Effect of Alternating Passage on Adaptation of Sindbis Virus to Vertebrate and Invertebrate Cells

Ivorlyne P. Greene, Eryu Wang, Eleanor R. Deardorff, Rania Milleron, Esteban Domingo, and Scott C. Weaver*

Center for Biodefense and Emerging Infectious Diseases and Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555-0609, and Centro de Biología Molecular “Severo Ochoa,” Consejo Superior de Investigaciones Científicas, Universidad Autonoma de Madrid, Cantoblanco, 28049 Madrid, Spain

Received 8 April 2005/Accepted 6 August 2005

Mosquito-borne alphaviruses, which replicate alternatingly and obligatorily in mosquitoes and vertebrates, appear to experience lower rates of evolution than do many RNA viruses that replicate solely in vertebrates. This genetic stability is hypothesized to result from the alternating host cycle, which constrains evolution by imposing compromise fitness solutions in each host. To test this hypothesis, Sindbis virus was passaged serially, either in one cell type to eliminate host alteration or alternatingly between vertebrate (BHK) and mosquito (C6/36) cells. Following 20 to 50 serial passages, mutations were identified and changes in fitness were assessed using competition assays against genetically marked, surrogate parent viruses. Specialized viruses passaged in a single cell exhibited more mutations and amino acid changes per passage than those passaged alternately. Single host-adapted viruses exhibited fitness gains in the cells in which they specialized but fitness losses in the bypassed cell type. Most but not all viruses passaged alternately experienced lesser fitness gains than specialized viruses, with fewer mutations per passage. Clonal populations derived from passaged viruses also exhibited adaptation to both cell lines, indicating that polymorphic populations are not required for simultaneous fitness gains in vertebrate and mosquito cells. Nearly all passaged viruses acquired Arg or Lys substitutions in the E2 envelope glycoprotein, but enhanced binding was only detected for BHK cells. These results support the hypothesis that arbovirus evolution may be constrained by alternating host transmission cycles, but they indicate a surprising ability for simultaneous adaptation to highly divergent cell types by combinations of mutations in single genomes.

Sindbis virus (SINV) is the type species of the genus Alphavirus, a group of RNA viruses with nonsegmented single-stranded genomes of approximately 11.7 kb (23, 25). The alphavirus genome is capped at the 5' end and contains a 3' poly(A) tail. The 5' two-thirds of the genome encode the nonstructural proteins 1 to 4, which are necessary for viral replication. The structural proteins, capsid and E1 and E2 envelope glycoproteins, are translated from a subgenomic mRNA (26S) which is identical in sequence to the 3' one-third of the genome (Fig. 1). The Sindbis virion contains an icosahedral nucleocapsid that consists of 240 copies of the capsid protein surrounded by a lipid envelope derived from the plasma membrane of infected cells, into which the glycoprotein E1/E2 heterodimers are embedded (4).

Nearly all alphaviruses rely on horizontal mosquito-borne transmission among vertebrate hosts, requiring alternating replication in highly divergent hosts and cell types (7, 34). Alphaviruses and other arthropod-borne viruses also appear to undergo lower rates of evolution than many other animal RNA viruses that replicate solely in vertebrates (6, 33, 35). Rates of SINV nucleotide substitution, deduced from oligonucleotide fingerprinting, are approximately $4 \times 10^{-4}$ substitutions per nucleotide per year (18, 36), similar to estimates for other alphaviruses and other arboviruses and approximately 10-fold lower than rates reported for many single-host RNA viruses (25).

Experimental validation of arbovirus genetic stability was first provided by studies of the bunyavirus La Crosse virus during horizontal (oral infection of Aedes triseriatus mosquitoes) and vertical (transovarial transmission in mosquitoes) transmission (1). No RNA sequence changes were detected by oligonucleotide fingerprinting in any of the passages, corroborating genetic stability in nature. Similar studies using transovarial transmission of the bunyavirus Toscana virus also revealed genetic stability during over 12 sandfly generations during a 2-year time period (2).

The factors responsible for arbovirus genetic stability are unknown. Possible explanations include the persistent infection of arthropod vectors, where replication is modulated within a few days or weeks by RNA interference (22) and possibly other mechanisms. Tick-borne flaviviruses, which undergo longer persistence in ticks than do mosquito-borne viruses in their vectors, appear to exhibit even slower evolutionary rates than their mosquito-borne relatives (6), consistent with this hypothesis. Another hypothetical explanation for the genetic stability of arboviruses is that vertebrate and invertebrate hosts impose different selective forces and that adaptation for optimal fitness in either host type involves compromises for fitness in the other. In other words, the alternating...
host transmission cycle may constrain arbovirus evolution by requiring these viruses to adopt a compromise fitness genome for alternating replication in each host (24, 36).

Arbovirus adaptation to different hosts and cells was examined by Taylor and Marshall using the alphavirus Ross River virus (26). Serial passage in cell cultures depressed virulence, while mouse passage raised the level of virulence in a step-wise manner. Biological clones from both the original virus population and the 10th passage in mice were heterogeneous with respect to virulence, indicating a quasispecies population. Alternate passages between A. aegypti mosquitoes and mice resulted in no detectable virulence change of two different virus strains (27), indicating that alternate passages stabilized the virulence phenotype.

More recently, direct evidence for the effect of host alteration on arboviral adaptation came from studies of the rhabdovirus, vesicular stomatitis virus (VSV), and from alphaviruses. When VSV was passaged alternately in sandfly and baby hamster kidney (BHK) cells, or when allowed to specialize on one cell type through serial passages, fitness increases were observed in all cases (17). Surprisingly, VSV passaged exclusively acutely in hamster cells also increased its fitness in sandfly cells, indicating that specialization did not result in cell-specific adaptation. However, persistent infection of insect cells resulted in fitness declines for acute infection of mammalian cells and appeared to dominate the evolution of VSV populations that alternated their replication with acute infection of mammalian cells (37). However, other studies focusing on vertebrate cells showed fitness declines in unselected cellular environments, suggesting that fitness trade-offs occur when VSV is allowed to specialize (30).

Studies with the alphavirus Eastern equine encephalitis virus (EEEV) yielded different results and conclusions (33). Specialization on BHK cells resulted in fitness losses for mosquito cells, and vice versa. However, viruses forced to alternate between cell types achieved fitness increases in both cell types comparable to that of each respective specialist, again contrary to the hypothesis that alternation constrains adaptation by arboviruses. However, rates of sequence change were lower in the alternating passage series, supporting the hypothesis that host alternation constrains evolutionary rates. Similar results with EEEV were also obtained using avian and mosquito cells (5).

Some studies with SINV have focused on the subgenomic promoter and its response to selective pressure for adaptation to hosts. To determine if promoter utilization varies in vertebrate versus mosquito cells, Hertz and Huang (10) passaged SINV containing a library of different promoter sequences in BHK and mosquito cells. Selection was faster and more rapid in mosquito cells, which selected a smaller number of promoter sequences. Extensive passaging in BHK cells led to a promoter consensus sequence that increasingly resembled the wild type, suggesting that the wild type and similar sequences are optimal for promoter function in hamster cells (9); similar results were obtained from mosquito cell passage (10). These studies suggest that SINV makes little or no evolutionary compromise in maintaining the ability to replicate alternately in the two disparate host organisms.

To test the effect of alternating host cell replication on genetic and fitness stability of SINV, we studied adaptation to BHK and C6/36 mosquito cells of two different strains. Following 20 or 50 serial passages, sequence stability and fitness gains were evaluated to compare the effects of specialization versus alternating replication transmission cycles.

### MATERIALS AND METHODS

**Cell cultures.** Monolayer cultures of BHK cells were grown at 37°C in Eagle's minimal essential medium (MEM) with 5% heat-inactivated bovine serum. Aedes albopictus C6/36 mosquito cells were grown at 32°C in MEM with nonessential amino acids and 10% heat-inactivated fetal bovine serum.

**Sindbis virus infections.** Two infectious cDNA clones were used to produce SINV stocks to initiate passage series. The pToto1101 clone (19) is a chimera of different SINV strains, some with extensive laboratory passage (19), and is attenuated in young mice compared to wild-type SINV strains. The pTR339 clone (15) is more representative of wild-type SINV and is virulent for young mice. Virus stocks were prepared from both clones by linearization with XhoI, and RNA was transcribed in the presence of MTG-5′-ppp-5′G cap (New England Biolabs, Beverly, MA) with SP6 RNA polymerase (Invitrogen, Carlsbad, CA), followed by electroporation of BHK cells using standard methods.

Three different types of passage series were done for each SINV strain: (i) serial (specialized) passage in BHK cells; (ii) serial (specialized) passage in C6/36 cells; and (iii) alternating passage between BHK and C6/36 cells. Infected cell cultures were incubated at 32°C to eliminate temperature sensitivity as a factor in the outcomes. Following harvest at 24 (BHK) or 48 (C6/36) hours after infection to achieve approximately maximum titers, cell culture media were diluted to initiate subsequent infections. A multiplicity of approximately 0.01 PFU/cell was maintained to minimize the influence of defective interfering particles. Duplicate, parallel passage series were done for each treatment to assess variance in outcomes and to identify common mutations resulting from positive selection. The 50th passage of each Toto1101 series and the 20th passage of each TR339 SINV series were used for genetic and phenotypic assays.

**RT-PCR amplons and sequencing.** The E2 envelope glycoprotein gene was sequenced from reverse transcription-PCR (RT-PCR) amplicons to evaluate mutations accompanying passage series. Viral RNA was extracted from 0.25 ml of cell-free supernatant by adding 0.75 ml of TRIzol LS (BRL, Bethesda, MD) according to the manufacturer's protocol. The cDNA was synthesized at 42°C with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and RNA was transcribed in the presence of M7G-5′cap (sense) and cDNA primer (antisense), designed to amplify genome position 8557 to 9335; and 5′-AGTGCAAGTGGCGCACACTAC-3′ (sense) and cDNA primer SIN-9004 (antisense) and cDNA primers 9229 to 9004. PCRs were done with 30 cycles as follows: heat denaturation at 95°C for 30 s, primer annealing at 53°C for 30 s, and extension at 72°C for 1 min. Amplicons were extracted from 1% agarose gels and sequenced directly using the PCR primers and the Applied
Marked surrogate parent virus. A marked, surrogate parent virus was generated for each SINV strain to use in competition assays. PCR primers SIN-2215 and SIN-2806 were used to amplify a 591-bp fragment corresponding to the N terminus of the nsP2 gene. Amplicons were ligated into the pBluescript vector (Stratagene) in the presence of Smal (5 U) restriction enzyme at 14°C for 12 to 18 h. Subclones were used as a template for mutagenesis PCR with the following primers: 5'-CTGAAAAAGATATCTGCACCAAGAC-3' (sense) and 5'-GTC TTGTGTGAGATATCATTITTTCAAGG-3' (antisense), which introduced two synonymous, third codon position mutations to eliminate the Ndel restriction site at genome position 2586. Subclones were screened by Ndel digestion followed by sequencing to confirm the desired mutations. Mutated subclones were ligated back into the pToto1101 and pTR339 clone backbones, and the resulting marked surrogate parent clones were screened via Ndel digestion and sequenced to confirm the mutations. The marked SINV strains were rescued as described above and used as surrogates for the parent strains in competition assays.

Competition assays. Serially passaged SINV strains were competed with surrogate parents in a modification of the assay developed by Holland et al. (11). Briefly, serially passed and marked, surrogate parent viruses were mixed in a 1:1 ratio and used to infect BHK or C6/36 cells at a multiplicity of infection (MOI) of 0.01 PFU/cell. Following incubation for 24 (BHK) or 48 (C6/36) hours, viral RNA was extracted from cell-free supernatant and a 439-bp RT-PCR amplicon was generated from the nsP2 gene as described above. Amplicons were digested with S U of Ndel for 2 to 16 h at 37°C and viewed on 1.5% agarose gels. Changes in the ratios of passed and marked surrogate parent genotypes following a single passage were estimated by quantifying the DNA content in uncut (439-bp) versus cut (338- and 101-bp) bands from digested RT-PCR amplicons derived from the inocula and competition harvests. Controls included marked and parent SINV strains to ensure proper digestion and identification of products of the correct size. Relative DNA amounts in each band were quantified using densitometry and the 1D Scan program (Scanalytics, Inc., Billerica, MA). Because the competing genotypes have very few nucleotide differences and therefore form heteroduplexes that behave like nicked DNA in gel electrophoresis, heteroduplexes that behave like nicked DNA in gel electrophoresis. Because the competing genotypes have very few nucleotide differences and therefore form the final denaturation steps of a PCR, genotype ratios were corrected using a binomial equation: w^2 + 2nm + m^2 = 1; w = wild type, m = marked, surrogate wild-type mutant resistant to NdeI digestion, where w2 represents completely cleaved, homodimer, wild-type, surrogate parent DNA and the frequency of w is determined by its square root; uncleaved fragments represent 2nm + m^2.

Cell binding assays. Virus stocks were radiolabeled with [35S]methionine (15 μCi/ml; Amersham Biosciences, Piscataway, NJ) during infection of BHK-21 or C6/36 cells at a multiplicity of infection of approximately 10 PFU/cell. Following 4 h of incubation at 32°C, the medium was replaced with methionine-free MEM for 3 h. Supernatant was collected at 24 (BHK) or 48 h (C6/36) and clarified by centrifugation at 3,000 × g for 10 min. Virus was precipitated with polyethylene glycol 8000 and NaCl at final concentrations of 7% and 2.3% (wt/vol), respectively, and pelleted at 6,000 × g for 30 min. The viruses were purified on continuous 20-to-70% (wt/vol) sucrose gradients in TEN buffer (0.05 M Tris-HCl, pH 7.2; 0.1 M NaCl; 0.001 M EDTA) and pelleted as described previously (32). Cell monolayers were rinsed twice with ice-cold binding buffer (phosphate-buffered saline [PBS] with 0.5 mM MgCl2, 1 mM CaCl2) with 0.5% bovine serum albumin. About 10^4 cpm of [35S]-labeled virus was added to each well in 150 μl of binding buffer, and plates were rocked at 4°C for 1 to 4 h. Following incubation, unbound virus was removed and the cells were washed three times with ice-cold binding buffer. The cells were then lysed in 1% sodium dodecyl sulfate, and counts per minute were assayed by liquid scintillation counting.

**FIG. 2.** Proportion wild-type and marked (NdeI-resistant), surrogate wild-type SINV in mixed populations as assayed by RFLP. Following RT-PCR amplification of mixtures, amplicon DNA was digested with NdeI and electrophoresed on a 1.5% agarose gel. Lanes are as follows: left lane, 100-bp marker; A, undigested, wild type alone; B, undigested mutant only; C, NdeI-digested wild type only; D, NdeI-digested mutant only; E, NdeI-digested wild type and mutant mixture, 1:5 mixture; F, NdeI-digested wild type and mutant mixture, 1:1 mixture; G, NdeI-digested wild type and mutant mixture, 5:1 mixture. Upper bands from mixed samples show a slightly slower migrating form, representing marked wild-type heteroduplexes that behave like nicked DNA in gel electrophoresis.

**Statistics.** Comparisons among mean fitness values were performed using a one-way analysis of variance with Dunn’s multiple comparison test for normally distributed data or the Kruskal-Wallis test for data not normally distributed. A chi-square test was used for assessing a nonrandom ratio of synonymous versus synonymous mutations. The GraphPad Prism program (San Diego, CA) was used for these tests.

**RESULTS**

Generation of the marked, surrogate parent strains. Eliminating the NdeI restriction enzyme site at nucleotide position 2586, in the nsP2 gene, created a marked surrogate parent virus. This genome position was chosen because it contains no RNA sequences known to be under direct selection (e.g., for secondary structures), and so synonymous mutations there were predicted to have less chance of adverse affects on viral fitness than those in other regions of known primary sequence importance. Competition of the marked surrogate parent viruses against Toto1101 or TR339 stocks in both BHK-21 and C6/36 cells resulted in no detectable fitness differences (no reproducible changes in frequencies of the two competitors following triplicate mixed infections; P > 0.05) (data not shown), indicating that the marked viruses were suitable surrogates for the parents in competition assays.

Competition fitness assays. To assess the accuracy of the restriction fragment length polymorphism (RFLP) assay in quantifying the ratios of wild-type and marked viral genomes, titers of stocks of each virus were determined and stocks were mixed to ratios of approximately 1:5, 1:1, and 5:1. After each mixture was made, titers of aliquots were determined by plaque assay to estimate the true ratios, and the PCR-RFLP assay was conducted to obtain an independent estimate. Each test was done twice to generate two pairs of estimates. An example of the RFLP gel is presented in Fig. 2, and the estimated ratios are presented in Table 1. Plaque and RFLP assays

**TABLE 1.** Proportion of wild-type virus in mixed populations, as estimated by plaque and PCR-RFLP assays

| Target ratio (WT:mutant) | Target proportion of WT (%) | Proportion (%) WT by: | Plaque assay | RFLP*
|--------------------------|----------------------------|----------------------|--------------|--------
| 1:5                      | 17                        | 12-18                | 17-25        |
| 1:1                      | 50                        | 41-52                | 48-53        |
| 5:1                      | 83                        | 78-84                | 79-83        |

* WT, wild-type; mutant, surrogate wild-type, marked mutant.

* Range of estimates derived from two independent assays.
yielded very similar ratios and both were within 9% of expected values, suggesting that the genetic assay is as reliable as the plaque assay for determining the frequencies of SINV strains in mixtures.

Competitions between all passage series and the marked, surrogate parent strains were done in both BHK (Fig. 3) and C6/36 (Fig. 4) cells. Alternating host cell passages were terminated in both cell types to assess the effect of the final host cell infection on SINV population fitness. In BHK cells, both pToto1101-derived SINV strains passaged 50 times in BHK cells (TO50K1.2) (Fig. 3A) and both alternating BHK-C6/36 series [ending with BHK (TO50KA-1.2) or C6/36 (TO50CA-1.2) cell passage] exhibited fitness increases ($P < 0.001$), while single-host-cell C6/36 series viruses (TO50C-1.2) experienced fitness declines ($P < 0.001$). The fitness increases of the single-host Toto1101-BHK series were approximately 20 to 50% greater than that of either of the alternating cell passage series, but this difference between the passage types was not significant ($P > 0.05$). The fitness trend was different in C6/36 cells (Fig. 4A). Both the single-host Toto1101-C6/36 series and the alternating series exhibited fitness increases in C6/36 cells ($P < 0.001$). However, the C6/36 specialists exhibited fitness increases approximately two to three times lower ($P < 0.05$) than the increases experienced by both alternating passage series. Both single-host BHK series exhibited fitness declines in C6/36 cells ($P < 0.05$). Because the cell origin (vertebrate versus insect) of alphaviruses affects glycosylation of the envelope glycoproteins (23), alternately passaged viruses were terminated in the 50th passage from either BHK or C6/36 cells for competition assays to control for carbohydrate differences. Some consistent fitness differences were noted between viruses terminated in each cell type, suggesting population shifts with each alternating passage or effects of cell-specific glycosylation (Fig. 3 and 4). However, these differences were not significant ($P > 0.05$).

To examine smaller numbers of SINV passages and earlier adaptation, as well as to initiate passages with another SINV strain without an extensive history of cell culture passages, strain TR339 was used for similar experiments limited to 20...
passes. Like the Toto1101 passage series, strain TR339 passed serially (specialized) in a single cell type exhibited fitness gains in that cell type ($P < 0.001$) but fitness declines in the bypassed cell type ($P < 0.05$ in BHK cells, not significant in C6/36 cells). The BHK passage and alternating series terminated in BHK cells underwent similar fitness gains in BHK cells (Fig. 3B), while BHK fitness gains for alternating passages terminated in C6/36 cells were five to seven times lower, a significant difference ($P < 0.05$). Unlike Toto1101, TR339 passed exclusively in C6/36 cells exhibited comparable fitness gains when compared to the alternating passages (Fig. 4B).

**Fitness of clonal populations.** The simultaneous adaptation of SINV to both vertebrate and mosquito cells during alternating passages suggested two mechanisms: (i) because SINV is stable and suboptimally fit virions may survive from one passage to the next without replication, a polymorphic population comprised of a mixture of mutants, each specialized for one cell or the other, may have developed; or (ii) individual SINV genomes may have accumulated combinations of mutations that conferred fitness gains for both cell types. The differences in fitness in the alternating passages terminated in BHK versus mosquito cells supported the former hypothesis. However, to address the second hypothesis, we obtained clonal pools of the TR339 virus from 20 alternating passages by harvesting two well-isolated plaques from both BHK and C7/10 mosquito cells (used instead of C6/36 cells because they produce more distinct plaques). The fitness of each clonal population was assessed using the competition assay as described above. All four clonal populations outcompeted the marked surrogate parent in both BHK and C6/36 cells in all competition replicates, with final/starting ratios of adapted/surrogate parent in both BHK and C6/36 cells in all competitions.

**Genetic changes.** The 50th Toto1101 passages in BHK and C6/36 cells and the final alternating passage terminated in each cell type were sequenced to detect mutations in the E2 envelope glycoprotein. This gene was selected to investigate mutations associated previously with adaptation to glycosaminoglycan binding in vitro (3, 12) and also to compare sequence stability with the various passage treatments. A total of 20 nucleotide changes, 18 of which were nonsynonymous, was detected (Table 2). This frequency of nonsynonymous mutations, though not significantly higher than expected based on random mutations in the E2 gene, suggested positive selection for amino acid changes involved in adaptation. With one exception, the synonymous substitutions were all exclusive to a given passage treatment (Table 3); four amino acid changes were common to both BHK continuous series, and one was found only in one series (no. 2). Again, the preponderance of mutations common to both series suggested convergent evolution resulting from positive selection. One of the amino acid substitutions common to both BHK cell passages, Gln55→His, involved a known determinant of SINV neurovirulence in mice (13); His55 increases binding of SINV to mouse neuroblastoma cells and promotes rapid internalization and degradation of viral proteins in BHK and mouse neuroblastoma cells (28).

Both C6/36 passage series showed an amino acid change that was also found in both BHK cell series, Glu251→Val. Virus populations from the alternating series terminated in BHK or C6/36 cells had identical consensus sequences, suggesting no major shift in the population makeup during cell alternation with respect to E2 sequences. Both alternating series (Table 3) shared three amino acid substitutions but, surprisingly, these did not include the Glu251→Val change common to both specialized passage series.

**Sequences of the strain TR339 passages identified 12 nucleotide changes resulting in six amino acid changes (Table 2). Both the continuous BHK and one of the alternating passage series had a mutation at E2 residue 1 (Ser→Arg) that is associated with increased binding to and infectivity of BHK cells (3, 12) (Table 2). A second mutation, Glu70→Lys, found in the alternating passage series terminated with C6/36 cells is also known to increase SIN binding to BHK cells (3, 12). The C6/36 cell passages included one common amino acid change.
at position 60 (Ser→Arg), also suggesting positive selection on this amino acid for binding to negative molecules on the cell surface.

Overall, the single host cell passage series exhibited a mean of four nucleotide and three amino acid changes in BHK cells and two nucleotide and one to two amino acid changes in C6/36 cells. The alternating passage series, which underwent replication comparable to the sum of the BHK and C6/36 specialized passages, exhibited a mean of only 3.5 nucleotide and 2.25 amino acid changes, less than the sum (mean of 6 nucleotides and 4.5 amino acids) of the specialized series. These results suggest that cell alternation may have a stabilizing effect on SINV evolution.

**Binding assays.** Vertebrate cell culture passage of SINV is associated with the generation of mutations that increase binding to cell surface glycosaminoglycans (3, 12). Two such mutations, Ser1→Arg, and Glu70→Lys, were found in TR339 viruses passaged in BHK cells. Another mutation (Ser60→Arg) involving increased E2 charge accompanied both C6/36 cell passages of TR339, suggesting similar selection by mosquito cells for glycosaminoglycan binding. To assess more directly the role of these adaptive mutations on binding to both host cells, we measured binding of purified, radiolabeled, passaged TR339 to each cell type. Both BHK-passaged series viruses bound BHK cells at higher rates than the parent virus (Fig. 5), consistent with artificial selection for glycosaminoglycan binding. Surprisingly, the C6/36-passaged TR339 strains also increased their affinity for BHK cells (Fig. 5). However, binding to C6/36 cells did not appear to change with passage in either BHK or C3/36 cells (Fig. 5). Because there was no consistent difference between the BHK or C6/36 cell-passaged strains in binding to either cell type, the binding properties of the alternating passages were not assessed.

**DISCUSSION**

**Effects of host cell alternation on SINV adaptation.** We hypothesized (i) that alphavirus evolution is constrained by the two-host transmission and replication cycle. A prediction of this hypothesis is that freeing SINV from the requirement for alternate replication in vertebrate and mosquito cells should facilitate adaptation to a given host or host cell line, resulting in faster adaptation and evolution in single host cell passages than in alternating passages. The second hypothesis tested was (ii) adaptation to a given host or cell is generally specific and results in fitness declines in other hosts or cells, as observed previously for EEEV (33). To test these hypotheses, SINV recovered from two different infectious clones was passaged 20 or 50 times, either continuously or alternately, in vertebrate and invertebrate cells. Following passages, viruses were assayed genetically by sequencing of the E2 envelope glycoprotein gene, and their fitness change was determined via competition against a marked surrogate parent virus.

Our results support the second hypothesis, but support for the first hypothesis is limited. As predicted, single-host passage series underwent more nucleotide and amino acid changes in their consensus sequences compared to viruses forced to replicate alternately in both cell types. Overall, 71% of mutations were nonsynonymous, providing no statistical evidence that positive selection predominated in our SINV passages. This ratio contrasts sharply with arbovirus mutations that occur in nature, where synonymous mutations vastly outnumber nonsynonymous mutations, suggesting purifying selection (31). The identification of several amino acid changes common to both passage series in the same cell line also suggests convergent evolution via positive selection.

Our results also contrast with those from experimental evolution studies of VSV, which undergoes similar numbers of mutations during alternating or specializing host cell passages (17). When we allowed SINV to specialize on a given host cell, it invariably lost fitness for replication in the bypassed cell line, supporting the second hypothesis. This result agrees with previous studies of another alphavirus, EEEV (5, 33), but contrasts with experimental adaptation studies of VSV, which undergoes fitness increases in both sandfly and BHK cells when allowed to specialize via serial BHK cell passages (17). When we allowed SINV to specialize on a given host cell, it invariably lost fitness for replication in the bypassed cell line, supporting the second hypothesis. This result agrees with previous studies of other alphaviruses, EEEV (5, 33), but contrasts with experimental adaptation studies of VSV, which undergoes fitness increases in both sandfly and BHK cells when allowed to specialize via serial BHK cell passages (17). Unlike our results with SINV, experimental evolution of VSV shows asymmetry in that specialization for acute infection of mammalian cells has no cost (results in no fitness decline) for persistent infection of insect cells, but persistent infection of insect cells has a dramatic cost for vertebrate cell infection (37). Persistent infections of alphaviruses are needed to generate comparative data. However, when passaged in different vertebrate cell lines, VSV exhibits fitness gains in the selected cell environment but suffers reduced competitiveness in the unselected host cell (30). The natural transmission cycles of VSV remain obscure, with limited evidence that the virus gen-

![Graph showing binding assays](image-url)
erates sufficient viremia in vertebrates for horizontal transmission of arthropod-borne RNA viruses (20). An exception to the pattern of greater fitness increases in SINV allowed to specialize compared to alternating passages were our results with adaptation to C3/36 cells. Alternately passaged strain Toto1101 exhibited fitness increases in C6/36 cells that were two to three times higher than increases following serial passage in C6/36 cells alone (specialization). Strain TR339 exhibited similar fitness increases when allowed to specialize on C3/36 cells or when forced to alternate between C3/36 and BHK cells. A possible explanation for the lack of faster adaptation with specialization by both SINV strains is that greater amounts of replication occur in BHK than in C6/36 cells (virus yields at harvest were greater [data not shown]), allowing SINV to sample more mutations in the alternating passage series than during serial C3/36 cell passages. While the alternating host cell-passaged SINV exhibited similar or greater fitness gains in C6/36 cells compared to the C6/36-specialized series, the final C6/36 passage in the alternating series appeared to have a strong effect on fitness; virus stocks from the alternating passage series terminated in C6/36 cells exhibited higher fitness than those terminated in BHK cells. These results suggest the development of polymorphic populations during alternating cell passages. However, the fitness of clonal populations derived from plaques from the alternating passage populations exhibited comparable fitness gains in both cells compared to large populations, indicating that individual genomes acquired mutations that increased their fitness for replication in both cell types. Clonal populations invariably exhibited higher fitness values than those of the parent population used to produce the plaques, suggesting that a reduction in the quasispecies genetic diversity may have increased fitness in the selected environment. Reverse genetic culture adaptation of SINV by enhancing binding to heparan sulfate on the cell surface (12, 15) and also result in murine attenuation (8, 21).

Our results indicate that strain TR339 passaged in BHK cells and containing positive charge amino acid substitutions binds BHK cells at a greater level than the parental strain. These mutations appeared in the consensus sequences of TR339 passaged both continuously in vertebrate cells and in alternating passages between vertebrate and mosquito cells. Following TR339 passage in mosquito cells, a similar amino acid substitution of Ser60→Arg suggested that adaptation for binding to distinct glycosaminoglycans was involved in adaptation to C3/36 cells. The enhanced binding of C3/36-passaged virus suggests that this mutation, the only one affecting the charge of the E2 protein following passage in mosquito cells, also enhances affinity for BHK cells. Surprisingly, our binding studies failed to demonstrate any change in affinity for C6/36 cells, even in the passage series that specialized in these mosquito cells. Elucidating the effect on replication in mosquito cells and binding to BHK cells of the Ser60→Arg substitution in the SINV E2 protein deserves further study.

**ACKNOWLEDGMENTS**

The pToto1101 SINV clone was kindly provided by Charles Rice of Washington University (currently at Rockefeller University), and the pTR339 SINV clone was kindly provided by William Klimstra and Robert Johnston of the University of North Carolina.

E.R.D. was supported by the CDC training grant 1T01/CCT622892. S.C.W. was supported by Fogarty Senior International Fellowship from the National Institutes of Health. This research was supported by grant AI049725 from the National Institutes of Health through the joint National Science Foundation/National Institutes of Health program on the Ecology of Infectious Disease, by grant BMC2001-1823-C02-01, and by the Fundacion Ramon Areces.

**REFERENCES**


