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Antagonistic pleiotropy involving promoter sequences in a virus

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Abstract

Selection of specialist genotypes, that is, populations with limited niche width, promotes the maintenance of diversity. Specialization to a particular environment may have a cost in other environments, including fitness tradeoffs. When the tradeoffs are the result of mutations that have a beneficial effect in the selective environment, but a deleterious effect in other environment, we have antagonistic pleiotropy. Alternatively, tradeoffs can result from the fixation of mutations that are neutral in the selective environment but have a negative effect in other environment, and thus the tradeoff is due to mutation accumulation. We tested the mechanisms underlying the fitness tradeoffs observed during adaptation to persistent infection of vesicular stomatitis virus in insect cells by sequencing the full-length genomes of twelve strains with a history of replication in a single niche (acute mammalian infection or persistent insect infection) or in temporally-heterogeneous niches, and correlated genetic and fitness changes. Ecological theory predicts a correlation between the selective environment and the niche width of the evolved populations, such that adaptation to single niches should lead to the selection of specialists and niche cycling should result in the selection of generalists. Contrary to this expectation, adaptation to one of the single niches resulted in a generalist and adaptation to a heterogeneous environment led to the selection of a specialist. Only one-third of the mutations that accumulated during persistent infection had a fitness cost that could be explained in all cases by antagonistic pleiotropy. Mutations involved in fitness tradeoffs included changes in regulatory sequences, particularly at the 3' termini of the genomes, which contain the single promoter that controls viral transcription and replication.

Introduction

One of the most fundamental questions in evolutionary biology is how diversity is generated and maintained. The availability of multiple niches, each one being exploited by a particular specialized genotype, promotes the preservation of diversity¹. Specialization allows the coexistence of multiple mutants because it limits competition, and it may result in adaptive radiation². In contrast, growth in heterogeneous environments should result in the selection of generalists: genotypes that can use multiple resources and, thus, grow in multiple niches³. The selection of specialists requires a cost in alternative niches, and this diminished ability to survive in alternative environments represents the cost of specialization. In contrast, a generalist that can survive in multiple niches may achieve this potential at the expense of

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suboptimal adaptation to each individual niche, and this limitation represents the cost of generalism (reviewed in ⁴). Costs can come from the shape of the fitness landscapes where the population is replicating; two environments may produce fitness peaks of different height, so if the fitness increase of the generalist in a given environment were not as much as the fitness increase in the specialist, the generalist would be unable to outcompete or even coexist with the specialist³. The second source of costs is tradeoff, which consists of fitness increase in one environment that correlates with fitness loss in another environment. Tradeoffs during adaptation may be the result of two mechanisms, antagonistic pleiotropy⁵ or mutation accumulation⁶. Antagonistic pleiotropy represents situations in which a mutation that has a beneficial effect in one environment has a deleterious effect in a different environment. In contrast, mutation accumulation occurs when there is fixation of mutations that are neutral in the selective environment, but deleterious in alternative environments. These neutral mutations could accumulate by chance rather than selection for two different reasons: random drift, and hitchhiking. Note that the fixation of non-selective mutations does not represent mutation accumulation unless those mutations have a deleterious effect in the alternative environment.

Microbes are excellent systems to study evolution^{7; 8}. They replicate very fast, their population sizes are large, and they can be manipulated in the laboratory under tightly-controlled conditions. RNA viruses are particularly suitable for experimental evolution due to their high mutation rates and small genome sizes. Mutation rates for RNA replication are between 10^{-3} and 10^{-5} substitutions per site and round of replication⁹, and experiments typically take tens of generations (compared to hundreds or thousands in bacteria). The small genomic size, about 10 Kb., allows the characterization of full-length genomes, so we can identify any mutation (at any locus) that becomes dominant during evolution. In addition, many plant, animal and human pathogens are RNA viruses, and evolution often results in their acquisition of new properties, such as antibody- and drug-escape, expanded host range, and increased virulence (reviewed in ¹⁰). All these potential adaptive changes have clear bearings on our ability to control diseases caused by RNA viruses.

Vesicular stomatitis virus (VSV) is one of the most commonly used systems for studies on microbial experimental evolution. VSV, the prototype of the *Rhabdoviridae* family¹¹, is an arthropod borne virus (arbovirus) that is maintained in an insect vector by either horizontal transmission (via co-feeding)¹² or vertical transmission from infected female to offspring coupled with a periodical vertebrate host infection¹³. Infection of insects in nature and insect cells in the laboratory leads to an initial phase of rapid replication and production of relatively high titers (acute phase) followed by the establishment of life-lasting, persistent infections¹⁴. Persistent infections are characterized by lack of cytopathic effect and low, but continuous, virus production. In cell culture virus replication continues for long as the cells continue growing and dividing, and thus, does not depend on the availability of fresh host cells. In contrast, infection of mammalian cells is generally acute and ends in hours or days with the death of the cell, and sustained replication requires the periodic passage of virus from killed monolayers to fresh ones. VSV is an enveloped, non-segmented, negative-stranded RNA virus whose genome is 11,161 nucleotides (nt) in length. The genome codes for five proteins, from 3' to 5': N nucleoprotein, P phosphoprotein, M matrix protein, G glycoprotein and L polymerase. The virus is effectively asexual¹⁵. VSV has been very useful as a model system to dissect the mechanisms of evolution in RNA virus populations (reviewed in ¹⁶).

Several laboratories have addressed the effects of adaptation in single vs. heterogeneous environments on viruses. Acute infection of vesicular stomatitis virus (VSV) in BHK-21 (hamster) mammalian cells and LL-5 (sand fly) insect cells does not involve tradeoffs, and adaptation to either environment and alternation between the two environments result in the selection of generalist virus populations with improved fitness in both BHK-21 and LL-5 cells¹⁷. In general, replication in different mammalian cell lines may or may not select for

specialists, but alternation between cells lines produces generalists¹⁸. Similar results have been observed in several studies with other species of RNA viruses^{19; 20; 21; 22; 23}. Adaptation of foot-and-mouth disease virus to BHK-21 cells resulted in expanded niche width, such that BHK-adapted strains gained the ability to infect cell lines of human and primate origin²⁴. In contrast, Crill and coworkers demonstrated tradeoffs due to antagonistic pleiotropy during DNA bacteriophage Φ X174 replication in *Escherichia coli* and *Salmonella enterica*, but during the alternation between the two niches the experimental regime allowed enough time at each niche to select for specialists for each environment²⁵. Some of the studies in which tradeoffs occurred in RNA viruses also addressed the identification of genetic changes accumulated during adaptation either by fingerprinting of whole genomes¹⁹ or by sequencing of candidate genes^{14; 19; 22}. Results typically showed that after adaptation to the selective environment there were specific mutations consistently present that were never found populations adapted to other environments, suggesting tradeoffs due to antagonistic pleiotropy.

In contrast to the absence of tradeoffs during adaptation of VSV to acute infection, adaptation to persistent infection of insect cells has a cost due to tradeoff, as shown by its low fitness in the mammalian environment (BHK-21 cells)²⁶. The evolution of fitness during alternation of an environment of persistent infection in insect cells and an environment of acute infection in mammalian cells is largely driven by the selective pressures that act during the persistent infection in the insect host cells¹⁴. Thus, VSV strains that had a history of persistent insect infection lost fitness in the acute mammalian environment, even when the populations were subjected to periodic acute infection of mammalian cells. Partial sequencing of sample genomes showed a pattern of genomic changes that was consistent with the results of fitness changes. In this report, we determined the full-length sequence of twelve genomes from populations of VSV replicating in single environments or alternating between persistent insect infection and acute mammalian infection, as well as four genomes from intermediate passages, and we correlated genomic changes with fitness changes. Our goal was to establish (i) the contribution of mutations that have accumulated during specialization to tradeoffs; (ii) whether antagonistic pleiotropy, mutation accumulation or a combination of both account for the cost of specialization; and (iii) potential molecular mechanisms that may explain the observed antagonistic pleiotropy.

Results

Parallel molecular evolution of VSV strains

We previously reported the fitness evolution of genotypes under three adaptation regimes: persistent infection in LL-5 sand fly cells, acute infection in BHK-21 mammalian cells and alternation between the two environments¹⁴. We measured fitness by direct competitions between evolved genotypes and a genetically-marked ancestor, following changes in the relative ratio between the two competitors (see Materials and Methods). We observed tradeoffs such that the genotypes replicating in LL-5 cells decreased their fitness in BHK-21 cells (Figure 1). Furthermore, while alternating passages of persistent infection in LL-5 cells with passages of acute infection in BHK-21 cells somewhat limited the fitness costs, there was an overall fitness loss in the mammalian environment (Figure 1). Based on the ability to perform in different environments, we can classify the different populations as generalists and specialists. The P25 genotypes, selected in homogeneous environments, can only perform well in the selective environment, so it is a specialist. This result is consistent with the predictions of ecological theory^{3; 27}. In contrast, the results from A25 and K25 populations were at odds with theoretical predictions. A25 populations were selected in heterogeneous environments, but only performed well in one of them, making these populations specialists instead of the expected generalist. The situation is reversed in the K25 populations, which were selected in

a homogeneous environment, but behave as a generalists with high fitness in both LL-5 and BHK-21 cells.

Figure 2 shows the mutations found in the consensus sequences of evolved VSV genomes. All or some of these mutations are responsible for the fitness increases observed in the environments to which the populations adapted (LL-5 cells), and presumably for the fitness decrease observed in other environments when that happened (BHK-21 cells). It is important to stress that the sequences were derived from total amplified cDNA and not from individual clones, so each nucleotide represent the most common (consensus) within the populations of genomes. We shall refer to genotypes with a history of 25 passages of persistent infection in sand fly cells as P25 (or P10 to refer to passage 10 genotypes), strains with a history of 25 passages of acute infection in mammalian BHK-21 cells as K25, and viruses with a history of alternation between the two environments as A25; letters A-D after the passage number refer indicate the replica (for details, see Materials and Methods). Parallel mutations were frequent within lineages following the same evolutionary regime, and between P25 and A25 strains. Parallel evolution describes the repeated occurrence of the same changes in independent replicas and it has been previously observed in VSV^{17; 28} and other viruses^{19; 29}. The accumulation of parallel mutations in P25, A25 and K25 populations correlated with parallel phenotypic changes (Figure 2), so A25 and P25 strains fixed many of the same mutations, and these mutations were different than those in K25 genotypes. Specifically, six single-nucleotide substitutions (positions A163G, C1817U, C2257U, C3421A, C3978A and G4122U) were identified in six or more of the twelve populations with a history of persistence (P25 and A25 genotypes). The K25 genotypes instead fixed a single nucleotide substitution, U2937C, which is the most commonly found mutation during adaptation to BHK-21 cells, and frequently the first change in the consensus sequence (unpublished results), but was not found in any of the A25 or P25 genotypes. Parallel evolution has two possible interpretations: fixation is the result of positive selection, or the hitch hiking of neutral (or deleterious) mutations in hot spots. None of these loci were mutated in strains with a history of repeated plaque-to-plaque bottleneck passages³⁰. Plaque-to-plaque passages allow the fixation of any non-deleterious mutations because of the effect of selection is minimized through the elimination of competition between mutants. Thus, fixation of mutations depends on chance (and therefore their frequency) rather than their selective value. Mutations in hotspots are expected to be overrepresented in a population, so the chance of fixation at these loci is increased and these mutations would be likely to appear under a regime of drift. The absence of the parallel mutations reported here in strains with a history of bottleneck passages suggests that they do not represent mutational hotspots, although the published dataset may be insufficient to rule out hitchhiking formally as an explanation for parallel evolution.

Mutations accumulated during specialization without cost

The phenotype of P25 and A25 genotypes was that of specialists, with high fitness in one environment (LL-5 cells) and low fitness in another environment (BHK-21 cells). Mutations that are deleterious in BHK-21 cells cannot be fixed during adaptation to those cells, and therefore, mutations found during acute BHK-21 infection and persistent LL-5 infections will not carry a cost in either environment. We identified several substitutions in A25 and P25 genotypes (nts A1377G, C2257U, C3421A, C4003M, G4122U, A4139U, U4434G, A6145C, G9110U and G11153U; Figure 2) that we had previously recognized in other strains with a history of adaptation to BHK-21 cells^{30; 31}, unpublished results). In previous experiments some of these mutations usually appeared after longer periods of replication (up to 80 passages) and may be absent from K25 genotypes described in this report due to lack of enough time to allow fixation, or to a lesser beneficial effect in BHK-21 cells than in LL-5 cells. Beneficial mutations do not need to achieve fixation before having a significant effect in the population. For instance, it may take 20 adaptive passages or more to find a fixed mutation, while significant fitness

differences can be observed after only 10 passages³⁰. Those mutations can be observed as minor peaks in sequencing chromatograms when their frequency reaches high enough levels. In conclusion, the appearance of genomic changes in P25 and A25 genotypes that are also found in regimes of adaptation to BHK-21 infections strongly suggests that these substitutions are beneficial, not deleterious, in both environments and would represent adaptation to multiple niches. Mutations that do not have a cost in BHK-21 cells included nine out of 15 parallel sites, and in one unique substitution (G11153U in P25D).

Cost of specialization due to antagonistic pleiotropy

To identify which one(s) of the mutations fixed in P25 and A25 populations caused the low fitness in BHK-21 cells we looked for changes present in A25 and P25 genotypes, but never found in regimes of adaptation to BHK-21 cells, including K25 genotypes. Within this group of mutations most or all are likely to be the result of selection, but some may be the result of hitch hiking. As indicated earlier, parallel mutations can be considered the product of selection because the likelihood of fixation in multiple replicas is extremely low. The probability of chance fixation for a neutral mutation is $1/N_e$ ³². N_e is the effective population size, and it can be calculated as the harmonic mean of the different population sizes during evolution³³. We can use the population size at transmission (the lowest value, 2×10^5 PFU) as N_e to calculate the maximum probability of fixation in a single replica of passages, so in our case the p value is 0.5×10^{-5} . The probability of fixing the same neutral mutation in n independent replicas would be $(0.5 \times 10^{-5})^n$ and thus we can ignore chance as a cause of parallel evolution. The presence of parallel mutations in the A25 and P25 genotypes, but not in BHK-21-adapted genotypes would represent antagonistic pleiotropy. In contrast, the presence of unique mutations can be interpreted in two different ways. First, they may be beneficial and also responsible for antagonistic pleiotropy. Second, they may be the result of hitch hiking, and cause tradeoffs due to mutation accumulation. Among the parallel mutations found in A25 and P25 strains, we found six mutations that never appeared in strains adapted to BHK-21 cells (Figure 2). Substitutions A163G and G253A (N gene), C1817U (P gene) and C3978A (G gene) were non-synonymous. The N and P proteins have a role in RNA synthesis¹¹ and mutations found in these proteins may have an impact in the observed phenotype. We also identified differences in the 3' termini of the genomes: a 43-nt. duplication of nts. 20-62 at position 20, and a single substitution U→C at position 50 whose pattern of fixation was the same as that described for the A163G, G253A, C1817U and C3978A substitutions. Even though each of the four replicas of alternating regimes had a different combination of mutations, all the mutations in A25A, A25B and A25C were parallel (e.g. found in more than one replica) and likely the product of selection. This result ruled out any significant contribution of mutation accumulation to tradeoffs in these three genotypes. A25D had one unique mutation in the L gene (C8668U). On the basis of sequence comparison alone, this change could represent tradeoffs for BHK-21 infection in the form of mutation accumulation. However, there was no statistical difference between the fitness of A25D and any virus whose genotype lacked this individual mutations, that is, A25A, A25B and A25C (t test, $p > 0.3$). Thus, even if this unique mutation in A25D had no selective value, we could not assign any cost to it. In contrast, all the P25 genotypes had individual mutations, and their fitness in BHK-21 cells was significantly lower than the fitness of A25 genotypes. These individual mutations could be the result of selection or hitch hiking and, in the latter case, represent tradeoffs due to mutation accumulation. To test whether this was the case, we determined the sequence in persistent populations at passage 10 (P10).

Between passage 10 and passage 25 the fitness of P strains in BHK-21 cells increased (Figure 1A). This increase was significant for replicas B, C, and D ($p < 0.03$), but not for replica A ($p = 0.23$). The comparison of mutations present in P10 and P25 genotypes showed that not all parallel mutations were found at passage 10 (Table 1). Most notably, substitution U50G,

present in all the P25 replicas, was absent from all the P10 replicas. This result is consistent with the small but significant fitness increases observed between passage 10 and 25 in replicas B, C and D of strains adapted to persistent infection, and suggests that biologically-significant fitness increases are still occurring in replica A as well, despite the lack of statistical support. All the unique mutations in genotypes adapted to persistent infection, except C16U in P25A, appeared after passage 10 (Table 1), during a period of fitness increase in BHK-21 (or at least no fitness loss, Fig. 1). Therefore, even though we do not know whether these mutations were fixed because of selection or because of hitch hiking (or even drift), we can conclude that they could not contribute to fitness losses in BHK-21 cells, and we can rule any role out mutation accumulation as a mechanism of tradeoff. In contrast, all mutations in the promoter (except U50C) as well as substitutions in the N and P ORFs were present at passage 10, further supporting our hypothesis that they play a major role in adaptation to persistent infection in LL-5 cells and they are deleterious for acute infection in BHK-21 cells. P10A is polymorphic at C16 and at the site of the insertion. The subsequent disappearance of the insertion and fixation of C16U suggest that this site is also under positive selection. The overall conclusion is that there is little or no contribution of mutation accumulation to tradeoff.

Sequence determinations of P10 genomes led to the identification of transient polymorphisms in several loci (Table 2). Each of the P10 populations had mutations that were present at substantial frequency but later disappeared. All transient polymorphisms except three (transitions A7710U, C8678 and U192C) were parallel changes. Substitutions A1377G and A6145C were also found in A25 genotypes. Most noteworthy was the presence of the 3' insertion in replicas A and B. Because P25 populations underwent most of the fitness gains in LL-5 cells and most of the fitness loss in BHK-21 cells during the first 10 passages, this result provides additional evidence that this change in the promoter has a beneficial effect in persistent infection and a deleterious effect in acute infection. In other words, this mutation is responsible for tradeoffs due to antagonistic pleiotropy.

Discussion

The selection of generalists and specialists in RNA viruses

In previous work showed that acute infection of insect (LL-5) or mammalian (BHK-21) cells led to the selection of generalists with increased fitness in both types of cells¹⁷, while persistent infection of insect cells led to the selection of specialists with a cost for acute replication in mammalian cells in the form of tradeoffs¹⁴. Moreover, we showed that alternation between acute mammalian infection and persistent insect infection produced specialists that were highly adapted to persistent infection of insect cells but had low fitness in acute infection of mammalian cells¹⁴. We do not know why the selective forces that persistent infections impose dominated the profile of adaptation, but there are some explanations that we can rule out. First, even though the period of virus replication during persistent infection in LL-5 cells (2 weeks) was much longer than the period of virus replication during acute infection in BHK-21 cells (24 hours), virus production during persistent infection (approximately 1.4×10^6 PFU/passage) was orders of magnitude lower than the level of replication in acute infections (at least 10^8 PFU/passage)¹⁴, and, therefore, we cannot explain the dominance of persistence in the evolution of these populations as a result of higher levels of replication. A second explanation that we can rule out is that most of the sample transferred from the acute BHK-21 infection to the next persistent LL-5 infection represented virus that never replicated, because replication in BHK-21 cells at each passage resulted in a 10^3 - to 10^5 -fold increase in viral titer. Finally, we ruled out the presence of defective interfering particles (DIPs) experimentally¹⁴.

There is a large body of evidence consistent with the prediction that microbial adaptation to a single niche should select specialists. Studies on bacterial evolution under single environments and temporally- or spatially-heterogeneous environments have shown that selection tends to

match the niche width of the genotype with the availability of resources and that specialization often has a cost. However, such cost is not always the result of tradeoffs due to antagonistic pleiotropy (reviewed in 2; 3; 27). The production of viral vaccines by adaptation to non-human hosts (as Pasteur's rabies vaccine) or to cell lines of non-human origin (for example, Sabin's attenuated polio vaccine) are remarkable examples of microbial specialization that due to antagonistic pleiotropy. Several arboviruses, including VSV, lose to some extent their ability to replicate in mammalian cells after adaptation to persistent infection in insect cells^{26; 34; 35; 36}. Nevertheless, adaptation of RNA viruses to a single niche may often result in the selection of generalists^{17; 18; 24}. Our results showed that even when adaptation to a novel niche involved tradeoffs in the original host, not all the mutations accumulated in evolved genotypes carried a cost.

Persistent infection in insect cells and acute infection in mammalian cells carried asymmetric costs, a situation that has been also observed in other virus species^{25; 37}. Asymmetric costs have also been reported in bacteria growing in different carbon sources³. Interestingly, the similar behavior of P25 and A25 strains indicated that there were no tradeoffs due to differences in the mode of transmission. While we do not know whether viral progeny reinfects LL-5 cells during persistent regimes (and if so, to what extent), viral survival is linked to cell growth, so when the cell divides the progeny cells carry the virus. Thus, transmission during regimes of persistent infection was vertical, while alternating regimes included vertical transmission during the persistent phase and horizontal transmission between hosts. At odds with the theoretical prediction — and previous observations^{17; 19; 20; 21; 22; 23} — that a temporally-heterogeneous environment should lead to the selection of a generalist, we observed that alternation between persistent and acute infection resulted in the selection of a specialist highly adapted to persistent infection of insect cells, but maladapted to acute infection of mammalian cells.

Molecular basis of the cost of specialization

Adaptation to a particular environment acquired at the expense of fitness in other environments constitutes the cost of specialization. Previous work on experimental populations of RNA viruses suggested that the cost of specialization is generally due to antagonistic pleiotropy. Studies on two alphaviruses^{19; 22} showed a correlation between specific mutations and narrowing of niche width. However, in these studies only part of the genomes were sequenced, and the authors could not rule out a contribution of mutation accumulation to the observed tradeoffs. Remold and coworkers³⁸ used full-length genome sequencing of VSV strains adapted to HeLa or MDCK cells to conclude that both antagonistic pleiotropy and mutation accumulation contribute to tradeoffs during specialization, but because of the loss of the genetic marker in evolved strains³⁸ and the use of preadapted wt reference for competition¹⁸ it is difficult to correlate genetic changes with fitness values. In the present report we obtained the full-length sequence of each genome and we can fully explain the tradeoffs during adaptation in terms of antagonistic pleiotropy caused by a small subset of the mutations found in population with a history of persistent infections (P25 and A25). Most of the parallel mutations found in A25 and P25 populations had no cost at all, as they were present in genotypes adapted to BHK-21 cells. Most of the loci with unique mutations found in A25 and P25 genotypes contained mixtures of wt and mutant nucleotides (Figure 2) that may represent transient polymorphisms produced by drift or beneficial mutations on their way to fixation. Regardless, they appear after passage 10, during a period in which there was no fitness loss in BHK-21 cells (Figure 1) and thus they do not contribute to tradeoffs.

The mutations at the termini, together with the N and P mutations, appeared during the period of highest fitness increase in LL-5 and fitness decrease in BHK-21 cells. These mutations appeared repeatedly in regimes that included persistent infection of LL-5 cells and were never

found in regimes of acute infection of BHK-21 cells. This pattern strongly suggests that these mutations are the cause of tradeoff and this tradeoff is the result of antagonistic pleiotropy.

Phenotypic effects of adaptive mutations

VSV is one of the best-characterized viruses because of its extensive use as a model to understand the biology of Mononegavirales: RNA viruses with monopartite, negative-strand genomes¹¹. This knowledge provides us with an additional advantage when we use VSV as a model for evolution, because we can often assign phenotypic effects to specific mutations.

Nucleotide A1377G is located at the beginning of the highly conserved U₇ tract on the negative strand between the N and P genes. The substitutions in Figure 2 and Tables 1 and 2 correspond to the positive strand (the antigenome or the mRNA), so this mutation corresponds to a U to C substitution in the genome. This poly-U region serves as a template for synthesis of the poly-A tail in mRNAs³⁹. Shortening the tract to six nucleotides results in total abrogation of mRNA termination in a minigenome system⁴⁰. However, we have found this mutation in clones that are adapted to BHK-21 cells⁴¹, and other mutations in this region shown lethal in the minigenome system have been identified in natural isolates of Indiana serotype⁴², suggesting that the intergenic sequence may be involved in epistatic interactions with other regions along the genome (or in the polymerase complex), thus overriding the otherwise negative effect of a shortened U₇ tract.

The G protein covers the surface of the viral particle and is responsible for receptor recognition and fusion of viral and cell membranes during entry¹¹. The G ORF contained more mutations than any other region of the genome, including a total of six sites that fixed parallel mutations, each present in three to eight genotypes. Three of the mutations are likely to have an effect in entry. The mutation in nt C3421A results in a threonine to leucine substitution in amino acid 115, which may have an effect on the function the fusion peptide (residues 116-139)⁴³. Mutation U4434G results in a phenylalanine to cysteine substitution of amino acid 453, and maps to the very conserved domain B of the G-protein membrane-proximal region. Jeetendra and coworkers analyzed seven VSV isolates and found that position 453 was a non-polar amino acid in all cases, whereas we observed a polar amino acid substitution, suggesting a potential effect on fusion⁴³. We found a substitution at nt C3978A that leads to a change of the glutamine at position 301 to lysine. Martínez and Wertz demonstrated a significant effect on the ability of VSV to maintain infection at pH below 6.8 with a glutamine to arginine substitution (another positively-charged amino acid) at position 301⁴⁴.

The most remarkable changes in VSV populations adapted to persistent infection were the modifications of the 3' termini. All the strains with a history of persistent infection had one or more mutations in that region, including a duplication of nts 20-63 found in all the persistent strains at passage 10 and two of the four replicas at passage 25. Furthermore, in an independent experiment, starting with a different progenitor and following a slight modification of the regime of persistent infection, we found that after seven weeks the evolved genotype had incorporated an 86-nt. insertion in the 3' terminus³⁹. The 3' terminus of VSV and other Mononegavirales contains the promoters for both transcription and replication. Nucleotides 1-18 are strictly conserved in non-persistent strains, and deletions through nucleotide 47 can significantly affect mRNA levels⁴⁵. In an acute infection, higher titer often correlates to a higher fitness, but that may not be the case for persistent infections where survival depends on the viability of the infected cell¹⁴. This causes a shift in selective pressure, and may favor more mutations in the regions of the genome that regulate replication and transcription. Antagonistic pleiotropy due to changes in regulatory sequences is a common event in bacteria³, and our results indicate that the same mechanism is also operational in viruses. Another example of antagonistic pleiotropy due to changes in regulatory sequences is the generation of live poliovirus vaccine. Attenuation is also consistent with specialization due in part to changes in

translation regulatory sequences⁴⁶. The genomic structure and mode of replication of poliovirus is radically different than that of VSV⁴⁷. Poliovirus has a single stranded, positive-sense RNA genome that can act as mRNA as soon as it is released into the cell. Translation is cap-independent because the genome carries a special sequence in the 5' terminus, the IRES, that mediates ribosome binding⁴⁸. Vaccine strains that are produced by replication in monkey cells carry mutations in the IRES that are directly responsible for attenuation in humans, and virulent revertants recovered from vaccines often show reversion of the mutations in the IRES^{49; 50}.

The changes in the 3' termini are likely to have an effect on transcription or replication. These mutations were reminiscent of the modifications found in DIPs and wt selected to escape DIPs during persistent infection of mammalian cells⁵¹. DIPs are truncated genomes that lack the full sequence needed to be independently infectious, but can replicate if full-length genomes co-infect the same cell and provide the necessary proteins in trans⁵². Two results led us to reject the presence of significant amounts of DIPs. First, we could not demonstrate interference using supernatants from persistent infections¹⁴; second, we demonstrated that clones with the consensus sequence, including the insertion, are fully functional⁴¹. Nevertheless, the mutations found in DIPs and resistant wt in other VSV strains act by modifying the binding of polymerase to the promoter.

The modifications in the 3' termini of A25 and P25 are likely to change the levels of RNA synthesis, which also requires P and N proteins (as well as L). The parallel P protein mutation found in A25 and P25 genotypes (Threonine 142→Methionine) map in the central domain that mediates oligomerization⁵³. Replication requires the formation of tetramers^{54; 55}, and mutations in this domain cause severe inhibition of P protein function⁵⁶. The N protein does not seem to be involved in the transcription (except as part of the template), but it is required for replication⁵⁷. N proteins tend to aggregate and their availability in a functional form depends on interactions with P protein⁵⁸. While there are no studies regarding RNA synthesis in sand fly cells, persistent VSV infection of *Drosophila* cells results in dramatic changes in the relative amount of mRNA and progeny genomes compared to those generated during replication in mammalian cells^{59; 60; 61; 62}. Production of leader RNA, which is involved in cellular gene expression shut-off, decreases nearly two orders of magnitude, and its transport into the nucleus is inhibited⁶⁰. Both synthesis of mRNA and synthesis of progeny genomes are downregulated after an initial burst and stay at low but constant levels for the duration of persistence. Overall, replication is promoted over transcription⁵⁹. Based on our results and these studies on RNA synthesis during insect cell infections we propose that adaptation to persistent infection is accomplished by the mutations found in the 3' termini together with the N and P parallel mutations through changes in the regulation of synthesis of different RNA species (mRNA, antigenomes and genomes). These changes in the pattern of RNA synthesis would be the cause of fitness loss in BHK-21 cells.

In summary, the use of experimental evolution, fitness determinations and full-length genomic sequencing has allowed us to test the theoretical predictions regarding the cost of specialization. There was no correlation between the selective conditions and niche width of evolved populations, a significant fraction of the mutations carried no cost, and tradeoffs were the result of antagonistic pleiotropy. Among the mutations most likely to result in antagonistic pleiotropy we identified extensive modifications of the regulatory sequences at the 3' end of the genome and in N and P proteins, so the cost of specialization may be the result of changes in the regulation of RNA synthesis.

Materials and Methods

Cells and viruses

Mammalian host cells were BHK-21 (Baby Hamster Kidney) from John Holland's laboratory (UCSD)⁶³, and insect host cells were LL-5 sand fly cells kindly provided by Bob Tesh (University of Texas)⁶⁴. Methods for cell culture and infections have been described elsewhere^{14, 65}. All the VSV populations are the same as in Zárate and Novella¹⁴ and derive from a wild type (wt) Mudd-Summers strain (Indiana serotype) that has been maintained in BHK-21 cells for an unknown (but limited) number of passages. We have assigned to wt fitness of 1 arbitrarily. Because of its past history, we can assume that wt is reasonably adapted to BHK-21 cells, but because of our results showing additional fitness increases, it is clear that wt is not optimized in these mammalian cells. The history of the evolved populations is as follows: (1) K25A-D strains were the progeny of wt that followed a regime of acute infection in mammalian (BHK-21) cells for 25 passages. For this regime we infected four T-25 flasks with 2×10^5 PFU, added 5 ml of MEM and allowed infection to proceed at 37°C for 24 hours, when cytopathic effect was complete. At this time we recovered the progeny, diluted it as needed and infected four new flasks with fresh monolayers under the same conditions. We continued this regime for 25 passages. (2) P25A-D strains were the progeny of wt populations that were maintained as persistent infections of LL-5 sand fly cells. The initial infection was also done in four flasks of cells (LL-5 in this case) with 2×10^5 PFU. Maximum titers were reached after 48-72 hours at 28°C (this period corresponds to the acute phase of LL-5 infection) but the virus was not recovered at this time. Instead, we fed the monolayers on day 4 with 5 ml of MEM, we replaced the medium on day 7, and we fed again the cells on day 11 with 5 ml of MEM. On day 14 we discarded the medium, washed the cells with 0.7% NaCl solution and we took 1/20 of the total cells from each of the four replicas into four new flasks. We repeated this process for 25 passages of cell splitting. (3) A25A-D strains were the progeny of wt that alternated between 2 weeks of persistent insect (LL-5 cells) infections and 24 hours of acute mammalian (BHK-21) infections. The first passage of this regime took place as described for persistent infections, but at the end of 14 days we did not discard the medium, and, instead, we diluted the virus released into the supernatant as needed to carry out an infection of BHK-21 cells as indicated above. After 25 cycles of persistent infection of LL-5 cells followed by acute infection of BHK-21 cells we recovered and analyzed the progeny. Fitness was determined by direct competition between a genetically-marked, antibody-resistant ancestor, MARM U⁶³. Fitness values were calculated as the ratio of relative ratios test:reference virus before and after competition. The kinetics of fitness changes under each regime can be found in Zárate and Novella¹⁴, but in that report each fitness determination for alternating and persistent viruses in LL-5 cells were the results of one single-passage competition. For the present report we carried out additional fitness determinations to gain a more realistic measurement of error, and we present fitness values calculated as the average of 2-4 fitness determinations. Most of the fitness determinations were carried out by mixing evolved wt and reference MARM U and using the mixture to determine their relative ratio before and after competition by triplicate plaque assay in the presence and absence of I1 monoclonal antibody (Mab), as described⁶³. However, wt in persistent and alternating regimes after passage 10 has such a high fitness in LL-5 cells that MARM was lost after competition. To circumvent this problem we started the competitions with excess of MARM U in the mixtures, but this approach had another problem: because plaque assay in the absence of I1 Mab allows growth of wt and MARM plaques, MARM excess over 80-90% resulted in plaque numbers in the presence and absence of I1 Mab that are very close and usually have a high error. To gain accuracy we titrated wt and MARM independently before preparing the competition mixtures. We must disclose that the earlier article¹⁴ had an error in the representation of fitness for A25 populations (values were overestimates) that has been corrected here. The additional determinations and the earlier

overestimations resulted in some differences between the data published previously and the data reported here. In this report, statistical analyses were done using the SPSS package.

Sequencing methods

To obtain the full-length sequence of each genome we employed standard methods³¹. Because of the very low titer of P10 and P25, we amplified them once in LL-5 cells, harvested the progeny at two days post-infection, and concentrated the virions about ten-fold with a YM-100 Centricon (Millipore). We extracted RNA from cell supernatants of each strain with the QIAamp Viral RNA kit (Qiagen) and reverse transcribed it with Superscript II (Invitrogen). The internal regions of the VSV genome were divided into 10 overlapping PCR fragments. In addition, we used rapid amplification of cDNA ends (RACE) to obtain the 3' or the 5' termini of the genomes. The primers we employed were those designed by Rodriguez and coworkers⁴² with some minor modifications to match the sequence of our reference wt, and their sequences are available upon request. For each amplified fragment 100 ng of total PCR product were used as a template for multiple readings using an ABI Prism 3730xl sequencer. We collected the data and assembled full-length genomes with the AssemblyLIGN program (Oxford Molecular Group PLC). We present the sequences of the positive strand, which corresponds to the sequence of mRNA in coding regions and to the sequence of the antigenomes in non-coding regions; we indicate changes in comparison to the wt sequence (Genebank accession number bankit1105686). We have previously reported the changes presents in nucleotides 63-4610 of genotypes K25A, A25A and P25A¹⁴.

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References

1. Schluter, D. The ecology of adaptive radiation. Oxford series in Ecology and Evolution. Oxford University Press; Oxford: 2000.
2. MacLean RC. Adaptive radiation in microbial microcosms. *J Evol Biol* 2005;18:1376–1386. [PubMed: 16313450]
3. Kassen R. The experimental evolution of specialists, generalists, and the maintenance of diversity. *J Evol Biol* 2002;15:173–190.
4. Wilson DS, Yoshimura J. On the coexistence of specialists and generalists. *Am Nat* 1994;144:692–707.
5. Levins, R. Evolution in changing environments. Princeton University Press; Princeton, NJ: 1968.
6. Kawecki TJ. Accumulation of deleterious mutations and the cost of being a generalist. *Am Nat* 1994;144:833–838.
7. Lenski RE, Elena SF. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nature Rev Genet* 2003;4:457–469. [PubMed: 12776215]
8. Travisano M. Evolution: Towards a genetical theory of adaptation. *Curr Biol* 2001;11:R440–R442. [PubMed: 11516667]
9. Drake JW, Holland JJ. Mutation rates among RNA viruses. *Proc Natl Acad Sci USA* 1999;96:13910–13913. [PubMed: 10570172]
10. Domingo E, Holland JJ. RNA virus mutations and fitness for survival. *Annu Rev Microbiol* 1997;51:151–78. [PubMed: 9343347]
11. Rose, JH.; Whitt, MA. Field's Virology. 4th. 1. Lipcott Williams and Wilkins; Philadelphia: 2001. *Rhabdoviridae*: the viruses and their replication; p. 1121-1244.

12. Mead DG, Ramberg FB, Besselsen DG, Mare CJ. Transmission of vesicular stomatitis virus from infected to noninfected black flies co-feeding on nonviremic deer mice. *Science* 2000;287:485–487. [PubMed: 10642550]
13. Letchworth GJ, Rodriguez LL, Barrera JDC. Vesicular stomatitis. *Vet J* 1999;157:239–260. [PubMed: 10328837]
14. Zarate S, Novella IS. Vesicular stomatitis virus evolution during alternation between persistent infection in insect cells and acute infection in mammalian cells is dominated by the persistence phase. *J Virol* 2004;78:12236–12242. [PubMed: 15507610]
15. Pringle CR, Easton AJ. Monopartite negative-strand RNA genomes. *Semin Virol* 1997;8:49–57.
16. Novella I. Contributions of vesicular stomatitis virus to the understanding of RNA virus evolution. *Curr Op Microbiol* 2003;6:399–405.
17. Novella IS, Hershey CL, Escarmis C, Domingo E, Holland JJ. Lack of evolutionary stasis during alternating replication of an arbovirus in insect and mammalian cells. *J Mol Biol* 1999;287:459–465. [PubMed: 10092452]
18. Turner PE, Elena SF. Cost of host radiation in an RNA virus. *Genetics* 2000;156:1465–1470. [PubMed: 11102349]
19. Weaver SC, Brault AC, Kang W, Holland JJ. Genetic and fitness changes accompanying adaptation of an arbovirus to vertebrate and invertebrate cells. *J Virol* 1999;73:4316–4326. [PubMed: 10196330]
20. Cooper LA, Scott TW. Differential evolution of eastern equine encephalitis virus populations in response to host cell type. *Genetics* 2001;157:1403–1412. [PubMed: 11290699]
21. Chen WJ, Wu HR, Chiou SS. E/NS1 modifications of dengue 2 virus after serial passages in mammalian and/or mosquito cells. *Intervirology* 2003;46:289–295. [PubMed: 14555849]
22. Greene IP, Wang E, Deardorff ER, Milleron R, Domingo E, Weaver SC. Effect of alternating passage on adaptation of sindbis virus to vertebrate and invertebrate cells. *J Virol* 2005;79:14253–14260. [PubMed: 16254360]
23. Coffey LL, Vasilakis N, Brault AC, Powers AM, Tripet F, Weaver SC. Arbovirus evolution in vivo is constrained by host alternation. *Proc Natl Acad Sci U S A* 2008;105:6970–5. [PubMed: 18458341]
24. Ruiz-Jarabo CM, Pariente N, Baranowski E, Davila M, Gomez-Mariano G, Domingo E. Expansion of host-cell tropism of foot-and-mouth disease virus despite replication in a constant environment. *J Gen Virol* 2004;85:2289–2297. [PubMed: 15269370]
25. Crill WD, Wichman HA, Bull JJ. Evolutionary reversals during viral adaptation to alternating hosts. *Genetics* 2000;154:27–37. [PubMed: 10628966]
26. Novella IS, Clarke DK, Quer J, Duarte EA, Lee CH, Weaver SC, Elena SF, Moya A, Domingo E, Holland JJ. Extreme fitness differences in mammalian and insect hosts after continuous replication of vesicular stomatitis virus in sandfly cells. *J Virol* 1995;69:6805–6809. [PubMed: 7474092]
27. Kassen R, Rainey PB. The ecology and genetics of microbial diversity. *Ann Rev Microbiol* 2004;58:207–231. [PubMed: 15487936]
28. Cuevas JM, Elena SF, Moya A. Molecular basis of adaptive convergence in experimental populations of RNA viruses. *Genetics* 2002;162:533–542. [PubMed: 12399369]
29. Bull JJ, Badgett M, Wichman HA, Huelsenbeck JP, Hillis DM, Gulati A, Ho C, Molineux JJ. Exceptional convergent evolution in a virus. *Genetics* 1997;147:1497–1507. [PubMed: 9409816]
30. Novella IS, Ebendick-Corpus BE. Molecular basis of fitness loss and fitness recovery in vesicular stomatitis virus. *J Mol Biol* 2004;342:1423–1430. [PubMed: 15364571]
31. Novella IS, Zarate S, Metzgar D, Ebendick-Corpus BE. Positive selection of synonymous mutations in vesicular stomatitis virus. *J Mol Biol* 2004;342:1415–1421. [PubMed: 15364570]
32. Kimura, M. The neutral theory of molecular evolution. Cambridge Press; Cambridge, UK: 1983.
33. Nei, M. Molecular evolutionary genetics. Columbia University Press; New York, NY: 1987.
34. Hurlbut HS. West Nile infection in arthropods. *Am J Trop Med Hyg* 1956;5:76–85. [PubMed: 13292654]
35. Singh KRP. Propagation of arboviruses in Singh's *Aedes* cells. *Curr Top Microbiol Immunol* 1971;55:127–133. [PubMed: 5142320]
36. Taylor WP, Marshall ID. Adaptation studies with Ross River virus: laboratory mice and cell cultures. *J Gen Virol* 1975;28:59–72. [PubMed: 808588]

37. Gao Q, Solomon H, Nagai K, Parniak MA, Wainberg MA. Generation of multiple drug resistance by sequential *in vitro* passage of the human immunodeficiency virus type 1. *Arch Virol* 1994;136:111–122. [PubMed: 8002779]
38. Remold SK, Rambaut A, Turner PE. Evolutionary genomics of host adaptation in vesicular stomatitis virus. *Mol Biol Evol* 2008;25:1138–47. [PubMed: 18353798]
39. Barr JN, Whelan SPJ, Wertz GW. Transcriptional control of the RNA-dependent RNA polymerase of vesicular stomatitis virus. *Biochim Biophys Acta* 2002;1577:323–353.
40. Barr JN, Whelan SPJ, Wertz GW. cis-acting signals involved in termination of vesicular stomatitis virus mRNA synthesis include the conserved AUAC and the U7 signal for polyadenylation. *J Virol* 1997;71:8718–8725. [PubMed: 9343230]
41. Novella IS, Ebendick-Corpus BE, Zarate S, Miller EL. Emergence of mammalian cell-adapted vesicular stomatitis virus from persistent infections of insect vector cells. *J Virol* 2007;81:6664–8. [PubMed: 17428845]
42. Rodriguez LL, Pauszek SJ, Bunch TA, Schumann KR. Full-length genome analysis of natural isolates of vesicular stomatitis virus (Indiana 1 serotype) from North, Central and South America. *J Gen Virol* 2002;83:2475–2483. [PubMed: 12237430]
43. Jeetendra E, Ghosh K, Odell D, Li J, Ghosh HP, Whitt MA. The membrane-proximal region of vesicular stomatitis virus glycoprotein G ectodomain is critical for fusion and virus entry. *J Virol* 2003;77:12807–12818. [PubMed: 14610202]
44. Martinez I, Wertz GW. Biological differences between vesicular stomatitis virus Indiana and New Jersey serotype glycoprotein: identification of amino acid residues modulating pH-dependent infectivity. *J Virol* 2005;79:3578–3885. [PubMed: 15731252]
45. Li T, Pattnaik AK. Overlapping signals for transcription and replication at the 3' terminus of the vesicular stomatitis virus. *J Virol* 1999;73:444–452. [PubMed: 9847350]
46. Wimmer E, Hellen CUT, Cao XM. Genetics of poliovirus. *Ann Rev Genet* 1993;27:353–436. [PubMed: 8122908]
47. Racaniello, VR. *Field's Virology*. 4th. 1. Lipcott Williams and Wilkins; Philadelphia: 2001. *Picornaviridae: the viruses and their replication*; p. 685–722.
48. Kitamura N, Semler BL, Rothburg PG, Larsen GR, Adler CJ, Dorner AJ, Emini EA, Hanecak R, Lee JJ, van der Werf S, Anderson CW, Wimmer E. Primary structure, gene organization and polypeptide expression of poliovirus RNA. *Nature* 1981;291:547–553. [PubMed: 6264310]
49. Svitkin YV, Maslova SV, Agol VI. The genomes of attenuated and virulent poliovirus strains differ in their *in vitro* translation efficiencies. *Virology* 1985;147:243–252. [PubMed: 3000069]
50. La Monica N, Meriam C, Racaniello VR. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. *J Virol* 1986;57:515–525. [PubMed: 3003384]
51. O'Hara PJ, Nichol ST, Horodyski FM, Holland JJ. Vesicular stomatitis virus defective interfering particles can contain extensive genomic sequence rearrangements and base substitutions. *Cell* 1984;36:915–924. [PubMed: 6323026]
52. Holland, JJ. *Fundamental Virology*. 2nd. Raven Press; New York: 1991. Defective viral genomes; p. 151–165.
53. Ding H, Green TJ, Luo M. Crystallization and preliminary X-ray analysis of a proteinase-K-resistant domain within the phosphoprotein of vesicular stomatitis virus (Indiana). *Acta Crystallogr D Biol Crystallogr* 2004;60:2087–90. [PubMed: 15502336]
54. Gao Y, Lenard J. Cooperative binding of multimeric phosphoprotein (P) of vesicular stomatitis virus to polymerase (L) and template: pathways of assembly. *J Virol* 1995;69:7718–23. [PubMed: 7494281]
55. Gao Y, Lenard J. Multimerization and transcriptional activation of the phosphoprotein (P) of vesicular stomatitis virus by casein kinase-II. *Embo J* 1995;14:1240–7. [PubMed: 7720714]
56. Das SC, Pattnaik AK. Role of the hypervariable hinge region of phosphoprotein P of vesicular stomatitis virus in viral RNA synthesis and assembly of infectious virus particles. *J Virol* 2005;79:8101–12. [PubMed: 15956555]
57. Qanungo KR, Shaji D, Mathur M, Banerjee AK. 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* 2004;101:5952–7. [PubMed: 15069200]

58. Howard M, Wertz G. Vesicular stomatitis virus RNA replication: a role for the NS protein. *J Gen Virol* 1989;70(Pt 10):2683–94. [PubMed: 2552006]
59. Blondel D, Petitjean JM, Dezelee S, Wyers F. Vesicular stomatitis virus in *Drosophila melanogaster* cells: regulation of viral transcription and replication. *J Virol* 1988;62:277–284. [PubMed: 2824851]
60. Dezelee S, Blondel D, Wyers F, Petitjean JM. Vesicular stomatitis virus in *Drosophila melanogaster* cells: lack of leader RNA transport into the nuclei and frequent abortion of the replicative step. *J Virol* 1987;61:1391–1397. [PubMed: 3033267]
61. Mudd JA, Leavitt RW, Kingsbury DT, Holland JJ. Natural selection of mutants of vesicular stomatitis virus by cultured cells of *Drosophila melanogaster*. *J Gen Virol* 1973;20:341–51. [PubMed: 4355329]
62. Wyers F, Blondel D, Petitjean JM, Dezelee S. Restricted expression of viral glycoprotein in vesicular stomatitis virus-infected *Drosophila melanogaster* cells. *J Gen Virol* 1989;70:213–218. [PubMed: 2543737]
63. Holland JJ, de la Torre JC, Clarke DC, Duarte E. Quantitation of relative fitness and great adaptability of clonal populations of RNA viruses. *J Virol* 1991;65:2960–2967. [PubMed: 2033662]
64. Tesh RB, Modi GB. Development of a continuous cell line from the sandfly *Lutzomyia longipalpis* (*Diptera: Psychodidae*), and its susceptibility to infection with arboviruses. *J Med Entomol* 1983;20:199–202. [PubMed: 6842527]
65. Duarte EA, Novella IS, Ledesma S, Clarke DK, Moya A, Elena SF, Domingo E, Holland JJ. Subclonal components of consensus fitness in an RNA virus clone. *J Virol* 1994;68:4295–4301. [PubMed: 8207804]

Figure 1.

Fitness changes during adaptation of wt VSV to replication in homogeneous environments (acute infection in BHK-21 cells or persistent infection in LL-5 cells) or in a heterogeneous environment (alternation between persistent infection in LL-5 cells and acute infection in BHK-21 cells). For each genotype label, the first letter corresponds to the environment where the virus adapted (P, persistent infection of LL-5 cells; A, alternation between persistent infection of LL-5 cells and acute infection of BHK-21 cells) and the second to the replica (A, B, C or D). We also indicate the environment where the fitness was determined (in K, in BHK-21 cells; in L, in LL-5 cells).

Nucleotide	Protein (amino acid)	Acute		Alternating				Persistent			
		BHK- adapted (1)	K25B	A25A	A25B	A25C	A25D	P25A*	P25B**	P25C	P25D
C16-U	Non-coding										
20 (43n dup.)	Non-coding										
U50-G	Non-coding										
A163-G	N (K34-E)										
G253-A	N (V64-I)										
A822-R	N (E275)										
A1377-G	Non-coding										
U1438-Y	P (S15-P)										
C1817-U	P (T142-M)										
C2257-U	M (S3-F)										
C2605-A	M (T98-N)										
G2798-A	M (L183)										
U2937-Y	M (stop249-R)										
A3169-M	G (K30-T)										
C3421-A	G (T115-K)										
U3750-K	G (Y226-D)										
C3978-A	G (Q301-K)										
C4003-M	G (A-309E)										
G4122-U	G (V349-F)										
A4139-U	G (P354)										
U4434-G	G (F453-C)										
A6145-C	L (S471)										
C8668-U	L (D1312)										
G9110-U	L (D1460-Y)										
G10337-K	L (E1869-stop)										
A10523-G	L (S1931-G)										
G11153-K	Non-coding										
Fitness in	BHK-21		2.7±0.04	0.58±0.01	0.59±0.06	0.38±0.17	0.58±0.04	0.13±0.06	0.16±0.04	0.16±0.04	0.15±0.02
Fitness in	LL-5		13±2	8500±1100	12000±3800	4700±510	8900±1300	9000±2900	7500±250	7800±160	3600±560

Figure 2.

Genetic and fitness changes in the VSV genome accumulated during adaptation to homogeneous environments (acute infection in BHK-21 cells or persistent infection in LL-5 cells) or in a heterogeneous environment (alternation between persistent infection in LL-5 cells and acute infection in BHK-21 cells). The labeling of strains shows first the selective environment (K, acute infection of BHK-21 cells; A, alternating persistent LL-5 infections and acute BHK-21 infections; P, persistent infection of LL-5 cells), followed by the number of passages (25) and then the replica (A-D). The presence of a mutation is indicated with a filled box. Black represents non-synonymous mutations; dark gray represents synonymous mutations; hatched boxes represent mutations in non-coding mutations. (1) Include mutations previously identified in other genotypes with high fitness on BHK-21 cells (30; ³¹ and unpublished results). Mixtures of nucleotides at polymorphic loci are indicated as follows: M = A+C; R = A+G; Y = C+U; K = G+U; for non-synonymous mutations at these sites the amino acid change corresponding to the mutant nucleotide is shown. The fitness of the wt progenitor is 1. Only one of the four replicas of K25 genotypes is shown as representative of the same outcome; all K25 genotypes incorporated the same mutation and had similar fitness values in BHK-21 cells (2.1±0.04, 2.8±0.04 and 2.8±0.25) and LL-5 cells (10.8±1, 12.2±0.9 and 13.1±1.5). * nts 1-4 not determined; and ** nts 1-16 not determined.

Table 1

Parallel and unique mutations accumulated in persistent genotypes between passage 10 and passage 25.

Strain	Substitutions
	Parallel substitutions
PA	U50G, A1377G, C3978A, U4434G
PB	U50G, G253A, C4003A, U4434G
PC	U50G, C4003A, U4434G
PD	U50G, G253A, C3878A, C4003A
	Unique substitutions
PA	None
PB	G10337K,
PC	A822R, U3750K
PD	U1438Y, C2605A, G2798A, A3169M, A10523G

Table 2

Transient polymorphisms in persistent genotypes at passage 10. In each loci a mixture of wt and the mutated sequence were present.

Strain	Mutations
PA	20[20-63 insertion], G3200A, A7710U, C7839U, A8379G
PB	20[20-63 insertion], C8678U
PC	A1377G, A3200G, A6145C, C7839U, A8379G
PD	U192C, A1377G, G3200A, A6145C, C7839U