation different from the original infecting virus. Thus, a replicating population of RNA viruses does not consist of identical genomes, but a distribution of many different genomes, or mutants. This collection of mutants is referred to as a quasispecies (Eigen 1992; Eigen 1996; Bull, Meyers et al. 2005; Wilke, Forster et al. 2006). A quasispecies consists of a multitude of variants, meaning that at any given time a population of RNA viruses can stochastically contain mutants conferring resistance to antibodies or antivirals, even if the virus population has never encountered these selective agents. This presence of resistant mutants can thus cause seemingly rapid development of resistance during infection or drug treatment, and has been hypothesized to be a factor in the pathogenesis and resistance to treatment of different RNA viruses such as poliovirus (Vignuzzi, Stone et al. 2006), rabies virus (Morimoto, Hooper et al. 1998), and hepatitis C virus (Pawlotsky, Germanidis et al. 1998; Farci, Shimoda et al. 2000), and also in safety concerns for vaccines (Novella, Domingo et al. 1995). The quasispecies theory is an extension of the mutation-selection balance model for high

mutation rates. The quantitative differences provided by differences in the mutation rates leads to qualitative differences in the prediction of how selection operates. Thus, quasispecies theory has implications for different evolutionary tracks than are predicted by simple population genetics theory. The general mutation-selection balance model predicts that the genome of highest fitness will outcompete the other mutants in the population and assume dominance. This is often termed “survival of the fittest”. In contrast, quasispecies theory predicts group selection, such that the fittest genomes may be outcompeted by genomes of lower fitness that can generate a better ensemble of progeny (Swetina and Schuster 1982; Schuster and Swetina 1988; Eigen 1996). The generation and survival of better combinations of progeny genomes is the result of selection of the most robust mutants; that is, the viruses most able to accept mutations without deleteriously affecting the virus (see section "Factors affecting evolution"). This concept is termed “survival of the flattest” (Wilke, Wang et al. 2001) to highlight the difference between quasispecies and the more general model of population genetics. Thus, quasispecies theory leads to predictions for how RNA virus populations will evolve that are different than those that apply to DNA-based organisms. Indeed, the quasispecies nature of RNA viruses has been demonstrated in studies that have shown that high fitness virus populations can lose to a population of lower fitness, but higher robustness (de la Torre and Holland 1990; Burch and Chao 2000).

The mutation rates in viral quasispecies are so high that they cannot be increased any further without loss of meaningful genetic information (Holland,

Domingo et al. 1990). Increased mutation rates during viral replication leads to the introduction of so many mutations into the genome that viral products are no longer functional and the virus cannot replicate. Thus, a new approach to antiviral treatment would be the use of RNA mutagens that affect mutation rates, as DNA replication would see fewer effects, due to its inherent proofreading abilities. This process, termed lethal mutagenesis, was demonstrated using mutagenic nucleotide analogs to treat HIV-infected cell cultures (Loeb, Essigmann et al. 1999).

Ribavirin is one of the most successful antivirals available and it is used in the treatment of a variety of viral infections, including hepatitis C virus and a variety of hemorrhagic fever viruses (Huggins 1989; Reichard, Andersson et al. 1991). The main effect of ribavirin is an increase in mutation rates of the viral RNA polymerases (Crotty, Maag et al. 2000; Parker 2005). Ribavirin increases mutation rates by mimicking both purine bases, causing it to bind equally well with both uracil and cytosine (Crotty, Maag et al. 2000). This leads to an increase in mutations and the loss of genetic information, and thus lethal mutagenesis (Crotty, Cameron et al. 2001; Graci and Cameron 2002). In the presence of ribavirin, RNA viruses such as poliovirus can acquire mutations which counter the effects of the drug by increasing the fidelity of the polymerase (Vignuzzi, Stone et al. 2005). Escape is the result of selection of polymerase mutations that slow down its elongation speed, so that there is more time for proper pairing between the nucleotide in the template and the newly-incorporated nucleotide (Pfeiffer and Kirkegaard 2003). However, while ribavirin-resistant mutants seem to grow as well as sensitive wild type, they show

defects in their ability to infect mice, and, unlike sensitive strains, they do not reach the central nervous system (CNS). This result suggests that full virulence depends on the generation of different mutants. Virulence of resistant mutants was fully restored by mutagenic treatment, a result that confirms the importance of generating different mutants during infection and, therefore, the relevance of quasispecies theory to explain and understand the evolution of RNA viruses.

Complementation

Quasispecies theory predicts group selection in viral populations, but there are other factors that also promote group selection. One of them is complementation. Viral proteins are not sequestered during an infection, meaning proteins from one virus are free to act with or be assembled with the genome of another virus present in the cell. In this manner, a virus with deleterious mutations causing defective proteins can survive and replicate normally if it coinfects a cell with a virus that can provide normal proteins. Weaker genomes can thus be maintained in the presence of neutral or beneficial genomes by using the beneficial proteins during replication. The work of Vignuzzi and coworkers discussed in the previous section is an excellent example of *in vivo* complementation (Vignuzzi, Stone et al. 2006). As discussed earlier, ribavirin-resistant mutants are unable to generate enough beneficial mutations to overcome immune surveillance. However, the study found that the mutant virus could infect neural tissues normally *in vivo* when administered along with wild-type poliovirus. This study shows that complementation can play an important role during infections, perhaps expanding on the high diversity by allowing viruses with different

mutations to interact with each other, and thus spreading the effects of beneficial mutations. Another study using vesicular stomatitis virus has also shown that complementation can affect how virus populations evolve, with a high amount of coinfection leading to even very weak genomes being able to propagate at rates seen by neutral viruses (Novella, Reissig et al. 2004). The degree of complementation correlates negatively with the rate of decline in the frequency of deleterious mutants, and positively with the frequency of the deleterious mutant at mutation-selection balance (Presloid and Novella, unpublished results). Since antibody-escape and drug-resistant mutants often are deleterious in the absence of the relevant selective pressure, complementation could explain the persistence of such mutations, even though they are less fit than neutral viruses.

Complementation plays a role in the evolution and propagation of many RNA viruses, such as Dengue virus (Aaskov, Buzacott et al. 2006), tobacco mosaic virus (TMV) (Holt and Beachy 1991), and tomato aspermy cucomovirus (TAV) (Moreno, Malpica et al. 1997).

Virus Adaptation and Vaccine Design

RNA virus mutations have also been manipulated in order to benefit humans. When a virus adapts to an environment, it often becomes a specialist in that environment, becoming extremely well-adapted to utilizing the resources available in that environment. However, this is often accompanied by a trade-off, or decrease in adaptation to other environments. This can be due to antagonistic pleiotropy or mutation accumulation (Kassen and Rainey 2004). Under antagonistic pleiotropy,

mutations beneficial in one environment are deleterious in another. Under mutation accumulation, mutations accumulated in one environment are neutral, while they are deleterious in a second environment. In both cases, the result is a virus that is well-adapted on the first environment, but less-adapted to another environment. Varying degrees of trade-offs have been shown in different RNA viral systems, such as Dengue virus (Chen, Wu et al. 2003), eastern equine encephalitis virus (Cooper and Scott 2001), Sindbis virus (Greene, Wang et al. 2005), and vesicular stomatitis virus (Novella, Hershey et al. 1999; Weaver, Brault et al. 1999; Turner and Elena 2000). In addition to affecting host range and evolution, trade-offs can play an important role in the treatment of diseases caused by RNA viruses. For example, one major area where this is useful is in the area of vaccine development.

There are two main types of viral vaccines that contain virus particles: inactivated and attenuated, sometimes called live-attenuated. Inactivated vaccines contain killed or otherwise chemically incapacitated infectious agents, but still contain enough viral material to provoke an immune response. Unfortunately, because the viral material is inactivated, the immune reaction is usually not strong enough to elicit a complete immune response. Immunologic memory may not be induced, and thus these vaccines have low efficacy and often require booster shots. Examples of inactivated vaccines are the Salk polio vaccine and the yearly flu vaccines (Kersten, Hazendonk et al. 1999; Nichol 2003). In contrast, attenuated vaccines contain living, but weakened infectious organisms. This weakening can be achieved by removing a virulent property, or by growing the organism in a different

environment to hinder replication in a human host. As discussed before, growing the virus in a different environment, such as avian or insect cells, can cause the virus to become poorly-suited to infect human cells due to trade-offs. Since these viruses are still able to replicate, although poorly, attenuated vaccines cause a more complete immune reaction when compared to inactivated vaccines, resulting in greater protection and long-lasting immune memory. Examples of attenuated vaccines include the vaccine against mumps, measles, and rubella (MMR) (Watson, Laufer et al. 1996), the Sabin polio vaccine (Sabin, Ramos-Alvarez et al. 1960), and Pasteur's rabies vaccine. The Sabin vaccine is particularly interesting because it has been extensively studied. It has been found to contain 57 separate mutations compared to the parental poliovirus strain, which represent the mutations beneficial to the new environment (Kew, Sutter et al. 2005). These trade-offs caused the vaccine to replicate normally in the gastrointestinal tracts, but very poorly in neural tissues. The replication in the stomach and intestines allowed for immune responses to occur, but the lack of neural replication prevented onset of polio disease. A disadvantage to such attenuated vaccines, however, is the reversion of the vaccine back to a virulent form of virus (Cann, Stanway et al. 1984; Nielsen, Oleksiewicz et al. 2001). Thus, ideally, an attenuated vaccine could be designed whose mutants were unlikely to revert back to a virulent form, offering greater safety.

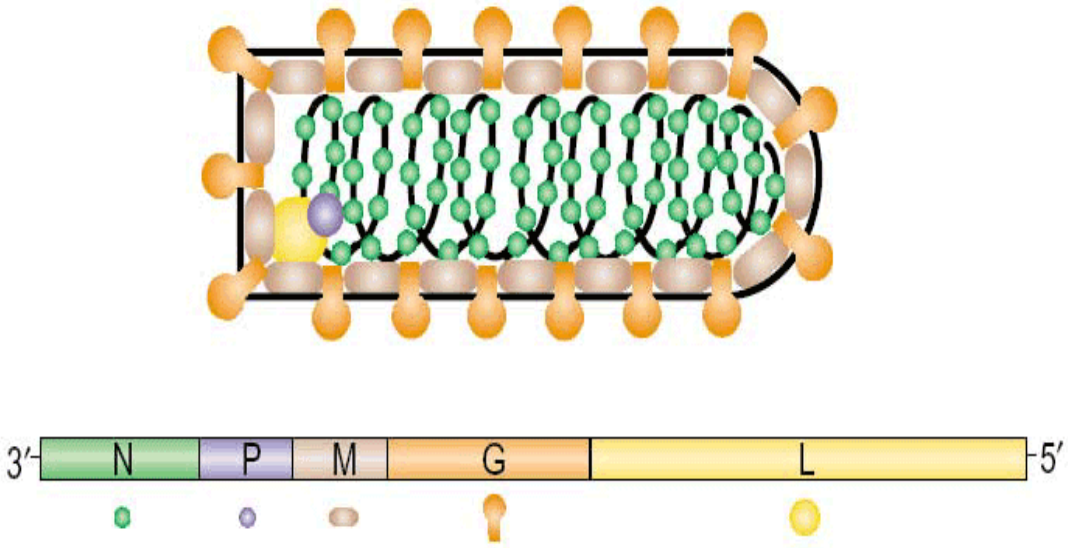
Vesicular Stomatitis Virus

Vesicular stomatitis virus (VSV) is a particularly useful model for studying the evolution RNA viruses for many reasons, which will be elaborated upon. VSV is

an enveloped RNA virus of the family *Rhabdoviridae*, in the group Mononegavirales, and it is a close relative of rabies virus. VSV contains a genome that consists of a single, nonsegmented, negative-sense strand of RNA. The virus particle is bullet-shaped, with a length of approximately 180 nm and a width of approximately 75 nm. The outer envelope consists of a lipid bilayer that is derived from the infected cell from which the virion has budded. The RNA is encapsidated by a viral protein that protects the genome from enzymatic degradation and that also controls replication (Rose and Whitt 2001).

The VSV genome is 11,161 nucleotides long, and encodes at least 5 proteins: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G), and large protein (L). The virion and genomic structures of VSV can be seen in figure 4:

Figure 4: The structure of the VSV genome and virion. N is complexed with the genomic RNA; P is complexed with L in the lower left hand section of the virus; M is located on the inner surface of the viral membrane, while G is inserted into the membrane and protrudes on the outer surface of the virus.



Mutations can occur anywhere in the genome of VSV, and thus an understanding of protein functions can help identify potential mutation effects. The N protein consists of 423 amino acids. The main function of the N protein is to encapsidate the genome, providing for structural support and protecting the genome from degradation. Inhibition of the N protein has been shown to cause an inhibition of genome replication, suggesting that N protein plays a vital role in replication (Cortay, Gerlier et al. 2006). The N protein also interacts with the P protein. It has been shown that the five carboxy-terminal amino acids of the N protein are required for interaction with the P protein (Takacs, Das et al. 1993). The N protein has been shown to consist of two lobes, with positive residues lining the cavity between the

lobes, allowing for interaction with negatively-charged RNA. Each monomer of N protein can interact with 9 bases of RNA. The monomers can also interact with each other, forming decamers that resemble a ring structure. These decameric rings can then be stacked on top of one another, with RNA winding around the stacks in the grooves between the lobes of the monomers. According to this proposed model, the lobes are slightly flexible, allowing for a tighter or looser arrangement of the RNA genome (Green and Luo 2006).

The P and L proteins are parts of the RNA-dependent RNA polymerase complex needed for viral transcription and replication. The P protein consists of 265 amino acids, while the L protein consists of 2,109 amino acids, and the sequence of its gene comprises over 60% of the VSV genome. The L protein also performs auxiliary transcriptional functions such as polyadenylation of mRNA transcripts (Banerjee 1987) and methyl transferase activity (Grdzelishvili, Smallwood et al. 2005). The L protein has six conserved domains, I – VI (Hunt and Hutchinson 1993). Domain II contains a positively-charged motif involved in the binding of RNA. Domain III contains a QGDNQ motif necessary for the formation of phosphodiester bonds, an important process for RNA synthesis. Mutational research has shown that the conservation of domains II and III are vital for RNA synthesis, as mutation in these domains abolishes this process (Sleat and Banerjee 1993). Domain VI plays a major role in the methyltransferase activities of the L protein, leading to capping of viral mRNAs (Grdzelishvili, Smallwood et al. 2005). The L protein interacts with phosphorylated P protein in a conserved region between amino acids 1638 and 1673

(Canter and Perrault 1996), and abolition of this interaction leads to inactivation of the L protein (Banerjee 1987). Additionally, the P protein at amino acids 260 and 262 interacts with the N protein at its C-terminal end (Takacs, Das et al. 1993), which is also required for replication (Das, Pattnaik et al. 1997; Pattnaik, Hwang et al. 1997). The P protein contains three conserved domains. The first two domains contain phosphorylation sites at serine-60, threonine-62, and serine-64 in domain I, and serine-226 and serine-227 in domain II (Takacs, Barik et al. 1992; Chen, Das et al. 1997). Phosphorylation of the domain I sites results in association of multiple P monomers, which in turn promotes association with the L protein. Three P monomers associate with one L protein, forming a complex which is vital for transcription (Das, Gupta et al. 1995; Gupta, Shaji et al. 2003). Phosphorylation of the domain II sites does not appear to affect transcription, but is required for efficient replication (Hwang, Englund et al. 1999). These results suggest that different domains of the P protein are involved in mRNA transcription and replication of genomic RNA. The third domain spans the extreme carboxy-terminal end of the protein, and contains multiple basic residues. It is believed to associate with the complex of N protein and genomic RNA (Banerjee 1987).

The M protein consists of 229 amino acids, and is primarily found on the inside of the virion membrane. It consists of a single domain, with alpha helices and beta sheets. The amino-terminal region of the M protein is rich in lysine residues that could aid in interactions with the viral membrane (Gaudier, Gaudin et al. 2002). The main structural function of the M protein is to help associate the nucleocapsid

with the cell membrane upon budding (Chong and Rose 1993). The M protein also serves to block nuclear export of cellular mRNAs, allowing viral mRNAs to monopolize cellular machinery for translation (Weck and Wagner 1978; Black and Lyles 1992; Petersen, Her et al. 2000; von Kobbe, van Deursen et al. 2000). In addition to blocking cellular mRNAs, the M protein can inhibit cellular mRNA production by inhibiting RNA polymerases I, II, and III (Ahmed and Lyles 1998). The M protein also appears to play a role in the cytopathic effects of VSV, such as cell rounding and formation of syncytia (Blondel, Harmison et al. 1990; Jayakar and Whitt 2002; Kopecky and Lyles 2003).

The G protein consists of 511 amino acids, and is located on the outer surface of the viral particle. It mediates receptor-recognition and binding to the surface of the host cell, and also the subsequent fusion of cellular and viral membranes, and it is thus the G protein that mediates viral entry into the cell (Zhang and Ghosh 1994; Fredericksen and Whitt 1995). Also, the G protein serves as the main antigenic site of VSV (Rose and Whitt 2001). The majority of the G protein, 463 amino acids, lies on the outside of the viral membrane. There are 20 amino acids in the transmembrane domain, and the remaining 28 amino acids are inside the viral particle (Doms, Ruusala et al. 1988). The outer membrane region is divided into four domains. The first domain spans amino acids 1 through 17 and 310 through 383, contains many beta sheets, and is suggested to play a role in interactions with cellular receptors (Roche, Bressanelli et al. 2006), although exact cellular receptors have not yet been identified. The second domain spans three segments of the G protein: amino acids 18

to 35, 259 to 309, and 384 through 405. This domain forms four alpha helices, which help to trimerize the glycoprotein. Domain III consists of amino acids 36 to 50 and 181 to 258, and appears to mediate interactions with the cellular membrane (Mashanov, Tacon et al. 2004). Additionally, several epitopes for neutralizing antibodies are found in domain III (Vandepol, Lefrancois et al. 1986). The fourth domain of the G protein consists of amino acids 51 to 180, and is vital for fusion of the viral and cellular membranes, which is needed for the release of the viral genome into the cell (Fredericksen and Whitt 1995).

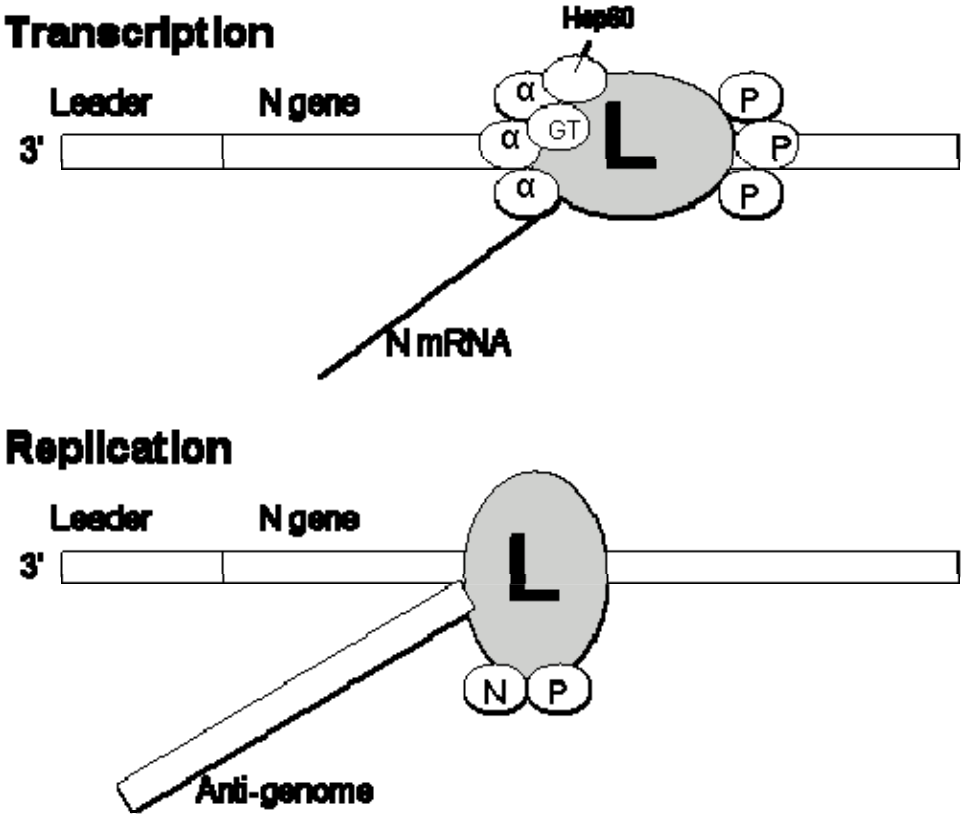
In addition to these five proteins, the P gene encodes in a -1 reading frame two other small proteins, C' and C, that are 55 and 65 amino acids in length, respectively. These proteins are found in infected cells but not in assembled virions. Even though their function is unknown and VSV mutants lacking the C' protein do not show a phenotype (Kretzschmar, Peluso et al. 1996), these overlapping open reading frames (ORFs) are conserved among most of the vesiculoviruses, suggesting that they play an important role during *in vivo* infections (Spiropoulou and Nichol 1993).

VSV infection begins with adsorption of the virion to the cell membrane. This is mediated by the G protein. Penetration of the membrane and uncoating of the virion follow adsorption. While adsorption can occur even at temperatures of 4°C, penetration requires physiological temperatures. VSV enters the cell via endocytosis and lowered pH in the endocytic vesicle triggers membrane fusion and release of the VSV nucleocapsid into the cytoplasm (Matlin, Reggio et al. 1982). Once the nucleocapsid-protected genome is inside the cytoplasm, transcription can occur. The

RNA-dependent RNA polymerase, which is packaged inside the virion, attaches at the 3’ end of the viral genome and processes along the RNA strand in a 3' to 5' order. VSV mRNAs are monocistronic and produced sequentially. Genes are separated by non-translated intergenic regions that carry cis-acting signals for the polymerase to stop transcription of the first mRNA and initiate transcription of the next mRNA. The intergenic regions also have a functional polyadenylation signal composing of 7 sequential uracil nucleotides. This causes the transcriptase to add a poly-A tail to the mRNA. After each transcriptional stop, the polymerase only restarts at the next gene about 70% of the time, and less often for L transcription (Wertz, Perepelitsa et al. 1998). This decline in transcriptional activity at each intergenic region is called attenuation and causes VSV to produce unequal amounts of each mRNA, with N being the most abundant mRNA, and L the least abundant mRNA (Abraham and Banerjee 1976; Rose and Whitt 2001). New viral mRNA is then translated by the cellular ribosomes. Leader RNA is thought to play an important role in the process of switching the polymerase from a transcriptase function to a replicase function. Leader RNA is a small, 47-nucleotide RNA whose sequence is encoded at the 3’ end of the VSV genome, before the N open reading frame. Although this leader RNA is not capped or polyadenylated (Colonno and Banerjee 1978), its importance is apparent from findings that showed the transcriptase entered at the 3’ end of the VSV genome, and produced leader RNA as its first RNA product (Emerson 1982). Since the transcriptase is known to have capping and polyadenylation functions, these results seemed contradictory. Studies were carried out to determine functional

differences between the replicase (genome and anti-genome production) and transcriptase (mRNA production) abilities of the VSV RNA polymerase complex. It was found that polymerase that was actively involved in transcription was bound to the cellular cofactors heat shock protein 60 (Hsp60), guanylyltransferase (GT), and elongation factor 1αβγ (EF-1αβγ). However, the polymerase involved in replication was not complexed with these cofactors, and replicated in the presence of N and P proteins (Qanungo, Shaji et al. 2004). An illustration of these two complexes is shown in figure 5.

Figure 5: Illustration of the transcriptase and replicase complexes for VSV. Note that the transcriptase enters at the N gene, while the replicase enters at the 3' end first, producing leader RNA. (reproduced from Qanungo, Shaji et al. 2004)



These results led to the creation of a new model for VSV replication. Under this model, the transcriptase consists of the L protein in complex with P protein, and cellular factors GT, Hsp60, and EF-1αβγ indicated by α in figure 5. This complex then enters directly at the beginning of the N open reading frame, and transcription of

the VSV mRNAs occurs, with capping and polyadenylation. After translation of viral mRNAs, the newly-made N and P proteins would then interact with L protein, causing it to disassociate from these cellular cofactors. This N-P-L complex would then be able to enter at the 3' end of the genome, allowing for replication of the genome. Thus, this model differs from the previous model in proposing the presence of two initiation sites, one for transcription and one for replication, as opposed to one site for both functions (Emerson 1982). The two-site model is supported by earlier studies, in which it was found that continuous production of N and P proteins in a 1:1 ratio was necessary for replication to begin (Peluso and Moyer 1988). Also, previous studies had suggested the presence of these two separate entry sites for the polymerase complex due to identification of mutants which produced greater than normal amounts of N mRNA when compared to leader RNA (Whelan and Wertz 2002). Thus in this manner, a full-length positive-sense RNA is formed, sometimes referred to as the anti-genome. The anti-genome is then used as a template by the replicase complex for production of full-length negative-sense genomes. This model for the change from transcription to replication is useful as it explains why replication is not seen early, and how the rate of replication increases with an increased concentration of translated proteins (Rose and Whitt 2001). As replication proceeds, the N protein associates with the P and L proteins, and surrounds the newly created genome. Once full-length replicated genomes are produced, the nucleocapsid binds to membrane-associated M proteins, which causes the encapsidated genome to coil tightly. After translation of mRNA, the G protein is glycosylated during trafficking

through the endoplasmic reticulum (ER) and Golgi apparatus, and is exported to the surface of the cell membrane. As a nucleocapsid associates near the membrane, the membrane begins to surround the nucleocapsid and bud off, and a virion is released.

Experimental Evolution of VSV

As an RNA virus, VSV does not undergo proofreading during replication, and thus any errors introduced during replication will be incorporated into the genome, leading to more mutations per base per round of replication than seen in organisms with a DNA genome. The average RNA virus mutation rate is estimated to be approximately 10^{-4} substitutions per nucleotide per round of replication (Drake and Holland 1999). In the case of VSV, this results in an average of one mutation found in each replicated progeny virus. This high mutation rate, coupled with rapid rates of replication, leads to observable population changes in phenotype and genotype in a relatively short amount of time (Holland, Spindler et al. 1982; Novella 2003), and makes VSV an excellent model for other rapidly-evolving RNA viruses such as influenza (Gorman, Bean et al. 1992) and HIV (Wain-Hobson 1993; Wain-Hobson 1996). Other factors that make VSV a useful tool for evolutionary studies are the relatively small size of the genome with only 11,161 nt (thus it is straightforward to sequence the entire genome) and the availability of reverse genetics systems to introduce individual mutations into the genome of VSV and produce viable virus, in order to test their effects (Lawson, Stillman et al. 1995; Whelan, Ball et al. 1995).

In nature, VSV is an arthropod-borne virus (arbovirus); arthropod vectors, such as insects, transmit the virus. Other arboviruses include West Nile virus and

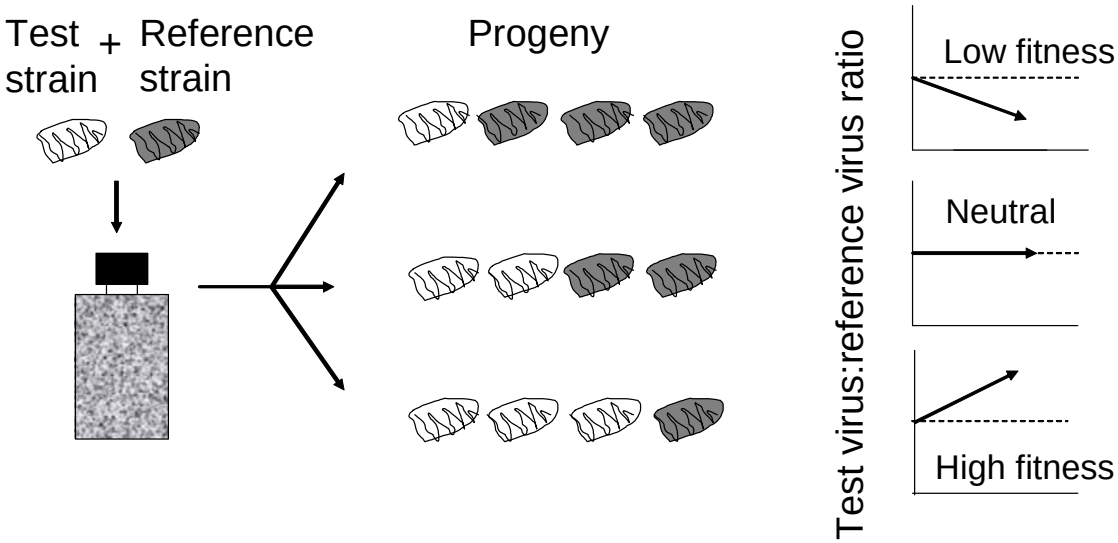
Dengue virus. In the case of VSV, the natural hosts include insects, such as sand flies, black flies and grasshoppers, as well as mammalian hosts, such as sheep, cattle, and swine (Mead, Ramberg et al. 2000). VSV is typically transmitted through blood feeding: infected insects can infect mammals when feeding, and an insect can acquire an infection when feeding upon an infected mammal (Mead, Mare et al. 1999; Mead, Ramberg et al. 2000). Additionally, mammals can become infected by the ingestion of infected insects, such as ingestion of grasshoppers while eating grass (Neumann, Whitt et al. 2002). Sandflies can also transmit the virus to their offspring when they lay eggs (Tesh, Chaniotis et al. 1972). This presents different environments for the virus to adapt to and evolve in, allowing for simple testing between different environments (Novella, Hershey et al. 1999; Novella, Quer et al. 1999). Additionally, VSV infects different cell types in different manners. Typically, mammalian cells are infected lytically with high amounts of cellular damage, including syncytia formation, cell rounding, and cell death. In contrast, VSV typically infects insect cells persistently with little or no cell damage (Tesh and Modi 1983). This wide range of host environments and viral infection mechanisms allows VSV to be used to explore many aspects of adaptation and evolution. Additionally, VSV is not a typical human pathogen, so it is safer to work with than other RNA virus pathogens such as influenza virus or HIV.

Factors Affecting Evolution: Fitness, Adaptability and Robustness

Fitness represents the relative ability of a virus to produce viable progeny when compared to another virus in a given environment, and thus it is a measurement

of its degree of adaptation. By definition, fitness measurements require a standardized virus to measure against. Typically, this reference virus is wild-type, to which a fitness of 1.0 is assigned arbitrarily. Fitness values are obtained during virus competitions. In a competition, a mixture containing two viruses is used to infect a culture of cells. When the virus sample is sufficiently diluted and agarose is added to the media, progeny virus can only infect surrounding cells, producing visually quantifiable plaques in the cell monolayer. Importantly, one virus in the mixture is antibody-susceptible, and the other virus is antibody-resistant. Thus, the amount of resistant virus can be measured by adding antibody to the assay, while the amount of susceptible virus can be calculated by subtracting the observed number of resistant viruses from the number of total viruses, found using no antibody in the assay. Ratios of the two viruses can then be calculated over a series of passages; by plotting these ratios on a logarithmic scale, a slope can be determined. This slope is the fitness of the tested virus (Holland, de la Torre et al. 1991). Thus, a horizontal line yields a fitness of 1.0, meaning the virus creates as much progeny as the reference virus. A slope greater than 1.0 means that the virus produces more progeny than wild-type VSV and corresponds to a high fitness virus; a fitness value higher then zero, but lower than 1.0 indicates that a virus produces less progeny compared to wild-type VSV and corresponds to a low fitness virus. A simple schematic of these fitness assays is shown in figure 6:

Figure 6: Representation of fitness assays, exhibiting how differences in progeny produced will result in differences in fitness values (Holland, de la Torre et al. 1991).



These fitness assays are reproducible, and since they are compared to a common wild-type strain of virus as a standard, a fitness value from one experiment can be compared with a fitness value from a different experiment.

In addition to fitness, other factors can affect how a population of VSV can evolve. One of these factors is adaptability, or the ability of the population to adapt. Essentially, it is a measure of a genome's ability to generate beneficial mutations. Adaptability can be observed or measured in different ways depending on the expected differences among populations. First, virus populations can be subjected to regimes of positive selection for a limited number of passages and the degree of fitness increases can be measured at the end of the experiment. This method is useful

to compare strains with dramatic differences in adaptability, which will correlate positively with the level of fitness increase. Viruses with adaptability defects may show no increase at all. Second, lesser differences can be revealed by extending the number of passages and using samples of intermediate passages to obtain estimates of the speed of adaptation, maximum fitness and the time it takes to achieve maximum fitness. Adaptability correlates positively with the speed of adaptation and negatively with time to maximum fitness. Finally, subtle differences in fitness can be observed doing long-term competitions between two viruses, provided that they have a similar relative fitness (e.g. they are relatively neutral); if the two competitors have similar adaptability, each virus will have an equal chance of winning the competition. In other words, if the competition is carried out multiple times, each virus should win half of the competitions. However, if one of the competitors has adaptability problems the number of wins in multiple competitions will be significantly below 50% (Quer, Huerta et al. 1996; Novella 2004).

Another factor that can also affect VSV evolution is robustness. Robustness is defined as the ability of a genome to accept mutations without affecting the phenotype (de Visser, Hermisson et al. 2003; Wilke and Adami 2003). This is especially important in RNA viruses, due to their high mutation rates. It is important to note that robustness, as well as adaptability and fitness, can be affected by epistasis. Epistasis would predict that certain mutations can alter the effects of secondary mutations. In this manner, mutations that are normally beneficial can be weakened, or even become deleterious, in the presence of other mutations. Work

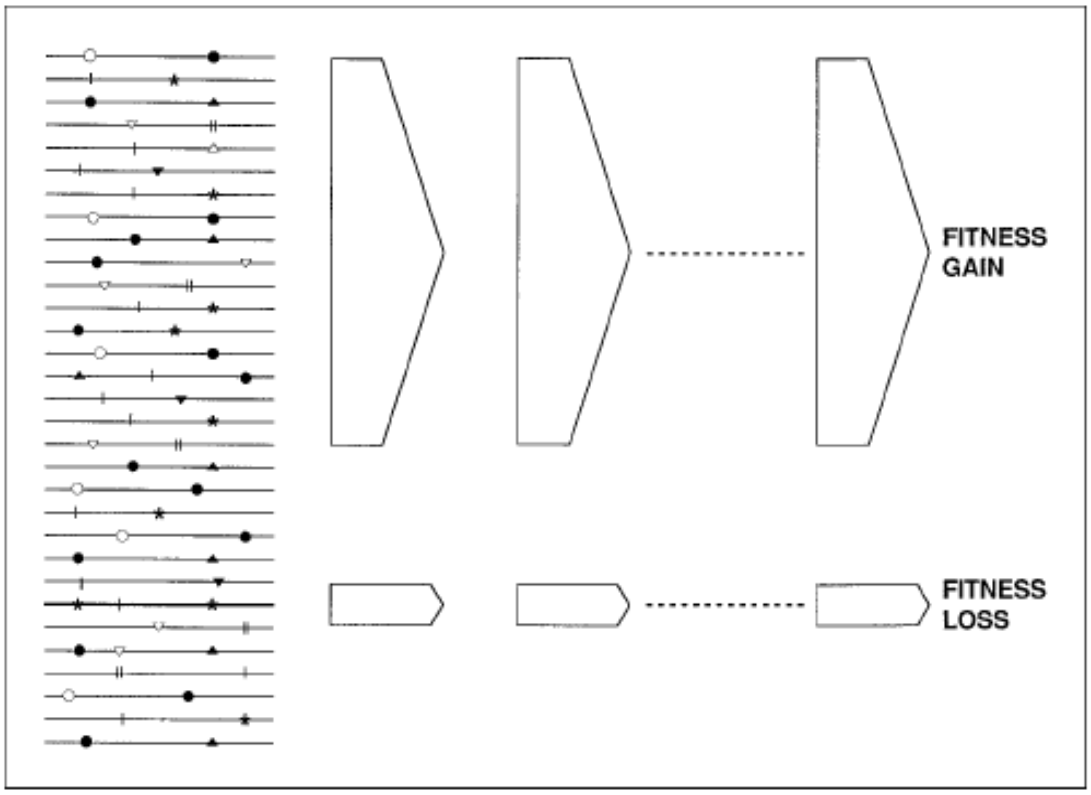
done with digital modeling (Wilke, Wang et al. 2001) suggests that genomes with higher robustness can outcompete genomes with a higher fitness at high mutation rates. Thus, robustness is also an important factor when considering the evolution of RNA viruses such as VSV.

Genetic bottlenecks and Muller's ratchet

VSV produces many mutants during each round of replication. Typically a large population passage, or passaging with large numbers of virus, will lead to an increase in fitness. This increase in fitness is because the population has a large collection of mutants that can compete, and the viruses most well-adapted to infecting the cells will replicate at a higher rate (Holland, Spindler et al. 1982; Novella, Duarte et al. 1995). Conversely, in small population passages, called bottlenecks, only a small number of viruses are available to compete, minimizing the effect of selection. Thus, viruses passaged repeatedly in small population passages typically experience losses in fitness in a process known as Muller's ratchet (Muller 1964). Bottlenecking a virus during transmission will cause random mutations to become fixed, increasing the chances of deleterious mutation fixation according to Muller's Ratchet. In a previous experiment, such bottlenecking was performed on a strain of VSV to the extent that only a single infectious unit was transmitted for each round of infection. This regime was repeated over several replicas, so that each replica experienced 20 bottleneck events. All replicas were performed from the same original starting virus population (Novella 2004). As predicted, after the serial bottlenecking most replicas were ultimately lower in fitness than the original population, reflecting the fixation of

more common deleterious mutants. This concept has been tested and supported experimentally on other RNA viruses such as phage φ6 (Chao 1990), phage MS2 (de la Pena, Elena et al. 2000), foot-and-mouth-disease virus (Escarmis, Davila et al. 1996) and HIV (Yuste, Sanchez-Palomino et al. 1999). In this way, by changing the population size during virus transmission, a virus strain can be manipulated to increase or decrease fitness (Clarke, Duarte et al. 1993; Novella, Elena et al. 1995). This concept is illustrated in figure 7:

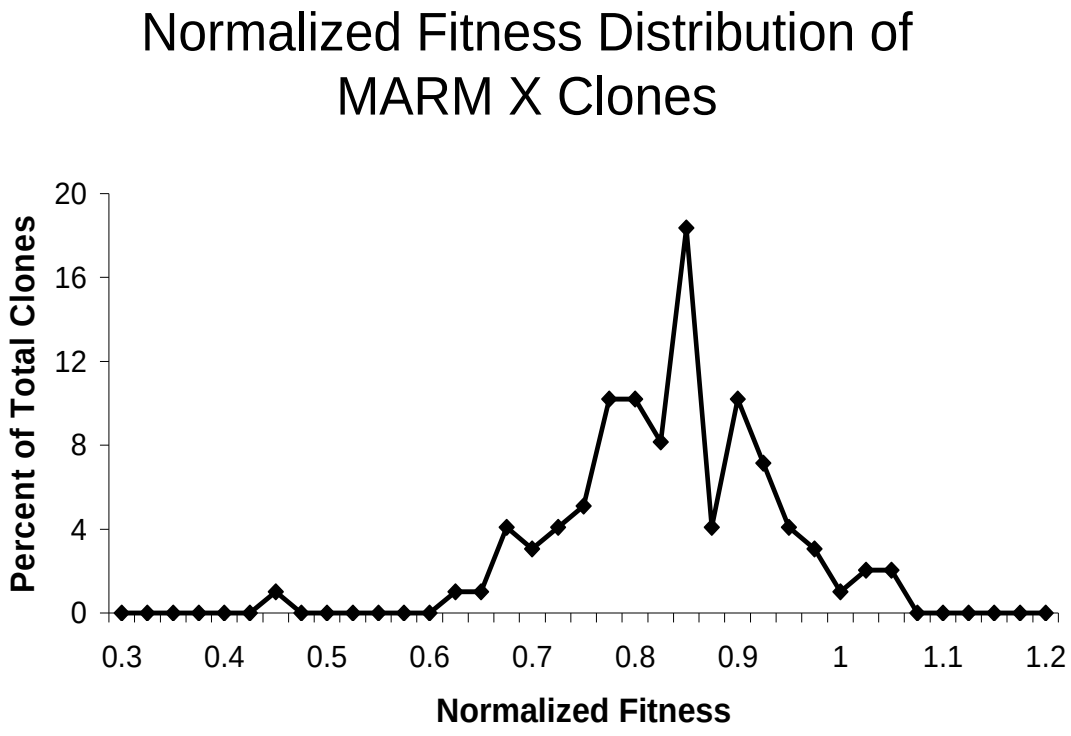
Figure 7: Fitness gain results from the passage of a large number of genomes because genomes with beneficial mutations can be selected. However, when few genomes are selected, typically only deleterious mutations will be passed, resulting in fitness loss. (Domingo, Menendez-Arias et al. 1997)



Biologically, the concept of bottlenecks has importance. Virus transmission can occur with a small number of particles, creating a bottleneck effect upon initial infection. This has been supported by findings that HIV diversity is much lower during initial stages of infection, which would indicate fewer initial infecting particles (Nowak, Anderson et al. 1991; Pang, Shlesinger et al. 1992).

The complexity within a quasispecies can be described by the frequency distribution of its genotypes or phenotypes. Sequences more closely related to the master sequence would be expected to be more numerous and thus represent the peak of the frequency distribution, while sequences which have more mutations and are thus more distant from the originating genome would be expected to be more rare, and thus form the smaller tails of the distribution. To determine the composition of a quasispecies, one must examine the individual components. In viruses, this can be done by examining clones. As described earlier, addition of agarose to the infection medium makes it thicker, inhibiting virus movement. Progeny virus budding from an infected cell can then only infect cells immediately surrounding it, eventually forming a plaque. Theoretically, each plaque originates from a single replicating virus, and so each plaque should represent a different infecting genome. Viruses acquired from these isolated plaques are referred to as individual virus clones. By analyzing these clones, one can analyze different individuals from a population. Through this type of analysis on a strain of VSV, Duarte et al (1993) found that most of the clones had lower fitness values than the population average, as shown in figure 8:

Figure 8: Clonal fitness distribution of MARM X, a high-fitness VSV population. Fitness values have been normalized to the population average. In this population, only a small portion of clones from the parental population are of higher fitness, and the average fitness from the tested clones can be different than the average population fitness (Clarke, Duarte et al. 1993)



This result shows that most mutations will have a deleterious effect on fitness. In addition to measuring fitness differences from these biological clones, other studies have shown a correlation between fitness and plaque size (Burch and Chao 2000). The Burch and Chao (2000) study also found that mutations and quasispecies content can affect the overall evolution of a virus. Here, a high-fitness clone was isolated from a virus population. This high-fitness clone evolved into a lower fitness virus

that had similar properties to the population from which the clone was originally isolated. This result, where the higher-fitness clone did not reach dominance and in fact was selected out of the population, supports quasispecies theory.

Bottlenecking and Adaptability Losses

Fitness losses after Muller's ratchet operation can be reversed by restoring selection in the system. Thus, transmission of large populations ensures that some of the variants will carry beneficial mutations and these will increase in frequency resulting in overall fitness gains (Novella, Duarte et al. 1995; Novella and Ebendick-Corpus 2004). However, one of the mutants (Monoclonal Antibody-Resistant Mutant C, or MARM C) that had recovered fitness after a regime of repeated bottleneck passages was shown to be consistently outcompeted by wild-type (Quer, Huerta et al. 1996) and it was unable to adapt to various environmental challenges (Quer, Hershey et al. 2001). To test whether this adaptability defect was a general consequence of repeated bottlenecks, some of the Muller's ratchet strains described above were chosen so that a range of starting fitness values were present: MRb (Muller's Ratchet strain b) had a neutral fitness (1.00 ± 0.05), MRy (Muller's Ratchet strain y) had a higher than normal fitness (1.23 ± 0.03), while MRd (Muller's Ratchet strain d) (0.82 ± 0.07), MRq (Muller's Ratchet strain q) (0.6 ± 0.1), MRr (Muller's Ratchet strain r) (0.43 ± 0.08), and MRm (Muller's Ratchet strain m) (0.15 ± 0.07) had a range of lower fitness values. The low-fitness replicas in the group were then passaged at large populations, so that they could recover in fitness to a neutral phenotype. A neutral fitness was desired so that

all virus strains would have similar starting points in the competitions, and the competitions would not be biased against the very low fitness phenotypes. The first significant observation was that MRr was unable to gain any fitness after six passages, indicating immediate adaptability problems. All other strains tested could be rescued up to neutral fitness in this time. The recovered replicas were then competed against wild-type VSV. In a long-term competition, one strain must eventually outcompete the other (Clarke, Duarte et al. 1994) as predicted by the competitive exclusion principle (Gause 1971). When starting from the same fitness level, each virus population should have an equal chance of excluding the other virus, as is seen with competitions between neutral virus MARM U and wild type. All the competitions carried out between wild-type and recovered neutral mutants (strains that had a history of bottlenecked passages) resulted in mutant extinction, even for a mutant such as MRy that initially had a slight fitness advantage. Typical results of these experiments are shown in figures 9 and 10:

Figure 9: Representative competition results between MARM U and wild-type VSV, depicting the change in ratio of MARM U to wild-type over time. Open and closed circles represent two independent competitions which are typical of all results. Overall, the results were not statistically different from MARM U winning 50% of the competitions, as is predicted (Novella 2004).

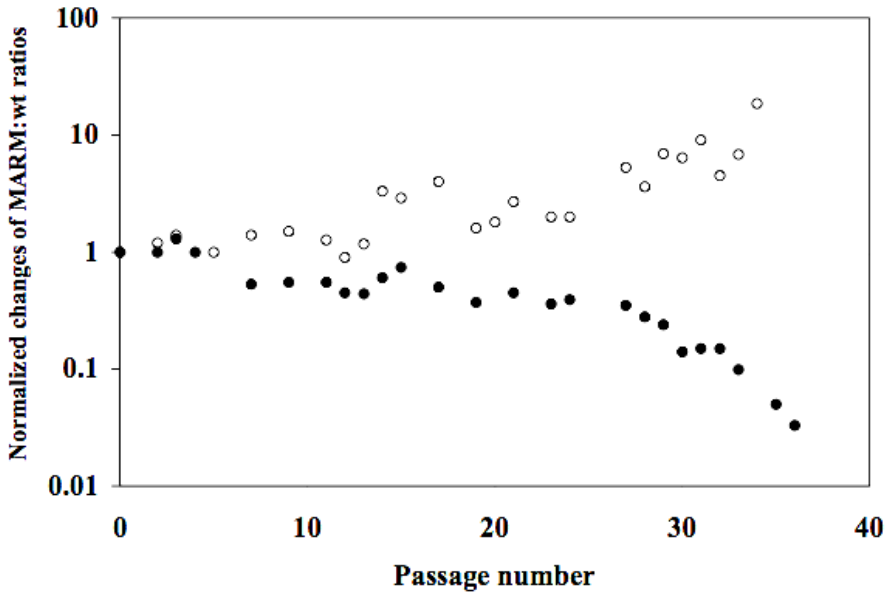
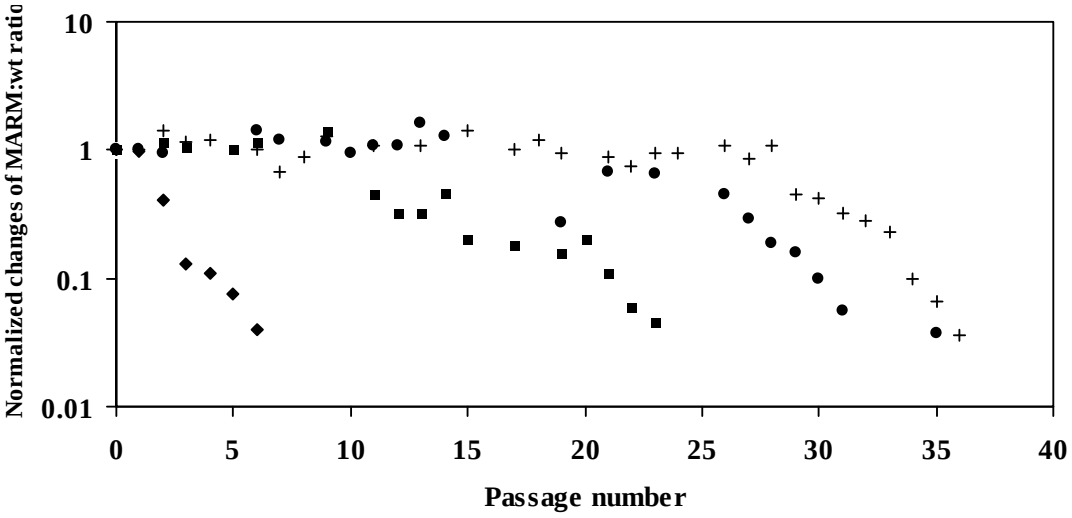


Figure 10: Representative competition results between bottlenecked and wild-type VSV strains, depicting the change in ratio of bottlenecked strain to wild-type over time. Each symbol represents a different strain that has undergone serial bottleneck passages, then recovery to a neutral fitness prior to competition with wild-type. Circles are MRb, squares are recovered MRd, crosses are recovered MRm, and diamonds are recovered MRq. All tested bottlenecked strains always lost in competitions with wild-type over the long-term (Novella 2004).



The inability to compete in the long-term that is seen in figure 10 suggests that bottlenecked strains suffer from adaptability defects in addition to their typical fitness losses. Also, the MRr strain was found unable to recover in fitness after 6 large population passages, pointing to adaptability problems so severe that the population

could not gain in fitness even when passaged without a competitor. Again, this result suggests that bottlenecking a virus strain could affect its adaptability.

In this study, I used quasispecies compositions to explore robustness in VSV populations. To gain an understanding why some viral strains have adaptability problems I selected two mutant populations (MRr and MRb) and two control populations (MARM U and wild-type) and analyzed their quasispecies composition and their behavior during adaptation. My first working hypothesis was that the adaptability problems that are found in bottlenecked virus strains could be due to a lower robustness. Robustness of a virus strain could be measured by determining the phenotypes of individuals within the quasispecies population. As described earlier, these clones would represent individual mutants of the parental virus, and a large number of deleterious variants would represent lower robustness. By measuring the phenotypes of the different quasispecies, I expected to find more low-fitness mutants and fewer high-fitness mutants in the bottlenecked, less-adaptable strains when compared to a strain with no adaptability issues, such as MARM U. My second hypothesis was that when evolving in isolation, strains with lower adaptability would gain less fitness than strains with higher adaptability. This hypothesis was tested by passaging many replicas of large population passages for both the test and control populations, and determining the fitness of the evolved viruses at different times depending on strain. By exploring the causes of adaptability problems in RNA viruses, it may be possible to use evolutionary techniques for making better vaccines, or reducing the likelihood of revertants.

MATERIALS AND METHODS

Cells

For all experiments carried out in this project, a line of baby hamster kidney cells, BHK-21, was used. These cells were supplied by John Holland, and are a standard cell line in VSV evolution studies because they allow for the rapid replication of VSV to very high titers. Cells were grown in minimal essential medium (MEM) supplemented with 7% heat-inactivated (60ºC, 30 min.) bovine calf serum (BCS), and 0.05% proteose peptone #3 (PP3) (Difco). For plaque assays, identical media was used except it did not contain any PP3, as PP3 inhibits viral replication. Virus stocks were generated in MEM containing no PP3, but containing 7% fetal bovine serum (FBS) instead of BCS. For neutralization, monoclonal antibody I1 was harvested from hybridoma cells, kindly provided by Douglas Lyles (Lefrancois and Lyles 1982).

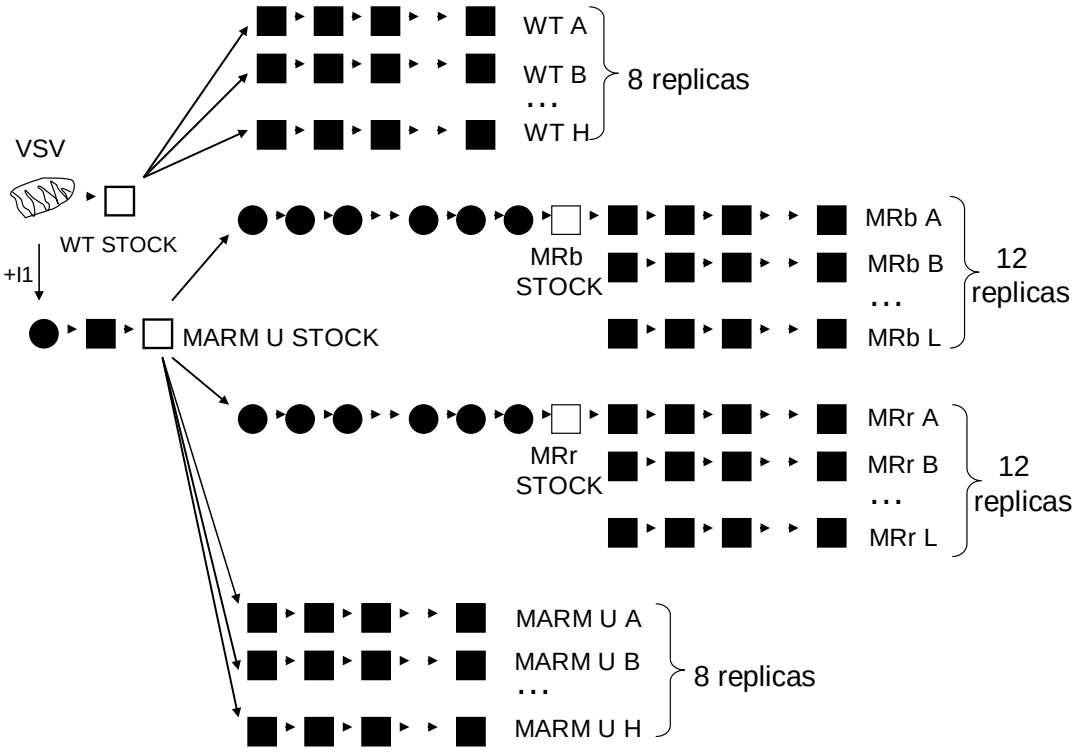
Viruses

All virus strains used in this project originated from an Indiana serotype, Mudd-Summers strain of wild-type VSV. To generate a population of genetically-marked VSV, wild-type was replicated in the presence of monoclonal antibody I1. The resulting clones were antibody-resistant, and were passaged twice at low multiplicity of infection to amplify the virus to a working stock. The resulting population is known as monoclonal antibody-resistant mutant U, or MARM U. Full genomic sequences show that MARM U differs from wild-type at only one nucleotide, U3853G, predicted to change amino acid 259 on the glycoprotein from

alanine to aspartic acid, and conferring resistance to the I1 antibody in the process (Holland, de la Torre et al. 1991). MARM U is neutral compared to wild-type (1.00 ± 0.05) (Novella 2004), and it can be used as a surrogate for wild-type virus during competition experiments with an antibody-sensitive strain of VSV.

MRb and MRr are two strains of VSV that had been previously isolated and characterized (Novella 2004; Novella and Ebendick-Corpus 2004). Both strains derive from MARM U after 20 plaque-to-plaque bottleneck passages. After this regime the last clone was amplified once in BHK-21 cells to produce a working stock. Fitness determinations showed that MRb was still neutral compared to wild-type (1.01 ± 0.05), even though it had accumulated two nucleotide substitutions: U4699C in the intergenic region between the G and L ORFs and a synonymous U7739C transition in the L ORF. MRr had a low-fitness phenotype when compared to wild-type (0.43 ± 0.08) and had accumulated three mutations, including synonymous transitions in the N ORF (G813A) and L ORF (C10491U), and a nonsynonymous substitution in the G ORF (U4399C). These strains were chosen because they each show adaptability problems. MRr shows immediate adaptability problems, as it is unable to gain in fitness when passaged alone for six passages. MRb was chosen because it has never outcompeted wild-type, even though it starts from a neutral fitness and would thus be predicted to win such a competition half of the time. A schematic for the histories of the virus strains used in this project is shown in figure 11:

Figure 11: History of viral strains used in this project. Black squares represent large population passages, black circles represent bottleneck passages, white squares represent stock population.



Fitness Assays

Fitness assays were performed as described previously (Holland, de la Torre et al. 1991). Essentially, a mixture of two viruses, one resistant and one susceptible

to monoclonal antibody, is used to infect a cell monolayer so the two viruses compete for resources. After cytopathic effect is complete, the progeny is diluted and used to initiate a second competition passage and, using the virus produced from this second passage, a third competition passage. In this experiment, wild-type is always used as the susceptible virus, and the other virus (MARM U, MRb, MRr) is resistant to the I1 antibody. The ratio of viruses added initially and obtained after each passage is measured in triplicate plaque assay in the presence and absence of monoclonal antibody. The changes in ratio can be quantified and plotted as the log of the ratio vs. time (passage number). The slope of this log-linear fit is the fitness value for the virus. Passages are done at low m.o.i. (0.1 plaque forming units/cell, or PFUs/cell) in order to prevent defective interfering particle (DIP) formation or complementation, which can alter fitness values (Wilke and Novella 2003; Wilke, Reissig et al. 2004). For clonal analysis competitions were done at m.o.i. of 0.01 was used to conserve virus stock, as only a limited amount of virus was available from each clone. Wild-type VSV was used as a reference virus for all fitness assays performed, except when evolved wild-type was tested. In this case, the stock of neutral MARM U was used as a reference virus.

To study the adaptation of the different virus strains, replicas of each strain (8 replicas for wild-type and MARM U, 12 replicas for MRb and MRr) were allowed to adapt and evolve on BHK-21 cells (Figure 11). Large population passages are carried out in T-25 flasks at low multiplicity of infection (m.o.i.) to avoid the emergence of DIPs (Holland 1991). Each replica was passaged alone in a T-25 flask containing a

monolayer of 2 x 10^6 BHK-21 cells. The viruses were passaged at a population size of 2 x 10^5 PFUs to yield an m.o.i. of 0.1 PFUs/cell. After 22-24 hours, when complete cytopathic effect and maximum titer (approximately 10^{10} PFU/mL) was reached, the progeny virus was removed and diluted, and then used to infect a new T-25 monolayer of BHK-21 cells under the same conditions as above. This process was continued for the specified number of passages (up to 25 passages for MRr, up to 50 passages for all other strains), and stocks of each replica were kept for further analysis. Fitness values for evolved replicas were determined at the following passages: wild-type at passages 25 and 50, MARM U at passages 25 and 50, MRb at passages 25 and 50, MRr at passages 10 and 25. Fitness changes in MRr were compared to fitness changes in MARM N because low-fitness strains tend to increase in fitness faster than neutral strains. MARM N had a fitness of 0.38 ± 0.01, similar to that of MRr (Novella, Duarte et al. 1995), and a single mutation compared to MARM U that was a nonsynonymous transversion of A8700C in the L ORF (Novella and Ebendick-Corpus 2004).

Measuring Phenotypic Clonal Distributions

To determine the quasispecies composition of the strains under investigation, individual plaques from each population were characterized. Two phenotypic traits were determined: plaque size and plaque fitness.

Plaque size is relevant because it correlates well with fitness (Burch and Chao 2000) and measurement of plaque size is much easier and less time consuming than fitness determinations. To measure plaque sizes, three T-175 flasks were infected

with a stock of MARM U, three with a stock of MRb, and three with a stock of MRr. Stocks were diluted so that each flask contained fewer than 500 plaques. The infections were allowed to continue for 48 hours as opposed to the normal 24 hours to allow visualization of extremely small plaques, and thus limit the bias against weaker phenotypes that very tiny plaques could represent. After 48 hours, the flasks were developed with crystal violet solution (0.5% crystal violet, 25% ethanol, 75% water). Following development, photographs were taken of the flasks and then enlarged. Measurements of the diameter of each plaque in each flask were then taken, and the plaque sizes were recorded. Data from all three flasks in each set were pooled together for plaque totals. In total, 710 plaques were measured for MARM U, 354 plaques were measured for MRb, and 593 plaques were measured for MRr.

To isolate individual clones of each virus strain, first the virus stock was diluted in such a manner that each flask was inoculated with less than 10 PFU (usually 1-5 PFU). Infections were allowed to continue for 48 hours, again to allow for the visualization of smaller plaques and to minimize bias. Upon visualization, all well-isolated plaques were picked. A well-isolated plaque is defined as a plaque which is more than two diameters away from any other plaque. In this manner, 250 clones were selected from each of the stocks of the MARM U, MRb, and MRr virus populations. A fitness value for each clone was determined during competition against wild-type VSV as described previously.

Statistical Analysis

Linear regression calculations used for fitness data, as well as other statistical calculations, were performed using Statistical Packages for Social Sciences (SPSS) 15.0 (SPSS, Chicago).

RESULTS

Long-term Adaptation

In these experiments, virus strains with adaptability problems were explored to pinpoint a cause for these problems. For the first part of the experiment, replicas of four different virus strains (wild-type, MARM U, MRb, and MRr) were passaged on BHK-21 cells for up to 50 passages to promote adaptation. Wild-type and MARM U strains serve as control populations, as neither strain has been shown to have adaptability defects during competitions. Eight replicas of wild-type and MARM U and 12 replicas of MRb and MRr were used. Fitness determinations were taken at intervals for each replica, and these results are summarized in tables 1 and 2:

Table 1: Summary of fitness values for replicas of evolved virus populations after 25 large population passages. NA = Not Applicable. WT = wild-type. All P values are results of Student's t-test, with all means compared to the mean for wild-type replicas. P < 0.05 represents a statistically significant difference.

Replica	WT	MARM U	MRb	MRr 10	MRr 25
A	3.1 ± 0.3	1.9 ± 0.4	2.5 ± 0.4	0.72 ± 0.04	1.7 ± 0.2
B	4.4 ± 1.4	2.4 ± 0.5	2.6 ± 0.3	0.51 ± 0.07	2.3 ± 0.5
C	4.0 ± 0.4	4.6 ± 0.6	2.5 ± 0.6	0.97 ± 0.2	2.1 ± 0.4
D	4.5 ± 1.2	3.2 ± 0.2	2.0 ± 0.3	0.70 ± 0.04	1.3 ± 0.2
E	3.9 ± 2.1	6.1 ± 1.4	3.1 ± 0.3	0.89 ± 0.1	1.4 ± 0.2
F	2.6 ± 0.7	5.6 ± 0.9	4.2 ± 0.6	0.82 ± 0.1	1.47 ± 0.08
G	5.2 ± 1.8	4.1 ± 1.6	2.5 ± 0.4	0.47 ± 0.09	1.9 ± 0.5
H	5.6 ± 1.5	3.9 ± 0.6	2.3 ± 0.2	0.74 ± 0.1	1.43 ± 0.09
I	NA	NA	3.1 ± 0.4	0.53 ± 0.1	1.8 ± 0.3
J	NA	NA	2.0 ± 0.4	0.49 ± 0.08	1.8 ± 0.3
K	NA	NA	2.6 ± 0.2	0.72 ± 0.1	1.1 ± 0.1
L	NA	NA	2.9 ± 0.4	0.85 ± 0.08	2.8 ± 0.6
Average:	4.2 ± 0.5	4.0 ± 0.3	2.7 ± 0.1 *	0.70 ± 0.03	1.8 ± 0.1 *
P:	-----	0.7367	0.0022	-----	0.0001

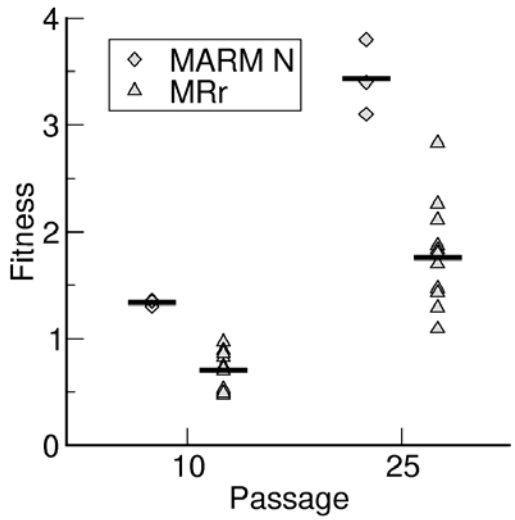
Table 2: Summary of fitness values for replicas of evolved virus populations after 50 large population passages. NA = Not Applicable. WT = wild-type. All P values are results of Student's t-test, with all means compared to the mean for wild-type replicas. P < 0.05 represents a statistically significant difference.

Replica	WT	MARM U	MRb
A	6.7 ± 0.5	6.7 ± 1.6	3.5 ± 0.4
B	6.2 ± 0.6	6.8 ± 2.1	2.6 ± 0.1
C	7.7 ± 3.2	9.4 ± 0.8	4.8 ± 1.1
D	7.1 ± 1.2	10.7 ± 1.5	4.5 ± 1.1
E	10.1 ± 2.1	6.56 ± 0.09	3.4 ± 0.2
F	8.6 ± 2.1	6.9 ± 1.9	9.7 ± 0.4
G	5.2 ± 0.2	8.0 ± 1.2	5.9 ± 2.5
H	8.1 ± 1.8	8.7 ± 1.4	6.0 ± 0.6
I	NA	NA	7.2 ± 1.1
J	NA	NA	6.2 ± 1.0
K	NA	NA	5.2 ± 1.7
L	NA	NA	6.8 ± 0.8
Average:	7.5 ± 0.6	8.0 ± 0.5	5.5 ± 0.3 *
P:	-----	0.5324	0.0041

These results show differences in fitness changes during large population passages for four strains of viruses: wild-type (WT), MARM U, MRb, and MRr. Strains with lower adaptability would not be able to reach the same levels of fitness gain as strains with normal adaptability. First, these results show the striking inability of MRr to gain in fitness. After 10 passages, there were still no replicas of MRr that had reached neutral fitness. This is unlike MARM N, which starts with a similar

fitness to MRr yet is able to gain fitness to levels that are above neutrality in the same amount of time, as seen in Figure 12:

Figure 12: Fitness gains in MARM N and MRr over time. Initial fitness: 0.38 ± 0.01 for MARM N (fitness data compiled from Novella, Duarte et al. 1995), 0.43 ± 0.08 for MRr.



This figure clearly shows that MARM N can gain in fitness normally when passaged alone, passing neutrality by 10 passages and obtaining a high-fitness phenotype by passage 25. This offers further support to correlate the failure of MRr to achieve such gains in fitness to its history of bottleneck events. Furthermore, there were still four out of the 12 replicas for which the fitness change was not statistically significant (replicas B, G, I and J). Low-fitness viruses typically gain in fitness very quickly

during large-population passages, so this result suggests severe adaptability defects in MRr.

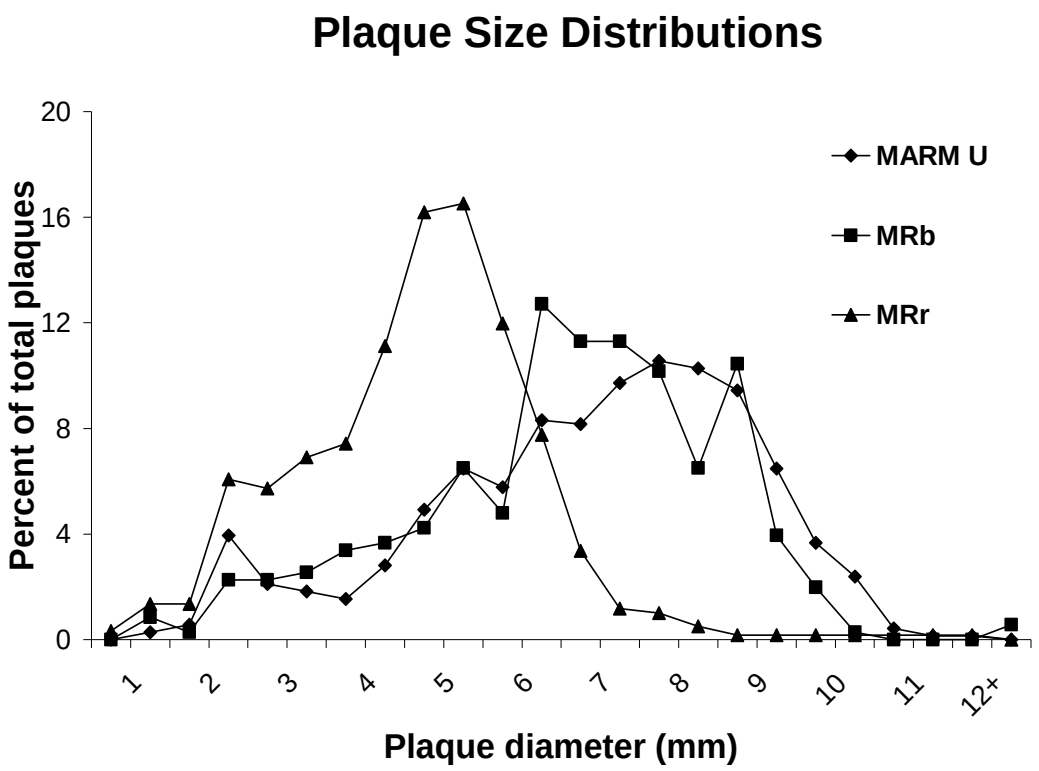
After 25 passages, the average fitness values of the replicas in each strain were as follows: 4.2 ± 0.5 for wild-type, 4.0 ± 0.3 for MARM U, 2.7 ± 0.1 for MRb, and 1.8 ± 0.1 for MRr. For comparison, others found that MARM N reaches fitness values of about 3 after 25 passages (Novella, Duarte et al. 1995). The student's t-test was used to measure the significance of any differences in the mean. According to the t-test, MARM U and wild-type are identical, but both MRb and MRr populations showed significantly lower fitness averages. These results confirm earlier findings that MRr has immediate adaptability problems (Novella 2004). However, MRb exhibits more subtle adaptability problems than MRr. MRb is able to sometimes compete against wild-type and survive after 25 and even 50 passages. Consistent with this result, some of the replicas of evolved MRb after 25 passages had fitness values that were similar to the fitness values of some wild type replicas. After an additional 25 passages fitness averages were again calculated for all replicas of wild-type, MARM U and MRb. Replica averages determined were 7.5 ± 0.6 for wild-type, 8.0 ± 0.5 for MARM U, and 5.5 ± 0.3 for MRb. Again, a t-test was implemented and it was found that only MRb had a significantly different average. Together, these results show that strains of MRb reached lower fitness values when adapted on BHK-21 cells under identical conditions when compared to wild-type and MARM U control strains. Once again, some of the replicas did gain as much fitness

as their wild-type and MARM U controls, as it would be expected from the fact that occasionally MRb still survives at passage 50 in competition.

Plaque Size Distributions

These results could possibly be explained by differences in robustness between the strains. At high deleterious mutation rates, less robust strains would accumulate more deleterious mutations than a strain of normal robustness, making it harder for the less robust virus to compete. The hypothesis was that wild-type and MARM U had normal robustness, while MRr had extremely low robustness, due to its inability to gain in fitness even outside of competitions. The long-term survival of MRb suggested that robustness of the clone would be the same as that of MARM U, and if any differences were observed, they would be small. Robustness was measured by assaying differences in phenotype, as mutations would be expected to cause small changes in viral phenotype. The first phenotype measured was plaque size. Generally speaking, viruses with a lower fitness will form smaller plaques. Thus, a sample of plaque sizes will provide an estimate for the overall distribution of beneficial or deleterious mutants in a virus population. The findings of this experiment are summarized in figure 13:

Figure 13: Plaque size distributions. Distribution of plaque sizes measured from three infected flasks for each strain. Total number of plaques for each strain: MARM U = 710, MRb = 354, MRr = 593.



This figure shows the differences in plaque sizes for the three virus strains. The average plaque size was 6.60 ± 0.07 mm for MARM U, 6.3 ± 0.1 for MRb, and 4.43 ± 0.06 for MRr. When a t-test is performed against the average of MARM U, which is the standard reference virus being used as a control, the results show that the average plaque size of both MRb and MRr are statistically different than the MARM U population with P values of 0.0139 and 0.0001 respectively. Also, the average

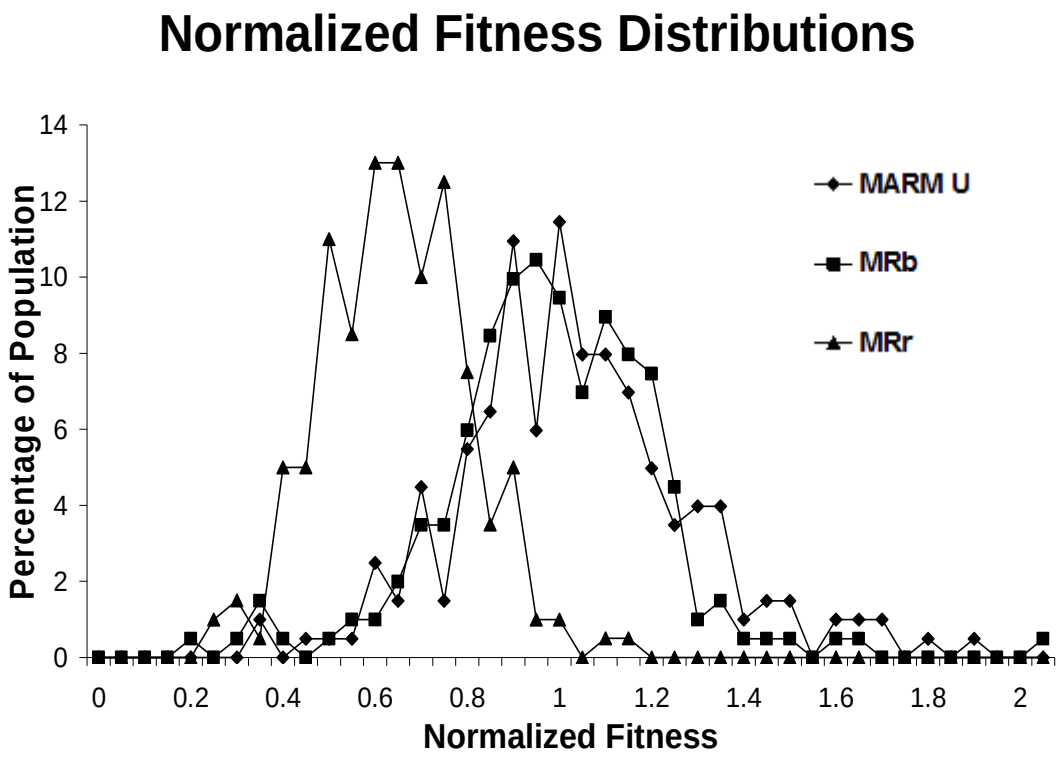
plaque sizes of MRb and MRr are significantly different from each other with a P value of 0.0001. While also statistically significant, the difference between MARM U and MRb was very small, as expected. Also as expected, the average fitness size was much smaller for MRr than for MARM U or MRb, but, surprisingly, in the distributions of plaque sizes, MRr has a higher frequency of average-sized plaques and a lower frequency of larger and smaller plaques, suggesting that MRr has a higher robustness than MRb and MARM U.

When normalized to average population plaque sizes, all three populations exhibit similar distributions (data not shown). In fact, MRr shows more larger-than-average plaques than MRb, and MRb shows more large plaques than MARM U. This implies that all three populations produce a majority of better-than-normal mutants and have similar robustness values. This finding could be explainable for MARM U and MRb, as both populations compete with wild-type similarly early in competitions and are both able to adapt normally at early passages. In contrast, the data shows that most of the clones of MRr are actually better than the average, which conflicts with previous findings of very low adaptability. These results do little to help explain why MRr has such problems adapting or explain differences between the strains, as the results seem to conflict previous results.

Fitness Distributions

Because of the conflicting results observed during the analysis of plaque sizes, a second experiment was designed to measure the actual fitness effects that random mutation had on the individual phenotypes of clones from each virus strain. Individual clones were isolated from three populations (MARM U, MRb, and MRr) and each clone was competed against an identical reference population of wild-type VSV. The resulting fitness values were than normalized to the average fitness values of each corresponding parental virus population (MARM U = 1.00 ± 0.05, MRb = 1.01 ± 0.05, MRr = 0.43 ± 0.08). The results can be seen in figure 14:

Figure 14: Distribution of clonal fitness values normalized against population fitness values. Total number of clones measured: MARM U = 201, MRb = 201, MRr = 200.



This figure shows the differences in normalized fitness values for the quasispecies of MARM U, MRb, and MRr. The average normalized fitness value was 1.01 ± 0.01 for MARM U, 0.96 ± 0.02 for MRb, and 0.62 ± 0.01 for MRr. Implementing the t-test against the average normalized fitness of MARM U yields P values of 0.0259 for MRb, and 0.0001 for MRr. These results are show that both MRb and MRr have significantly different normalized fitness averages than MARM

U. Additionally, MRr has a P value of 0.0001 when compared to MRb, meaning that it also has a significantly different average normalized fitness than MRb. However, the differences between MARM U and MRb were small, while differences between those two strains and MRr were much larger. There was also a major difference between the results from plaque size measurement and the results from fitness determinations. The average normalized fitness of clones (1.01 ± 0.01) was identical to the average fitness of the population for MARM U (1.00) and similar for MRb (0.96 ± 0.02 versus 1.00). In contrast, the average normalized fitness of MRr clones (0.62 ± 0.01) was significantly lower than the average fitness of the original population (1.00). This result clearly demonstrated that MRr has a lower robustness than MRb and MARM U. This implies that for the MRb and MRr distributions, a majority of clonal values are actually less than the mean fitness value, confirming earlier data that showed that most mutations are indeed deleterious (Duarte, Novella et al. 1994). This conflicts with the data on plaque size distributions, but can better explain the adaptability differences seen in these strains both during competitions and long-term adaptation studies. These results indicate that the quasispecies for MRb and MRr strains contain more deleterious variants when compared to the quasispecies of MARM U, and thus a lower robustness; MRb slightly more, and MRr a great deal more. It also illustrates the problems that using plaque size a surrogate of fitness may pose, as plaque size determinations seem to underestimate deleterious effects during competitions.

DISCUSSION

As an RNA virus, VSV experiences a high mutation rate that allows for rapid generation of variants, and thus rapid adaptation. However, this only holds true at large population sizes, when there are enough variants to ensure at least some beneficial mutations will be carried to the next infection. When a small population is transferred, the probability is high that a deleterious mutation will be fixed into the population or, at least, that the fitter mutants will be lost. This bottlenecking is biologically relevant, as often viruses are transmitted in small numbers. Bottlenecking can also have a large effect on adaptation through epistasis. The fixation of random mutations can alter the effects of additional mutations, preventing fitness gains. VSV strains with a history of bottlenecking previously produced in the lab have shown adaptability defects that are explored in this set of experiments.

First a quantification of fitness differences after adaptation was performed. This was done by adapting each virus strain to BHK-21 cells using large population sizes for infection. As discussed previously, the inclusion of large numbers of variants increases the probability of a beneficial high-fitness mutant being available for selection. Several replicas were performed for each strain (8 for wild-type and MARM U, 12 for MRb and MRr) to counter the effects of random drift. Wild-type is the standard reference strain of virus in studies of VSV, so its behavior is set as the control measurement. MARM U is a genetically-marked wild-type that has an identical sequence with the exception of the single nucleotide substitution that confers resistance to I1 antibody. In this experiment, the populations of MRb and MRr

originated from serial bottlenecking of MARM U clones, so that MARM U is an ancestor of the MRb and MRr strains. Previous experiments have shown that MARM U has a similar neutral fitness phenotype compared to wild-type and also can adapt and compete normally with wild-type (Novella 2004). Thus, MARM U also serves as a useful control in these experiments.

Past results with fitness competitions have shown that 25 passages give the virus population enough time to fix at least one mutation in most replicas, so that was the first point of measurement. After these first 25 passages, it was clear that differences could be seen between the virus strains. Wild-type and MARM U strains averaged statistically identical fitness values, but the fitness of evolved MRb was significantly lower than wild-type and MARM U, and the fitness of evolved MRr was significantly lower than all three other strains, as well as the fitness of MARM N evolved under the same conditions. This result confirms that MRr and MRb are exhibiting adaptability defects. This decrease in adaptability makes the virus strains less able to improve and gain in fitness dring adaptation, as is typical for VSV strains such as wild-type and MARM U. While the strains do increase in fitness, the increases are significantly less than those seen in the control strains of wild-type and MARM U. Also, MRr gains even less in fitness than MRb, and actually it did not gain any fitness at all in a single replica of six large-population passages (Novella 2004), and it does not always increase fitness significantly after as many as 10 large-population passages.

The evolution of MRb during long-term competitions against wild-type is predictable in that MRb is always the loser. However, the specific kinetics vary within replicas, and MRb can sometimes coexist with wild-type for more than 50 passages. Because MARM U reaches the top of its fitness peak at about passage 50, I chose this time point to look at differences in fitness among MARM U, wild-type and MRb. MRr was not carried out to this point as severe problems were already consistently seen at passage 10 and 25. As expected, at passage 50 there were no significant differences in fitness levels gained by the wild-type and MARM U populations. However, the average fitness gain of the MRb populations was significantly lower than that of both wild-type and MARM U.

The results of evolved virus fitness at passages 25 and 50 reinforce the findings that the bottlenecked strains (MRb and MRr) have adaptability defects that cause them to be unable to gain fitness as rapidly as control strains, wild-type and MARM U. Interestingly, one replica of MRb (MRbF) shows a fitness at passage 50 which is higher than the average fitness values seen in the wild-type and MARM U populations. This indicates that it is possible for these strains, or at least MRb, to obtain the types of fitness gains seen in strains without adaptability defects. However it also appears to be less likely to occur. Also worth noting is that in previous experiments, MRb could last during some replicas to passage 60 before losing out in competition to wild-type (Novella 2004). Thus, the presence of one high-fitness replica does not disprove the observation that these bottlenecked strains have an overall adaptability defect.

Once the adaptability defects of MRr and MRb were confirmed and quantified, the next question was what led to these differences. The simplest explanation is that MRr and MRb have increased fidelity and their polymerases do not introduce as many mutations as the polymerases of wild-type and MARM U. Since adaptation to the environment is based on the selection of beneficial mutants, if the genome produces less mutants overall, MRr and MRb would not be able to generate enough mutations, including beneficial mutations. This mechanism leading to adaptability defects has been shown in previous experiments (Vignuzzi, Stone et al. 2006). However, I ruled out this explanation because the polymerase complexes of MRr and MRb can be assumed to be identical to those of wild-type and MARM U, due to the lack of any mutations predicted to alter amino acid sequences of polymerase proteins. Thus, adaptability differences must be the result of differences in the beneficial mutation rates. Changes in the beneficial mutation rate can be the result of fewer beneficial mutations been generated or beneficial mutations that are generated in similar numbers, but with lower value (e.g. less beneficial). These two explanations are not mutually exclusive, and can be tested by determining the number of mutations accumulated in the consensus sequence of each evolved replica. Virus samples from each replica at passage 25 and passage 50 were taken. The full-length consensus sequence of all 56 strains was determined with the assistance of lab members Ranendra N. Dutta, Sarah D. Smith, Bonnie E. Ebendick-Corpus and Siming Yang, and the sequences of the evolved genomes were then compared with the consensus sequences of the original populations. Pending confirmation of a few

genomic regions in some of the replicas, averages were taken for the number of mutations per genome at both passage 25 and passage 50. At passage 25, these averages were 1.0 ± 0.3 for wild-type, 1.8 ± 0.5 for MARM U, and 1.4 ± 0.6 for MRr. The averages of MARM U and MRr were not found to be significantly different than the average of wild-type, with P values of 0.1917 and 0.6143, respectively. At passage 50, the mutation averages were 3.8 ± 0.7 for wild-type, 5.6 ± 0.4 for MARM U, and 3.4 ± 0.5 for MRb. While the number of mutations at passage 50 is significantly higher for MARM U than for wild-type (P = 0.0424), it is insignificant when MRb is compared to wild-type (P = 0.6382). This data showed that at both passages 25 and 50, both MRb and MRr exhibited statistically identical rates of mutation to that of wild-type. This suggests that both bottlenecked strains are not deficient in their ability to generate beneficial mutations to promote adaptation. An alternative explanation is that these beneficial mutations are not as beneficial in MRb or MRr background as they are in MARM U or wild-type backgrounds, and this change in the value of beneficial mutations can explained by epistasis. Epistatic interactions represent non-additive effects and can cause mutations to alter the phenotypic effects of other mutations in the genome.

Extensive epistasis was confirmed with the results from fitness distributions, which showed that the relative frequencies of deleterious, neutral and beneficial mutations were different in the different strains. Particularly, deleterious mutants were significantly overrepresented in the case of MRr, indicating that more mutations

in the MRr background have a deleterious effect and that the deleterious effects are more pronounced.

Plaque size was generally a good predictor of fitness. MRr generally had a smaller plaque phenotype when compared to MARM U or MRb. Additionally, MRb also appears to have statistically smaller plaques than MARM U, although the MRb plaques are still statistically larger than plaques from the MRr population. This had been estimated in past work with MRr (Novella 2004), but had never been quantified. These results are not surprising, because MRr has a much lower starting fitness (0.43 ± 0.08) than MARM U (1.00 ± 0.05), and thus is expected to have smaller plaques as a result. Interestingly, during the experiments MRb plaques appeared to be nearly identical to the plaques from MARM U. However, this test showed there was a small yet statistically significant difference between the two plaque sizes, and that plaques from MRb are indeed also smaller on average than plaques from MARM U. This result was not completely surprising because the averages from distributions were within the error of fitness measurements for MRb and MARM U, 1.01 ± 0.05 and 1.00 ± 0.05, respectively.

While plaque size may be a reasonable estimate of fitness, some of the results from the analysis of plaque size distributions were puzzling. First, the finding that most clones were better than the average contradicted previous findings (Duarte, Novella et al. 1994); second, the apparent higher robustness of MRr compared to MRb and MARM U was difficult to correlate with MRr's inability to increase in fitness during large-population passages. Therefore, the fitness values of individual

clones from each quasispecies were tested. MRr and MRb clones were individually isolated, with care taken to ensure that extremely small plaques were not overlooked or biased against, and each clone was competed against the laboratory reference strain of wild-type. Again, MARM U served as a control quasispecies in this experiment, as it is neutral compared to wild-type, exhibits no adaptability problems, and is the ancestor of both MRb and MRr. The fitness values obtained from this experiment were normalized against the population values. This was done to standardize differences between the strains; since MRr is a low-fitness strain, it inherently has a lower-fitness distribution than the two neutral strains tested. By normalizing clonal fitness values, it is also simpler to measure departures from the population fitness value, as absolute values will represent a different fraction of change depending on the original value. These results are plotted in figure 14. Again, the quasispecies of MRr clearly has a different distribution when compared to MRb and MARM U. Statistical analysis confirms this result, as the quasispecies of MRr has significantly lower average normalized fitness than either MARM U or MRb. Interestingly, the quasispecies of MRb also has significantly lower average normalized fitness compared to MARM U, although not nearly at the scale as seen in MRr. This suggests that even at an early stage where MRb does not exhibit phenotypic adaptability differences compared to MARM U, fitness distributions can point to a slightly lowered robustness, and perhaps predict more severe problems later in competitions or adaptability experiments.

The results of fitness determinations emphasize the importance of considering the viral populations as a whole and the limitations of using plaque size measurements as surrogate of fitness. Plaque size distributions suggested a higher robustness in MRr than in MARM U and MRb, and they also indicated that most mutations in all three strains are neutral or beneficial. Fitness distributions showed that these conclusions are incorrect and the advantages of measuring plaques compared to measuring fitness are insufficient to make it the method of choice.

One more result that needed explanation was the consistent extinction of MRb in long-term competitions when it survived passage 50. At this time, MRb should have gained as much fitness as wild-type and would have a 50:50 change of becoming dominant, but that never happened. A plausible explanation is that at passage 50 the two populations are at the top of their fitness peaks and there will be no further fitness gains because beneficial variation is exhausted. Instead, survival will depend on robustness to the effect of deleterious mutations, so strains that can generate mutations with a lower deleterious effect will win the competition. To test this hypothesis Kim Lust, a summer student in the laboratory, selected evolved virus strains and carried out mutation-accumulation experiments. Mutation-accumulation experiments are an alternative to the determination of fitness distributions when testing the robustness of a virus. During plaque-to-plaque passages mutations accumulate randomly, so their more beneficial or more deleterious effects will translate into a lower or higher level of fitness change. Two strains of evolved MARM U (G and H) and three strains of evolved MRb (F, I, and L) were isolated

after 50 large population passages. Each strain was then passaged through serial plaque-to-plaque bottlenecking. This process was continued for 20 passages, and with six replicas of each sample. After 20 passages, the progeny of each replica from each strain was competed against wild-type, and fitness values were obtained. Again, these results were normalized to starting values to be able to see relative changes in fitness during the experiment. These results are displayed in table 3:

Table 3: Normalized fitness results after serial bottlenecking. Two evolved MARM U strains (MARM U_G and MARM $_H$) and three evolved MRb strains (MRb_F, MRb_I, and MRb_L) were subjected to 20 plaque-to-plaque transfers. Six replicas were performed for each strain, and fitness values were determined and normalized against the fitness of the original population.

	MARM U_G	MARM U_H	MRb_L	MRb_I	MRb_F
A	**0.115**	**0.337**	**0.110**	**0.536**	**0.268**
B	**0.203**	**0.316**	**0.269**	**0.516**	**0.233**
C	**0.529**	**0.349**	**0.086**	**0.146**	**0.100**
D	**0.354**	**0.534**	**0.107**	**0.038**	**0.108**
E	**0.892**	**0.613**	**0.087**	**0.583**	**0.519**
F	**0.898**	**1.047**	**0.457**	**0.207**	**0.608**
Avg.	**0.50 ± 0.14**	**0.53 ± 0.11**	**0.19 ± 0.06**	**0.34 ± 0.10**	**0.31 ± 0.09**

These values represent the residual fitness for each strain compared to their ancestors, so a value of 1 indicates no fitness change, a value larger than 1 indicates fitness gains, and an average between zero and 1 indicate fitness losses. All averages

and most individual values were significantly below 1. This is expected, especially as all strains tested already had high fitness values. Comparison of the progeny of MRbL and MRbI with MARM U controls was marginally significant at best, but comparison between all of the MARM U-derived and all MRb-derived populations showed average values of 0.52 ± 0.09 for MARM U strains and 0.28 ± 0.05 for MRb strains, and these averages are significantly different from each other, with a P value of 0.0179 according to the student's t-test.

This experiment supports many findings. First, it again demonstrates that bottlenecking a virus typically causes deleterious accumulations, especially if the original virus is already of high fitness. This has been seen and reported previously (Novella, Elena et al. 1995). Second, this experiment supports previous findings that bottlenecking events are highly stochastic in nature (Novella 2004), in that a number of randomly determined events may happen at any passage. This is supported because there is a wide range of fitness losses, and even one instance of a slight fitness gain, experienced by the bottlenecked replicas. More importantly, this experiment helps to support the hypothesis that the adaptability defects seen in bottlenecked strains are due, at least in part, to problems with robustness. These viruses were taken after 50 adaptation passages, a time at which any MRb strains that have not already lost are optimized. So it is at this time that MRb will ultimately exhibit its lower-adaptability phenotype. The bottlenecking thus serves as a rough estimate of the effect mutations have in the quasispecies at passage 50. Because the mutants are chosen at random, accumulation of deleterious mutants with lower fitness

implies that there are more low-fitness mutants present in the quasispecies. This result confirms out hypothesis that long-term survival of the virus, when beneficial variation is exhausted, depends on the robustness of the genomes.

This work shows that the accumulation of mutations during bottlenecking has effects that go beyond the immediate fitness losses that result from the operation of Muller's ratchet. Selection of mutations during bottleneck regimes is relaxed, and mutations are allowed to accumulate that lower the robustness of the virus. Complementation during high m.o.i. infections also relaxes selection (see "Complementation" section) and the result is also the survival of less robust genomes that would otherwise be eliminated (Montville, Froissart et al. 2005). These results confirm that quasispecies theory is a better descriptor of RNA virus evolutionary dynamics than general mutation-selection balance models developed for DNA-based organisms. The evolution and adaptation of bottlenecked strains is further compromised by their lower beneficial mutation rates. Lower mutation rates are not the result of lower overall mutation rates or fewer beneficial mutations, but of similar number of mutations that have a lower beneficial effect. The overall behavior and loss of adaptability and robustness after bottlenecking can best be explained assuming extensive epistatic interactions along the genome, so unselected mutations accumulated in bottlenecked populations change the value of other mutations generated during replication. Widespread epistasis has been reported in live-attenuated strains of several viruses (Burch, Turner et al. 2003) and in HIV-1 (Bonhoeffer, Chappey et al. 2004).

Understanding the molecular basis of adaptability and robustness may be useful for the development of safer live-attenuated vaccines. The most important problem of these vaccines is their ability to revert and cause disease in vaccine recipients (Cann, Stanway et al. 1984). Because attenuation often correlates with fitness loss, and virulence with fitness recovery, mutations that affect robustness and adaptability could be used to generate safer vaccines that are crippled in their ability to recover.

REFERENCES

Aaskov, J., K. Buzacott, et al. (2006). "Long-term transmission of defective RNA viruses in humans and Aedes mosquitoes." Science **311**(5758): 236-8.

Abraham, G. and A. K. Banerjee (1976). "Sequential transcription of the genes of vesicular stomatitis virus." Proc Natl Acad Sci U S A **73**(5): 1504-8.

Ahmed, M. and D. S. Lyles (1998). "Effect of vesicular stomatitis virus matrix protein on transcription directed by host RNA polymerases I, II, and III." J Virol **72**(10): 8413-9.

Banerjee, A. K. (1987). "Transcription and replication of rhabdoviruses." Microbiol Rev **51**(1): 66-87.

Banks, J., E. Speidel, et al. (1998). "Characterisation of an avian influenza A virus isolated from a human--is an intermediate host necessary for the emergence of pandemic influenza viruses?" Arch Virol **143**(4): 781-7.

Beach, J. W. (1998). "Chemotherapeutic agents for human immunodeficiency virus infection: mechanism of action, pharmacokinetics, metabolism, and adverse reactions." Clin Ther **20**(1): 2-25; discussion l.

Black, B. L. and D. S. Lyles (1992). "Vesicular stomatitis virus matrix protein inhibits host cell-directed transcription of target genes in vivo." J Virol **66**(7): 4058-64.

Blondel, D., G. G. Harmison, et al. (1990). "Role of matrix protein in cytopathogenesis of vesicular stomatitis virus." J Virol **64**(4): 1716-25.

Bonhoeffer, S., C. Chappey, et al. (2004). "Evidence for positive epistasis in HIV-1." Science **306**(5701): 1547-50.

Bright, R. A., D. K. Shay, et al. (2006). "Adamantane resistance among influenza A viruses isolated early during the 2005-2006 influenza season in the United States." JAMA **295**(8): 891-4.

Bull, J. J., L. A. Meyers, et al. (2005). "Quasispecies made simple." PLoS Comput Biol **1**(6): e61.

Burch, C. L. and L. Chao (2000). "Evolvability of an RNA virus is determined by its mutational neighbourhood." Nature **406**(6796): 625-8.

Burch, C. L., P. E. Turner, et al. (2003). "Patterns of epistasis in RNA viruses: a review of the evidence from vaccine design." J Evol Biol **16**(6): 1223-35.

Cann, A. J., G. Stanway, et al. (1984). "Reversion to neurovirulence of the live-attenuated Sabin type 3 oral poliovirus vaccine." Nucleic Acids Res **12**(20): 7787-92.

Canter, D. M. and J. Perrault (1996). "Stabilization of vesicular stomatitis virus L polymerase protein by P protein binding: a small deletion in the C-terminal domain of L abrogates binding." Virology **219**(2): 376-86.

Chao, L. (1990). "Fitness of RNA virus decreased by Muller's ratchet." Nature **348**(6300): 454-5.

Chen, J. L., T. Das, et al. (1997). "Phosphorylated states of vesicular stomatitis virus P protein in vitro and in vivo." Virology **228**(2): 200-12.

Chen, W. J., H. R. Wu, et al. (2003). "E/NS1 modifications of dengue 2 virus after serial passages in mammalian and/or mosquito cells." Intervirology **46**(5): 289-95.
Cheung, C. L., J. M. Rayner, et al. (2006). "Distribution of amantadine-resistant H5N1 avian influenza variants in Asia." J Infect Dis **193**(12): 1626-9.
Chong, L. D. and J. K. Rose (1993). "Membrane association of functional vesicular stomatitis virus matrix protein in vivo." J Virol **67**(1): 407-14.
Clarke, D. K., E. A. Duarte, et al. (1994). "The red queen reigns in the kingdom of RNA viruses." Proc Natl Acad Sci U S A **91**(11): 4821-4.
Clarke, D. K., E. A. Duarte, et al. (1993). "Genetic bottlenecks and population passages cause profound fitness differences in RNA viruses." J Virol **67**(1): 222-8.
Colonno, R. J. and A. K. Banerjee (1978). "Complete nucleotide sequence of the leader RNA synthesized in vitro by vesicular stomatitis virus." Cell **15**(1): 93-101.
Cooper, L. A. and T. W. Scott (2001). "Differential evolution of eastern equine encephalitis virus populations in response to host cell type." Genetics **157**(4): 1403-12.
Cortay, J. C., D. Gerlier, et al. (2006). "Selection of single-chain antibodies that specifically interact with vesicular stomatitis virus (VSV) nucleocapsid and inhibit viral RNA synthesis." J Virol Methods **131**(1): 16-20.
Crotty, S., C. E. Cameron, et al. (2001). "RNA virus error catastrophe: direct molecular test by using ribavirin." Proc Natl Acad Sci U S A **98**(12): 6895-900.
Crotty, S., D. Maag, et al. (2000). "The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen." Nat Med **6**(12): 1375-9.
Daniel, N., V. Schneider, et al. (2003). "Emergence of HIV-1 mutated strains after interruption of highly active antiretroviral therapy in chronically infected patients." AIDS **17**(14): 2126-9.
Das, T., A. K. Gupta, et al. (1995). "Role of cellular casein kinase II in the function of the phosphoprotein (P) subunit of RNA polymerase of vesicular stomatitis virus." J Biol Chem **270**(41): 24100-7.
Das, T., A. K. Pattnaik, et al. (1997). "Basic amino acid residues at the carboxy-terminal eleven amino acid region of the phosphoprotein (P) are required for transcription but not for replication of vesicular stomatitis virus genome RNA." Virology **238**(1): 103-14.
de Jong, M. D., T. T. Tran, et al. (2005). "Oseltamivir resistance during treatment of influenza A (H5N1) infection." N Engl J Med **353**(25): 2667-72.
de la Pena, M., S. F. Elena, et al. (2000). "Effect of deleterious mutation-accumulation on the fitness of RNA bacteriophage MS2." Evolution Int J Org Evolution **54**(2): 686-91.
de la Torre, J. C. and J. J. Holland (1990). "RNA virus quasispecies populations can suppress vastly superior mutant progeny." J Virol **64**(12): 6278-81.

de Visser, J. A., J. Hermisson, et al. (2003). "Perspective: Evolution and detection of genetic robustness." Evolution Int J Org Evolution **57**(9): 1959-72.
Domingo, E., L. Menendez-Arias, et al. (1997). "Viral quasispecies and the problem of vaccine-escape and drug-resistant mutants." Prog Drug Res **48**: 99-128.
Doms, R. W., A. Ruusala, et al. (1988). "Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein." J Cell Biol **107**(1): 89-99.
Drake, J. W. and J. J. Holland (1999). "Mutation rates among RNA viruses." Proc Natl Acad Sci U S A **96**(24): 13910-3.
Duarte, E. A., I. S. Novella, et al. (1994). "Subclonal components of consensus fitness in an RNA virus clone." J Virol **68**(7): 4295-301.
Eigen, M. (1992). Steps Toward Life. Oxford, Oxford University Press.
Eigen, M. (1996). "On the nature of virus quasispecies." Trends Microbiol **4**(6): 216-8.
Emerson, S. U. (1982). "Reconstitution studies detect a single polymerase entry site on the vesicular stomatitis virus genome." Cell **31**(3 Pt 2): 635-42.
Escarmis, C., M. Davila, et al. (1996). "Genetic lesions associated with Muller's ratchet in an RNA virus." J Mol Biol **264**(2): 255-67.
Farci, P., A. Shimoda, et al. (2000). "The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies." Science **288**(5464): 339-44.
Fredericksen, B. L. and M. A. Whitt (1995). "Vesicular stomatitis virus glycoprotein mutations that affect membrane fusion activity and abolish virus infectivity." J Virol **69**(3): 1435-43.
Gaudier, M., Y. Gaudin, et al. (2002). "Crystal structure of vesicular stomatitis virus matrix protein." Embo J **21**(12): 2886-92.
Gause, G. F. (1971). The Struggle For Existence. New York City, Dover Publications.
Gorman, O. T., W. J. Bean, et al. (1992). "Evolutionary processes in influenza viruses: divergence, rapid evolution, and stasis." Curr Top Microbiol Immunol **176**: 75-97.
Graci, J. D. and C. E. Cameron (2002). "Quasispecies, error catastrophe, and the antiviral activity of ribavirin." Virology **298**(2): 175-80.
Grdzelishvili, V. Z., S. Smallwood, et al. (2005). "A single amino acid change in the L-polymerase protein of vesicular stomatitis virus completely abolishes viral mRNA cap methylation." J Virol **79**(12): 7327-37.
Green, T. J. and M. Luo (2006). "Resolution improvement of X-ray diffraction data of crystals of a vesicular stomatitis virus nucleocapsid protein oligomer complexed with RNA." Acta Crystallogr D Biol Crystallogr **62**(Pt 5): 498-504.
Greene, I. P., E. Wang, et al. (2005). "Effect of alternating passage on adaptation of sindbis virus to vertebrate and invertebrate cells." J Virol **79**(22): 14253-60.
Gubareva, L. V., L. Kaiser, et al. (2000). "Influenza virus neuraminidase inhibitors." Lancet **355**(9206): 827-35.

Gubareva, L. V., M. N. Matrosovich, et al. (1998). "Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus." J Infect Dis **178**(5): 1257-62.
Gupta, A. K., D. Shaji, et al. (2003). "Identification of a novel tripartite complex involved in replication of vesicular stomatitis virus genome RNA." J Virol **77**(1): 732-8.
Hatta, M., P. Gao, et al. (2001). "Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses." Science **293**(5536): 1840-2.
Hatta, M. and Y. Kawaoka (2002). "The continued pandemic threat posed by avian influenza viruses in Hong Kong." Trends Microbiol **10**(7): 340-4.
Holland, J. (1991). Defective viral genomes. Fundamental Virology. B. F. a. D. Knipe. New York, Raven Press**:** 151-165.
Holland, J., K. Spindler, et al. (1982). "Rapid evolution of RNA genomes." Science **215**(4540): 1577-85.
Holland, J. J., J. C. de la Torre, et al. (1991). "Quantitation of relative fitness and great adaptability of clonal populations of RNA viruses." J Virol **65**(6): 2960-7.
Holland, J. J., E. Domingo, et al. (1990). "Mutation frequencies at defined single codon sites in vesicular stomatitis virus and poliovirus can be increased only slightly by chemical mutagenesis." J Virol **64**(8): 3960-2.
Holt, C. A. and R. N. Beachy (1991). "In vivo complementation of infectious transcripts from mutant tobacco mosaic virus cDNAs in transgenic plants." Virology **181**(1): 109-17.
Huggins, J. W. (1989). "Prospects for treatment of viral hemorrhagic fevers with ribavirin, a broad-spectrum antiviral drug." Rev Infect Dis **11 Suppl 4**: S750-61.
Hunt, D. M. and K. L. Hutchinson (1993). "Amino acid changes in the L polymerase protein of vesicular stomatitis virus which confer aberrant polyadenylation and temperature-sensitive phenotypes." Virology **193**(2): 786-93.
Hurt, A. C., P. Selleck, et al. (2007). "Susceptibility of highly pathogenic A(H5N1) avian influenza viruses to the neuraminidase inhibitors and adamantanes." Antiviral Res **73**(3): 228-31.
Hwang, L. N., N. Englund, et al. (1999). "Optimal replication activity of vesicular stomatitis virus RNA polymerase requires phosphorylation of a residue(s) at carboxy-terminal domain II of its accessory subunit, phosphoprotein P." J Virol **73**(7): 5613-20.
Jayakar, H. R. and M. A. Whitt (2002). "Identification of two additional translation products from the matrix (M) gene that contribute to vesicular stomatitis virus cytopathology." J Virol **76**(16): 8011-8.
Johnson, V. A., F. Brun-Vezinet, et al. (2007). "Update of the drug resistance mutations in HIV-1: 2007." Top HIV Med **15**(4): 119-25.
Kassen, R. and P. B. Rainey (2004). "The ecology and genetics of microbial diversity." Annu Rev Microbiol **58**: 207-31.

Kersten, G., T. Hazendonk, et al. (1999). "Antigenic and immunogenic properties of inactivated polio vaccine made from Sabin strains." Vaccine **17**(15-16): 2059-66.
Kew, O. M., R. W. Sutter, et al. (2005). "Vaccine-derived polioviruses and the endgame strategy for global polio eradication." Annu Rev Microbiol **59**: 587-635.
Kiso, M., K. Mitamura, et al. (2004). "Resistant influenza A viruses in children treated with oseltamivir: descriptive study." Lancet **364**(9436): 759-65.
Kopecky, S. A. and D. S. Lyles (2003). "The cell-rounding activity of the vesicular stomatitis virus matrix protein is due to the induction of cell death." J Virol **77**(9): 5524-8.
Kretzschmar, E., R. Peluso, et al. (1996). "Normal replication of vesicular stomatitis virus without C proteins." Virology **216**(2): 309-16.
Kurosaki, M., N. Enomoto, et al. (1994). "Evolution and selection of hepatitis C virus variants in patients with chronic hepatitis C." Virology **205**(1): 161-9.
Lalezari, J. P., K. Henry, et al. (2003). "Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America." N Engl J Med **348**(22): 2175-85.
Lamb, R. A. and R. M. Krug (1996). Paramyxoviridae: The Viruses and Their Replication. Field's Virology, 3rd edition. B. N. Fields, Lippincott-Raven. **1:** 1177-1204.
Lawson, N. D., E. A. Stillman, et al. (1995). "Recombinant vesicular stomatitis viruses from DNA." Proc Natl Acad Sci U S A **92**(10): 4477-81.
Le, Q. M., M. Kiso, et al. (2005). "Avian flu: isolation of drug-resistant H5N1 virus." Nature **437**(7062): 1108.
Lefrancois, L. and D. S. Lyles (1982). "The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. I. Analysis of neutralizing epitopes with monoclonal antibodies." Virology **121**(1): 157-67.
Leslie, A. J., K. J. Pfafferott, et al. (2004). "HIV evolution: CTL escape mutation and reversion after transmission." Nature Medicine **10**: 282-289.
Loeb, L. A., J. M. Essigmann, et al. (1999). "Lethal mutagenesis of HIV with mutagenic nucleoside analogs." Proc Natl Acad Sci U S A **96**(4): 1492-7.
Manzin, A., L. Solforosi, et al. (1998). "Evolution of hypervariable region 1 of hepatitis C virus in primary infection." J Virol **72**(7): 6271-6.
Mashanov, G. I., D. Tacon, et al. (2004). "The spatial and temporal dynamics of pleckstrin homology domain binding at the plasma membrane measured by imaging single molecules in live mouse myoblasts." J Biol Chem **279**(15): 15274-80.
Matlin, K. S., H. Reggio, et al. (1982). "Pathway of vesicular stomatitis virus entry leading to infection." J Mol Biol **156**(3): 609-31.
Mead, D. G., C. J. Mare, et al. (1999). "Bite transmission of vesicular stomatitis virus (New Jersey serotype) to laboratory mice by Simulium vittatum (Diptera: Simuliidae)." J Med Entomol **36**(4): 410-3.

Mead, D. G., F. B. Ramberg, et al. (2000). "Transmission of vesicular stomatitis virus from infected to noninfected black flies co-feeding on nonviremic deer mice." Science **287**(5452): 485-7.
Montville, R., R. Froissart, et al. (2005). "Evolution of mutational robustness in an RNA virus." PLoS Biol **3**(11): e381.
Moreno, I. M., J. M. Malpica, et al. (1997). "A mutation in tomato aspermy cucumovirus that abolishes cell-to-cell movement is maintained to high levels in the viral RNA population by complementation." J Virol **71**(12): 9157-62.
Morimoto, K., D. C. Hooper, et al. (1998). "Rabies virus quasispecies: implications for pathogenesis." Proc Natl Acad Sci U S A **95**(6): 3152-6.
Muller, H. J. (1964). "The Relation of Recombination to Mutational Advance." Mutat Res **106**: 2-9.
Najera, I., A. Holguin, et al. (1995). "Pol gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy." J Virol **69**(1): 23-31.
Nelson, M. I. and E. C. Holmes (2007). "The evolution of epidemic influenza." Nat Rev Genet **8**(3): 196-205.
Neumann, G., M. A. Whitt, et al. (2002). "A decade after the generation of a negative-sense RNA virus from cloned cDNA - what have we learned?" J Gen Virol **83**(Pt 11): 2635-62.
Nichol, K. L. (2003). "The efficacy, effectiveness and cost-effectiveness of inactivated influenza virus vaccines." Vaccine **21**(16): 1769-75.
Nielsen, H. S., M. B. Oleksiewicz, et al. (2001). "Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations." J Gen Virol **82**(Pt 6): 1263-72.
Novella, I. S. (2003). "Contributions of vesicular stomatitis virus to the understanding of RNA virus evolution." Curr Opin Microbiol **6**(4): 399-405.
Novella, I. S. (2004). "Negative effect of genetic bottlenecks on the adaptability of vesicular stomatitis virus." J Mol Biol **336**(1): 61-7.
Novella, I. S., E. Domingo, et al. (1995). "Rapid viral quasispecies evolution: implications for vaccine and drug strategies." Mol Med Today **1**(5): 248-53.
Novella, I. S., E. A. Duarte, et al. (1995). "Exponential increases of RNA virus fitness during large population transmissions." Proc Natl Acad Sci U S A **92**(13): 5841-4.
Novella, I. S. and B. E. Ebendick-Corpus (2004). "Molecular basis of fitness loss and fitness recovery in vesicular stomatitis virus." J Mol Biol **342**(5): 1423-30.
Novella, I. S., S. F. Elena, et al. (1995). "Size of genetic bottlenecks leading to virus fitness loss is determined by mean initial population fitness." J Virol **69**(5): 2869-72.
Novella, I. S., C. L. Hershey, et al. (1999). "Lack of evolutionary stasis during alternating replication of an arbovirus in insect and mammalian cells." J Mol Biol **287**(3): 459-65.
Novella, I. S., J. Quer, et al. (1999). "Exponential fitness gains of RNA virus populations are limited by bottleneck effects." J Virol **73**(2): 1668-71.

Novella, I. S., D. D. Reissig, et al. (2004). "Density-dependent selection in vesicular stomatitis virus." J Virol **78**(11): 5799-804.
Nowak, M. A., R. M. Anderson, et al. (1991). "Antigenic diversity thresholds and the development of AIDS." Science **254**(5034): 963-9.
Pang, S., Y. Shlesinger, et al. (1992). "Rapid generation of sequence variation during primary HIV-1 infection." AIDS **6**(5): 453-60.
Parker, W. B. (2005). "Metabolism and antiviral activity of ribavirin." Virus Res **107**(2): 165-71.
Pattnaik, A. K., L. Hwang, et al. (1997). "Phosphorylation within the amino-terminal acidic domain I of the phosphoprotein of vesicular stomatitis virus is required for transcription but not for replication." J Virol **71**(11): 8167-75.
Pawlotsky, J. M., G. Germanidis, et al. (1999). "Evolution of the hepatitis C virus second envelope protein hypervariable region in chronically infected patients receiving alpha interferon therapy." J Virol **73**(8): 6490-9.
Pawlotsky, J. M., G. Germanidis, et al. (1998). "Interferon resistance of hepatitis C virus genotype 1b: relationship to nonstructural 5A gene quasispecies mutations." J Virol **72**(4): 2795-805.
Peluso, R. W. and S. A. Moyer (1988). "Viral proteins required for the in vitro replication of vesicular stomatitis virus defective interfering particle genome RNA." Virology **162**(2): 369-76.
Petersen, J. M., L. S. Her, et al. (2000). "The matrix protein of vesicular stomatitis virus inhibits nucleocytoplasmic transport when it is in the nucleus and associated with nuclear pore complexes." Mol Cell Biol **20**(22): 8590-601.
Pfeiffer, J. K. and K. Kirkegaard (2003). "A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity." Proc Natl Acad Sci U S A **100**(12): 7289-94.
Piot, P., M. Bartos, et al. (2001). "The global impact of HIV/AIDS." Nature **410**(6831): 968-73.
Plotkin, J. B. and J. Dushoff (2003). "Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus." Proc Natl Acad Sci U S A **100**(12): 7152-7.
Potter, C. W. (2006). "A History of Influenza." Journal of Applied Microbiology **91**: 572-579.
Qanungo, K. R., D. Shaji, et al. (2004). "Two RNA polymerase complexes from vesicular stomatitis virus-infected cells that carry out transcription and replication of genome RNA." Proc Natl Acad Sci U S A **101**(16): 5952-7.
Quer, J., C. L. Hershey, et al. (2001). "Contingent neutrality in competing viral populations." J Virol **75**(16): 7315-20.
Quer, J., R. Huerta, et al. (1996). "Reproducible nonlinear population dynamics and critical points during replicative competitions of RNA virus quasispecies." J Mol Biol **264**(3): 465-71.
Reichard, O., J. Andersson, et al. (1991). "Ribavirin treatment for chronic hepatitis C." Lancet **337**(8749): 1058-61.

Reid, A. H., T. G. Fanning, et al. (1999). "Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene." Proc Natl Acad Sci U S A **96**(4): 1651-6.

Rimmelzwaan, G. F., A. C. M. Boon, et al. (2004). "Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes." Virus Res **103**(1-2): 97-100.

Roche, S., S. Bressanelli, et al. (2006). "Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G." Science **313**(5784): 187-91.

Rose, J. H. and M. A. Whitt (2001). Rhabdoviridae and their replication. Field's Virology, 4th edition. B. N. Fields, Lipcott Williams and Wilkins. **1:** 1121-1244.

Sabin, A. B., M. Ramos-Alvarez, et al. (1960). "Live, orally given poliovirus vaccine. Effects of rapid mass immunization on population under conditions of massive enteric infection with other viruses." JAMA **173**: 1521-6.

Schuster, P. and J. Swetina (1988). "Stationary mutant distributions and evolutionary optimization." Bull Math Biol **50**(6): 635-60.

Shafer, R. W., S. Y. Rhee, et al. (2007). "HIV-1 protease and reverse transcriptase mutations for drug resistance surveillance." AIDS **21**(2): 215-23.

Shih, A. C., T. C. Hsiao, et al. (2007). "Simultaneous amino acid substitutions at antigenic sites drive influenza A hemagglutinin evolution." Proc Natl Acad Sci U S A **104**(15): 6283-8.

Shiraishi, K., K. Mitamura, et al. (2003). "High frequency of resistant viruses harboring different mutations in amantadine-treated children with influenza." J Infect Dis **188**(1): 57-61.

Sleat, D. E. and A. K. Banerjee (1993). "Transcriptional activity and mutational analysis of recombinant vesicular stomatitis virus RNA polymerase." J Virol **67**(3): 1334-9.

Smith, D. J., A. S. Lapedes, et al. (2004). "Mapping the antigenic and genetic evolution of influenza virus." Science **305**(5682): 371-6.

Smith, G. J., X. H. Fan, et al. (2006). "Emergence and predominance of an H5N1 influenza variant in China." Proc Natl Acad Sci U S A **103**(45): 16936-41.

Sole, R. V., R. Ferrer, et al. (1999). "Red queen dynamics, competition and critical points in a model of RNA virus quasispecies." J Theor Biol **198**(1): 47-59.

Spiropoulou, C. F. and S. T. Nichol (1993). "A small highly basic protein is encoded in overlapping frame within the P gene of vesicular stomatitis virus." J Virol **67**(6): 3103-10.

Stouffer, A. L., R. Acharya, et al. (2008). "Structural basis for the function and inhibition of an influenza virus proton channel." Nature **451**(7178): 596-9.

Suzuki, Y. (2006). "Natural selection on the influenza virus genome." Mol Biol Evol **23**(10): 1902-11.

Swetina, J. and P. Schuster (1982). "Self-replication with errors. A model for polynucleotide replication." Biophys Chem **16**(4): 329-45.

Takacs, A. M., S. Barik, et al. (1992). "Phosphorylation of specific serine residues within the acidic domain of the phosphoprotein of vesicular stomatitis virus regulates transcription in vitro." J Virol **66**(10): 5842-8.
Takacs, A. M., T. Das, et al. (1993). "Mapping of interacting domains between the nucleocapsid protein and the phosphoprotein of vesicular stomatitis virus by using a two-hybrid system." Proc Natl Acad Sci U S A **90**(21): 10375-9.
Tesh, R. B., B. N. Chaniotis, et al. (1972). "Vesicular stomatitis virus (Indiana serotype): transovarial transmission by phlebotomine sandlies." Science **175**(29): 1477-9.
Tesh, R. B. and G. B. Modi (1983). "Development of a continuous cell line from the sand fly Lutzomyia longipalpis (Diptera: Psychodidae), and its susceptibility to infection with arboviruses." J Med Entomol **20**(2): 199-202.
Thompson, W. W., D. K. Shay, et al. (2004). "Influenza-associated hospitalizations in the United States." JAMA **292**(11): 1333-40.
Tomaras, G. D., X. Shen, et al. (2006). "Antibody responses and virus escape: implications for HIV-1 vaccine and therapeutic design." Future Virology **1**(1): 79-88.
Turner, P. E. and S. F. Elena (2000). "Cost of host radiation in an RNA virus." Genetics **156**(4): 1465-70.
Ungchusak, K., P. Auewarakul, et al. (2005). "Probable person-to-person transmission of avian influenza A (H5N1)." N Engl J Med **352**(4): 333-40.
Van Valen, L. (1973). "A New Evolutionary Law." Evolutionary Theory(1): 1-30.
Vandepol, S. B., L. Lefrancois, et al. (1986). "Sequences of the major antibody binding epitopes of the Indiana serotype of vesicular stomatitis virus." Virology **148**(2): 312-25.
Vignuzzi, M., J. K. Stone, et al. (2005). "Ribavirin and lethal mutagenesis of poliovirus: molecular mechanisms, resistance and biological implications." Virus Res **107**(2): 173-81.
Vignuzzi, M., J. K. Stone, et al. (2006). "Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population." Nature **439**(7074): 344-8.
von Kobbe, C., J. M. van Deursen, et al. (2000). "Vesicular stomatitis virus matrix protein inhibits host cell gene expression by targeting the nucleoporin Nup98." Mol Cell **6**(5): 1243-52.
Wain-Hobson, S. (1993). "The fastest genome evolution ever described: HIV variation in situ." Curr Opin Genet Dev **3**(6): 878-83.
Wain-Hobson, S. (1996). "Running the gamut of retroviral variation." Trends Microbiol **4**(4): 135-41.
Watson, B. M., D. S. Laufer, et al. (1996). "Safety and immunogenicity of a combined live attenuated measles, mumps, rubella, and varicella vaccine (MMR(II)V) in healthy children." J Infect Dis **173**(3): 731-4.
Weaver, S. C., A. C. Brault, et al. (1999). "Genetic and fitness changes accompanying adaptation of an arbovirus to vertebrate and invertebrate cells." J Virol **73**(5): 4316-26.

Webster, R. G., W. J. Bean, et al. (1992). "Evolution and ecology of influenza A viruses." Microbiol Rev **56**(1): 152-79.
Weck, P. K. and R. R. Wagner (1978). "Inhibition of RNA synthesis in mouse myeloma cells infected with vesicular stomatitis virus." J Virol **25**(3): 770-80.
Wertz, G. W., V. P. Perepelitsa, et al. (1998). "Gene rearrangement attenuates expression and lethality of a nonsegmented negative strand RNA virus." Proc Natl Acad Sci U S A **95**(7): 3501-6.
Whelan, S. P., L. A. Ball, et al. (1995). "Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones." Proc Natl Acad Sci U S A **92**(18): 8388-92.
Whelan, S. P. and G. W. Wertz (2002). "Transcription and replication initiate at separate sites on the vesicular stomatitis virus genome." Proc Natl Acad Sci U S A **99**(14): 9178-83.
Wilke, C. O. and C. Adami (2003). "Evolution of mutational robustness." Mutat Res **522**(1-2): 3-11.
Wilke, C. O., R. Forster, et al. (2006). "Quasispecies in time-dependent environments." Curr Top Microbiol Immunol **299**: 33-50.
Wilke, C. O. and I. S. Novella (2003). "Phenotypic mixing and hiding may contribute to memory in viral quasispecies." BMC Microbiol **3**: 11.
Wilke, C. O., D. D. Reissig, et al. (2004). "Replication at periodically changing multiplicity of infection promotes stable coexistence of competing viral populations." Evolution Int J Org Evolution **58**(4): 900-5.
Wilke, C. O., J. L. Wang, et al. (2001). "Evolution of digital organisms at high mutation rates leads to survival of the flattest." Nature **412**(6844): 331-3.
Yuste, E., S. Sanchez-Palomino, et al. (1999). "Drastic fitness loss in human immunodeficiency virus type 1 upon serial bottleneck events." J Virol **73**(4): 2745-51.
Zhang, L. and H. P. Ghosh (1994). "Characterization of the putative fusogenic domain in vesicular stomatitis virus glycoprotein G." J Virol **68**(4): 2186-93.

ABSTRACT

Vesicular stomatitis virus (VSV) is an RNA virus commonly used for the study of RNA virus evolution. Like other RNA viruses, VSV has a high mutation rate, averaging one mutation per genome per round of replication. This high mutation rate leads to a genetically heterogeneous population; each produced virus will have at least one new mutation. This collection of mutants is termed a quasispecies. Bottlenecking of a population results in the fixation of random mutations, which are more likely to be deleterious than those that are fixed through selection. Previous studies have found bottlenecked virus strains with adaptability defects that are unable to adapt and gain fitness normally. In the set of experiments presented here, I explored the quasispecies of these adaptability-deficient strains. Plaque sizes were measured for each strain as a surrogate of fitness values, and then fitness values for at least 200 clones of each strain were measured. Results suggested a lack of robustness in adaptability-deficient strains, as these strains produced a greater amount of deleterious mutants. In addition, these strains were less able to generate beneficial mutations. A lack of robustness would explain data collected both previously and in these experiments. This could have implications in vaccine design and treatment of RNA virus diseases.