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Genetic and Fitness Changes Accompanying Adaptation of an Arbovirus to Vertebrate and Invertebrate Cells

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The alternating host cycle and persistent vector infection may constrain the evolution of arboviruses. To test this hypothesis, eastern equine encephalitis virus was passaged in BHK or mosquito cells, as well as in alternating (both) host cell passages. High and low multiplicities were used to examine the effect of defective interfering particles. Clonal BHK and persistent mosquito cell infections were also evaluated. Fitness was measured with one-step growth curves and competition assays, and mutations were evaluated by nucleotide sequencing and RNA fingerprinting. All passages and assays were done at 32°C to eliminate temperature as a selection factor. Viruses passaged in either cell type alone exhibited fitness declines in the bypassed cells, while high-multiplicity and clonal passages caused fitness declines in both types of cells. Bypassed cell fitness losses were mosquito and vertebrate specific and were not restricted to individual cell lines. Fitness increases occurred in the cell line used for single-host-adaptation passages and in both cells for alternately passaged viruses. Surprisingly, single-host-cell passage increased fitness in that cell type no more than alternating passages. However, single-host-cell adaptation resulted in more mutations than alternating cell passages. Mosquito cell adaptation invariably resulted in replacement of the stop codon in nsP3 with arginine or cysteine. In one case, BHK cell adaptation resulted in a 238-nucleotide deletion in the 3′ untranslated region. Many nonsynonymous substitutions were shared among more than one BHK or mosquito cell passage series, suggesting positive Darwinian selection. Our results suggest that alternating host transmission cycles constrain the evolutionary rates of arboviruses but not their fitness for either host alone.

Arthropod-borne viruses (arboviruses) are transmitted among vertebrate hosts by insect and tick vectors. Although some can persist by vertical transmission from female arthropods to their offspring, most must replicate alternately in vertebrates and vectors in horizontal transmission cycles. Eastern equine encephalitis virus (EEEV) and several other mosquito-borne alphaviruses appear to undergo lower rates of evolution than many other animal RNA viruses that replicate only in vertebrate hosts, such as human immunodeficiency virus, hepatitis C virus, and poliovirus (37, 38). The factors responsible for alphavirus genetic stability have not been addressed definitively. One hypothesis is that alphavirus evolution is constrained by innate properties of their genome replication, while others involve strong purifying selection imposed by the alternating host transmission cycle or other factors related to their transmission that minimize genetic drift and founder effects (38). None of these hypotheses have been tested experimentally.

Despite high mutation frequencies, the sequences of many RNA viruses remain stable in nature and under certain laboratory conditions, such as serial, low-multiplicity passages in vitro (28). However, other conditions promote genetic disequilibrium and rapid experimental evolution (29). One such factor is the presence of defective interfering (DI) particles, which are preferentially amplified during serial, high-multiplicity passages and interfere with standard virus replication. Selective pressure for resistance to interference leads to rapid genome evolution of vesicular stomatitis virus (VSV) (28). The presence of DI particles also contributes to rapid evolution of VSV in persistently infected cells (16). Another factor known to promote rapid phenotypic changes in RNA viruses is the founder effect or genetic bottleneck. When subjected to clonal (plaque-to-plaque) passages, bacteriophage ϕ6 (3), VSV (9), and foot-and-mouth-disease virus (13) undergo fitness losses due to Muller's ratchet (25). Deleterious mutations presumably accumulate in the genomes of these viruses because forward mutation rates exceed those of back mutations. Clonal passages can also prevent a more-fit variant present in the original population from being selected during passage and reduce the probability of regenerating mutation-free genomes through genetic recombination or reassortment (2).

Multiplicities of infection and DI virus may be important factors in the regulation of alphavirus evolution (38). Multiplicities in mosquito vectors may be limited by infectious virus titers in vertebrate blood and by the small volume (generally only a few microliters) of blood ingested. During progression of virus from the mosquito midgut to the salivary glands, basal laminae appear to interfere with alphavirus movement and may limit multiplicities of infection in other tissues (33, 40). Titers of alphaviruses in mosquito saliva also decrease after about 1 week of infection (1, 40), limiting inocula and multiplicities of infection in vertebrate hosts and increasing opportunities for founder effects. Because the life cycle of alphaviruses includes persistent infection of mosquito vectors, the potential for rapid evolution under the influence of DI virus has also been suggested (38).

To investigate the influences of alternating host replication, infection multiplicity, founder effects, and DI particles on genetic and fitness changes of an arbovirus, we used a cell culture model system to study the evolution of EEEV. Alternating host cell replication generally resulted in lower rates of genetic change but similar fitness increases compared single-host-cell passages. Undiluted passages, favoring the accumulation of DI

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particles, resulted in severe fitness declines and moderate genetic change, as did persistent infection of mosquito cells. Clonal passages resulted in little genetic change but reductions in fitness in both cell types.

MATERIALS AND METHODS

Cell cultures. Monolayer cultures of BHK21 cells were grown at 37°C in Eagle's minimal essential medium (MEM) containing 5% heat-inactivated calf serum. *Aedes albopictus* C6/36 mosquito cells were grown at 32°C in MEM supplemented with nonessential amino acids and 10% heat-inactivated calf serum.

EEEV infections. An unpassaged strain of EEEV, 2061-88, isolated in 1988 from field-collected *Culiseta melanura* mosquitoes in Pocomoke Swamp, Maryland, was used for experimental infections. The original triturated mosquito pool was diluted and inoculated into a bottle of BHK cells to yield a single plaque, and the harvested clonal pool was amplified once in BHK cells to generate the stock used for all subsequent infections.

The plaque harvest described above was used to initiate serial infections of cell cultures and persistent infections. All passage series were carried out 100 times, with the exception of the clonal (plaque-to-plaque) series, which was carried out 40 times, and persistent infections of mosquito cells, which were maintained for 200 days. All virus passages were carried out at 32°C to eliminate temperature as a selection factor. Virus (diluted in MEM or undiluted) was adsorbed to cells at room temperature for 30 min, followed by addition of MEM containing 5% serum and incubation at 32°C. Plaque assays were carried out at 32°C with BHK or Vero cells and 0.4% agarose in MEM for overlays. Randomly isolated plaques (no more than three in a 25-cm^2 bottle) were harvested by using a Pasteur pipette to obtain clonal pools of EEEV. Persistent infections of C6/36 mosquito cells were initiated by infection at a multiplicity of 0.1. Following 7 days of incubation at 32°C, cells were detached by vigorous shaking and diluted 1:20 with fresh medium. Thereafter, cells were split 1:20 at 10-day intervals.

One-step growth curves. To examine changes in replication kinetics after different passages, one-step growth curves were done in triplicate. Virus inocula were diluted to yield a multiplicity of infection of 0.01. MEM was aspirated from 25-cm^2 bottles containing cell monolayers, and 0.25 ml of virus was added at 5°C. Following adsorption for 1 h with frequent rocking, cells were washed three times with phosphate-buffered saline, and MEM was added at 32°C. Supernatant samples were taken at selected intervals and plaque assayed on Vero cells to determine replication kinetics.

Competition fitness assays. Competition fitness assays were a modification of those described previously (14). Each passaged virus was competed against the same standard, the alternating host cell, diluted-passage-series virus (see Table 1) that had undergone a mutation in an *Xho*I restriction site within the nsP4 gene. Mixtures of viruses were prepared in defined ratios and passaged in triplicate series with dilutions (10^{-4} for BHK cells and 10^{-2} for C6/36) to maintain multiplicities of ca. 0.01. Following incubation for 24 h (BHK) or 48 h (C6/36), RNA was extracted from cell culture media and the nsP3-nsP4 fragment was amplified by reverse transcription (RT)-PCR as described below. Approximately 500 ng of amplicons was purified by eluting DNA from 1% agarose gels and digested with *Xho*I (5 units) for 2 h at 37°C. The ratios of viruses were estimated by quantifying the DNA in uncut (542 bp) versus cut (398 and 144 bp) bands with densitometry of ethidium bromide-stained gels and the 2-D Scan program (Scanalytics, Inc., Billerica, Mass.).

T_1 oligonucleotide fingerprinting. Plaque clones and passaged EEEV populations were amplified by infection of BHK monolayers at 32°C with multiplicities of infection of 0.01 to 0.1. Viral RNA was intrinsically labeled by adding 0.1 to 0.2 mCi of ^{32}P, as inorganic phosphate, per ml to cell culture bottles during virus amplification. Cell culture supernatants were harvested after 24 h, and viral RNA was purified as described previously (39). Ribonuclease T_1 digestion of genomic RNA and two-dimensional electrophoresis of oligonucleotides were performed as described by Holland et al. (16). All RNA fingerprints were compared to those obtained for the starting plaque clone of strain 2061-88. Missing oligonucleotides, as well as newly appearing oligonucleotides, were recorded and assembled in a master map.

RT-PCR amplification and sequencing. Viral RNA was extracted from 0.25 ml of cell culture supernatants by adding 0.75 ml of Trizol LS (BRL, Bethesda, Md.) and processed according to the manufacturer's protocol. Yeast tRNA (0.2 μg) was added to enhance RNA precipitation. cDNA was synthesized at 42°C with Superscript reverse transcriptase (BRL) by using a primer of sequence 5′-T_{19}V-3′, and PCR was conducted with the following three primer pairs: 5′-CGTGG ACTTAATCACGTTTGACAG-3′ (sense) and 5′-CAGAGAGGTATGAGCCT AT-3′ (antisense), designed to amplify genome positions 5428 to 5969, covering the C terminus of nsP3 and the N-terminus of nsP4 (sequence positions according to reference 36); 5′-GGAGTAAAGGCACCGTACTTTTG-3′ (sense) and 5′-AATGGAACGTCTCAGGTCCTC-3′ (antisense), designed to amplify genome positions 7196 to 7735, covering the C terminus of the nsP4 gene, the promoter region and untranslated region (UTR) of the 26S mRNA 5′ region, and the N terminus of the capsid gene; and 5′-TTACCTGCAAAGGRGATT G-3′ (sense) and 5′-GAAATATTAAAAACAAAATA-3′ (antisense), designed to amplify genome positions 11118 to 11678, covering the C terminus of the E1 gene and the 3′ UTR. PCRs were done according to a previously described protocol (4), with 30 amplification cycles as follows: heat denaturation at 95°C for 30 s, primer annealing at 49°C for 30 s, and extension at 72°C for 1 min. PCR amplicons were extracted from 1% agarose gels and sequenced directly with the PCR primers and the Applied Biosystems (Foster City, Calif.) Prism automated DNA sequencing kit and sequencer, according to the manufacturer's protocol.

RESULTS

One-step growth curves to determine passage dilutions. To determine dilutions and passage times necessary to obtain serial EEEV passages of predictable multiplicity, one-step growth curves were determined for the parental strain 2061-88. BHK infectious titers reached about 10^{10} PFU/ml within 24 h of infection, when cytopathic effects (CPE) were 3+ to 4+, whereas titers in C6/36 cells neared their maximum of about 10^9 after 48 h of incubation (data not shown). Infected C6/36 cells also showed a slight clustering and elongation at that time. Diluted passages 20 and 50 showed similar kinetics. Therefore, to insure adequate inoculum titers during serial passages and multiplicities of about 0.01, BHK infections were incubated for 24 h and C6/36 infections were incubated for 48 h at 32°C for each passage.

Serial passages and DI particle generation. EEEV was subjected to 13 different passage series in BHK and/or C6/36 cells, including both diluted and undiluted, as well as clonal (plaque-to-plaque) passages in BHK cells and persistent infection of C6/36 cells (Table 1). All passage series were carried out 100 times with the exception of clonal (plaque-to-plaque) series, which were carried out 40 times, and persistent infections of mosquito cells, which were maintained for 200 days. To evaluate the infectious titers of these passages and confirm the presence of DI particles in the undiluted passages, culture media from the first 13 undiluted and diluted passage series were evaluated by plaque assay. Diluted passages maintained relatively stable, high titers (ca. 10^9 to 10^{10} PFU/ml) in both BHK and C6/36 mosquito cells. PFU titers were nearly identical on both BHK and Vero cell monolayers (data not shown). Titers of undiluted passages dropped by about 100- to 10,000-fold by passage 4 (C6/36) or 7 (BHK), followed by fluctuations during subsequent passages. These results were consistent with generation and amplification of DI particles in the undiluted passage series, leading to fluctuations in titer (cycling) as DI particles suppressed wild-type virus replication, followed by drops in DI replication due to inadequate amounts of helper virus (26). To confirm the presence of interfering activity in the undiluted passage series, 10^7 PFU of undiluted BHK and C6/36 passages 7 were mixed with standard helper virus (original clonal pool with one diluted BHK passage) at a multiplicity of 10 and used to infect BHK and C6/36 cells in triplicate. Infectious virus titers were measured at times listed below for one-step growth curves. Replication in either BHK or C3/36 cells was suppressed at least 3-fold ($P < 0.01$ [Student's t test]) at early time points and at least 30-fold ($P < 0.001$ [Student's t test]) at late time points by both BHK and C6/36 undiluted-passage viruses, relative to diluted passage 7 or first-passage controls, indicating the presence of interfering activity. BHK cell-generated DI particles interfered more with helper virus replication in BHK cells than in C6/36 cells, and C6/36 cell-generated particles interfered more in C6/36 cells.

One-step growth curves to evaluate fitness changes after serial passages. Initial fitness testing of all passage series comprised one-step growth curves in both BHK and C6/36 cells at 32°C. Infectious titers after 5 and 20 h of replication in BHK cells, or 20 and 44 h in C6/36 cells, are presented in Fig. 1. Diluted passages (100) in BHK cells resulted in little or no change in BHK replication measured at 5 or 20 h. However,

TABLE 1. Oligonucleotide fingerprint comparisons of serial passages to starting clone[a]

Cell type	Dilution	No. of passages	Oligonucleotides missing	Newly appearing oligonucleotides	Total changes
BHK	10^{-4}	100	3, 8, 18	A, *C, F, G	7
	10^{-6}		3, 4, 5, 8, 13, 18, 19, 39	B, C, G, N	12
	Undiluted		2*	A, C, *I, *F*	5
C6/36	10^{-4}	100	2*, 8, 9*, 34	F, T, U	7
	Undiluted		2, 7, 22*, 36	V, W, X	7
Alternate	Undiluted	100		A, M	2
	10^{-4}			Y	1
BHK	Clonal	40	2, 35, 40	S, N, 3[b]	6
					0
					0
					0
C6/36	Persistent	200 days	29		1
					0

[a] Oligonucleotide numbers and letters refer to those in Fig. 5. *, oligonucleotide represents only a fraction of genomes.
[b] Oligonucleotide doubled in intensity compared to starting clone.

the passaged virus exhibited 3- to 300-fold declines in C6/36 cell replication after 20 and 44 h compared to the parent. Likewise, diluted passage in C6/36 cells resulted in little or no change in C6/36 cell replication after 20 or 44 h but in 5- to more than 1,000-fold declines in BHK cell replication after 5 to 20 h. When viruses were passaged undiluted to enhance DI particle accumulation, replication kinetics declined in both cell types, with 10- to 1,000-fold decreases at both early and late time points, except for the BHK passage series tested in BHK cells at the 20-h incubation time. Alternating passage with dilutions resulted in both increases and declines in replication kinetics, depending on the cell type and time point in one-step growth curves. Undiluted, alternating passage resulted in reductions of 10- to 100-fold in replication in both cell types, except for BHK cells at the 20-h incubation time.

Clonal (40 plaque-to-plaque) passage in BHK cells resulted in 10-fold reductions in early replication in BHK cells and 3- to 1,000-fold reductions in early C6/36 replication, with smaller reductions or increases at later sampling points. Reductions were generally greater in C6/36 cell replication than in BHK cell replication, probably reflecting some selection for BHK cell replication during plaque formation. Persistent infection of C6/36 cells resulted in large reductions in fitness for acute infection of both BHK and C6/36 cells, with 4- to over 1,000-fold reductions in virus titers at both early and late time points of one-step growth curves. Reductions were generally greater in BHK than in C6/36 cells, again consistent with some selection for maintenance of fitness in mosquito cells.

To determine whether fitness decreases in bypassed cells were specific to the individual vertebrate and mosquito cell lines used for adaptation, we also measured one-step growth curves for the diluted passage series, as well as one persistent C6/36 cell infection series, in Vero monkey kidney and *Anopheles albimanus* mosquito cells. The results are presented in Fig. 2. As with BHK and C6/36 cell replication, the BHK-adapted virus replicated more poorly than the parent in the *Anopheles albimanus* mosquito cells, and the C6/36-adapted virus also exhibited a fitness decline in Vero cells. Fitness levels of BHK-adapted virus, as well as the alternating passage virus, were similar to the parent in Vero cells, and the C6/36-adapted and alternating viruses also had similar replication kinetics to the parent in *Anopheles albimanus* cells. These results indicate that the fitness declines in the bypassed cell lines were probably vertebrate and mosquito specific and not restricted to a single host cell line. The persistent C6/36-adapted virus also exhibited declines in replication in both cell types during acute infection, with the exception of the 20-h time point in Vero cells, when infectious titers were similar to those produced by the parent (Fig. 2).

Competition passages to evaluate fitness changes after serial passages. Competition relative fitness assays representing a modification of those used previously to study VSV evolution (14) were developed to overcome several limitations of one-step growth curves: (i) limited accuracy due to the dependence of virus replication on inoculum titers and the inherent imprecision of plaque assays, (ii) the possibility that plaque assays underestimate C6/36 cell-adapted viruses that may lose the ability to form visible plaques in vertebrate cells, and (iii) inconsistent results we sometimes observed comparing relative virus production at early versus late time points (Fig. 1). The loss of an *Xho*I restriction site within the nsP3-nsP4 amplicon sequence of the alternate-host-cell, diluted-passage series was used as a genetic marker; this alternate-passaged virus was used as a fitness standard and mixed with either the parent, BHK diluted (10^{-4}), or C6/36 diluted (10^{-2}) series, as well as the clonal passage viruses (series A to D), and competed in BHK or C6/36 cells in triplicate series with dilutions (10^{-4} or 10^{-2} for BHK and C6/36, respectively) to minimize the influence of DI particles. Proportions of viruses following each passage were monitored by RT-PCR amplification of the nsP3-nsP4 genome region from the culture supernatant, followed by *Xho*I digestion of the purified amplicon and agarose gel electrophoresis to determine ratios of the two genotypes. All viruses used in competition assays were also passaged alone four times to ensure stability of the marker (*Xho*I site present or absent). An example of this assay is presented in Fig. 3. The alternating passaged virus showed a consistent competition fitness advantage over the parent in both cells, as did each single-cell passaged virus in the adapted cell line (Fig. 4). Surprisingly, the single-host-cell-adapted viruses showed little or no advantage over the alternating-cell-passage virus when competed in either BHK or C6/36 cells. Clonally passaged viruses exhibited varying degrees of fitness declines, which tended to be more severe in the C6/36 cell environment.

Genetic analyses. All 13 passage series were analyzed by RT-PCR amplification and sequencing of three genome re-

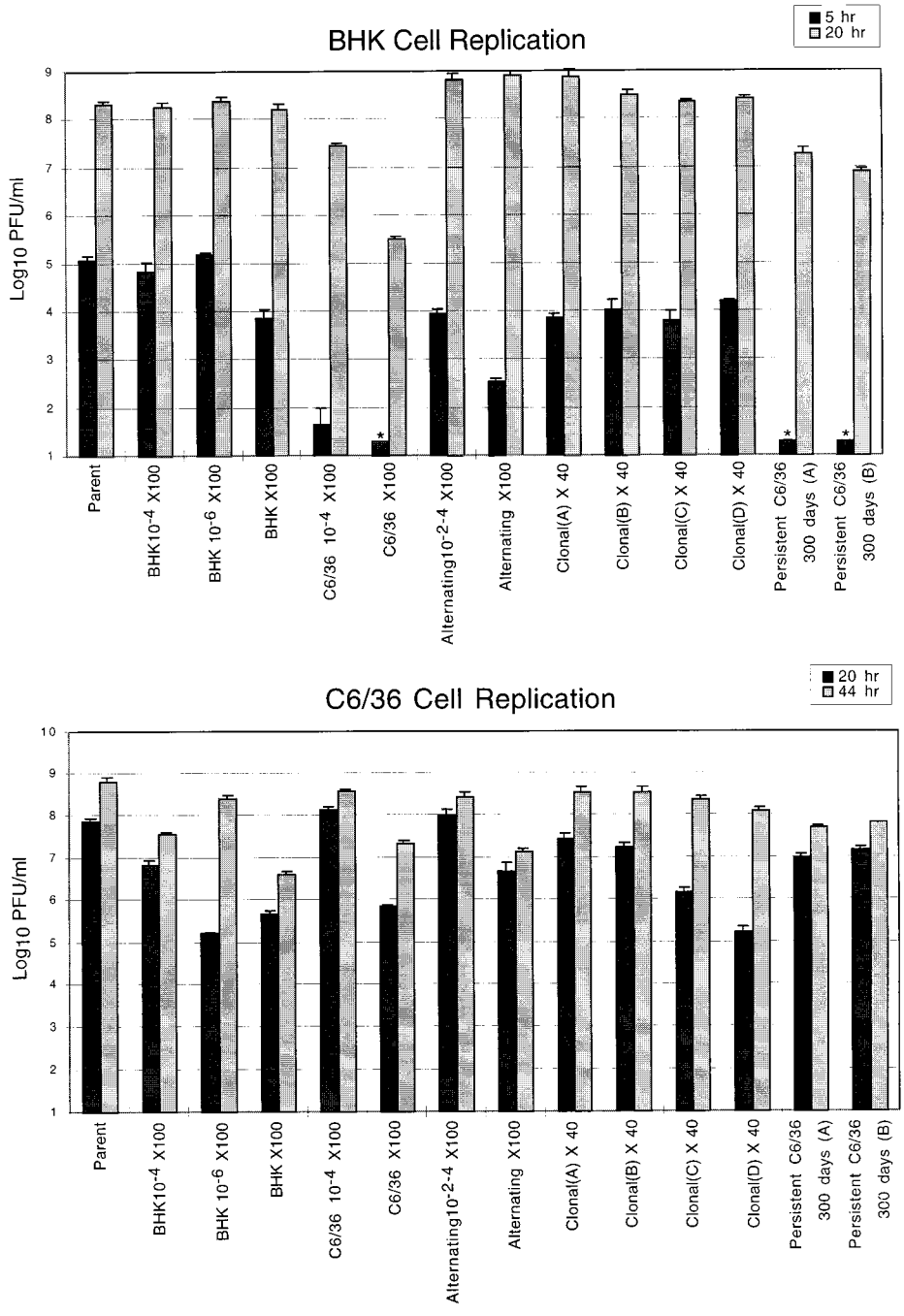


FIG. 1. Titers of EEEV in cell culture supernatant after one-step infection of BHK or C6/36 cells. The passage histories of viruses are shown below the graphs (all series underwent 100 passages with the exception of the clonal [plaque-to-plaque] series, which were carried out 40 times, and persistent infections of mosquito cells, which were maintained for 200 days). *, <1.3 $\log_{10}$ PFU/ml (below the detection limit of the plaque assay). Error bars show standard deviations of mean titers.

gions totaling ca. 1,500 nucleotides and by T_1 RNA fingerprinting, which samples all EEEV genes (34). The results of the fingerprinting analysis are presented in Table 1 and Fig. 5. For BHK cell passages, the 10^{-6} dilution series showed the most change, with 12 oligonucleotide differences versus the parent fingerprint. With a previous estimate of 13% sampling of the EEEV genome in the large, unique, T_1-resistant oligonucleotides evaluated in fingerprints (34), this change corresponded to about 0.9% sequence divergence from the starting clone. The undiluted BHK series was more stable, with only five oligonucleotide differences, or about 0.4% sequence change, while the 10^{-4} dilution series showed seven oligonucleotide changes, or about 0.5% divergence. Several of the oligonucleotides in the 10^{-4} and undiluted series were lower in molar amount than nearby (similar-length) oligonucleotides (Table 1), indicating that they were not present in all genomes of the virus population and suggesting the development of quasispecies populations. The C6/36 cell passages showed seven oligonucleotide changes versus the parent, while the alternating passaged viruses showed only one or two oligonucleotide

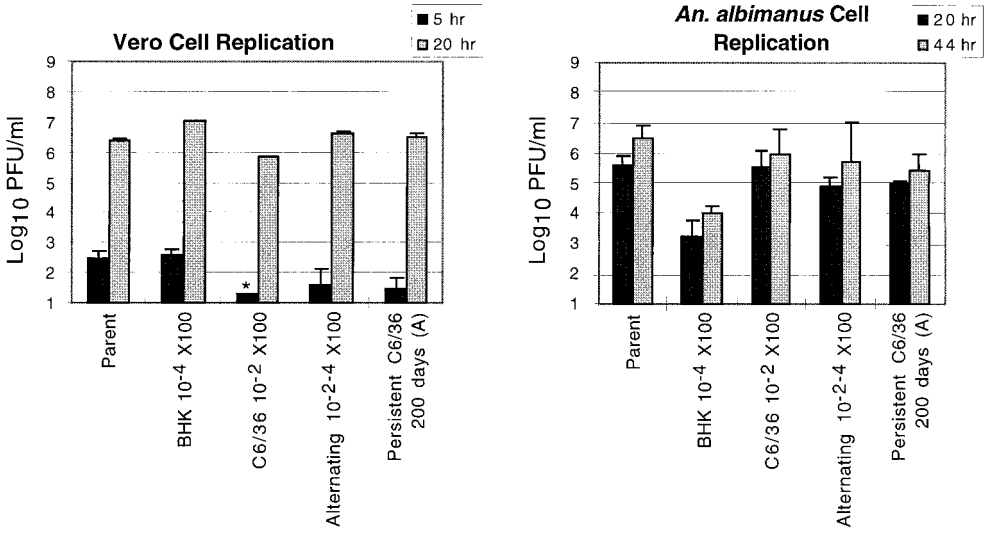


FIG. 2. Titers of EEEV in cell culture supernatant after one-step infection of Vero or *Anopheles albimanus* cells. *, <1.3 $\log_{10}$ PFU/ml (below the detection limit of the plaque assay). Error bars show standard deviations of mean titers.

changes. One clonally passaged virus had six oligonucleotide changes, while the persistent infections showed only one and no changes, respectively.

Sequence analyses included three regions of the EEEV genome, each encompassing ca. 500 nucleotides excluding primer sequences: genome positions 5428 to 5969, including the C terminus of nsP3, the N terminus of the nsP4 gene, and the termination codon; genome positions 7196 to 7735, including the C terminus of the nsP4 gene, the promoter region and UTR of the 26S RNA 5′ region, and the N terminus of the capsid gene; and genome positions 11118 to 11678, including the C terminus of the E1 gene and the 3′ UTR. Consensus sequences of PCR amplicons revealed nucleotide changes in all passage series except for two of the four clonal passages (Fig. 6; Table 2). The nsP3-nsP4 region showed the most change, with a total of 21 nucleotide changes, 19 of which were nonsynonymous. The nsP4-capsid region had 12 changes, 6 of which were nonsynonymous, and the E1 3′ UTR showed 17 changes, 2 of which were nonsynonymous changes in the E1 gene. Eleven of the 3′ UTR changes represented mixed nucleotide populations, especially in the undiluted BHK passage series (Fig. 6).

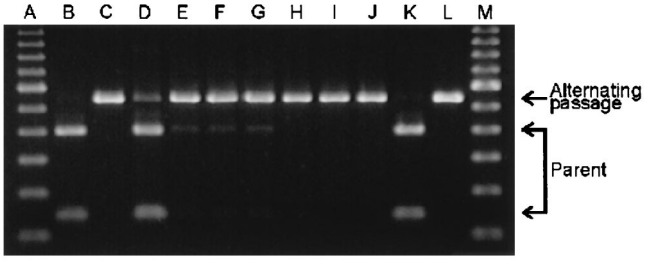


FIG. 3. Agarose gel electrophoresis showing results from an EEEV competition fitness assay. Parent and alternately passaged (100 times) viruses were mixed in a ratio of approximately 13:1 and passaged serially in triplicate in C6/36 cells with 100-fold dilution to reduce the multiplicity of infection. RNA was extracted from 250 μl of the original virus mixture as well as from the passages, and the nsP3-nsP4 genome region was amplified by RT-PCR. The resulting 542-bp amplicon was eluted from a 1% agarose gel and digested with *Xho*I to produce DNA fragments of 398 and 144 bp for the parental genotype only. A, 100-bp ladder; B, parent virus used in competitions; C, alternately passaged virus used in competitions; D, mixture of parent and alternately passaged viruses used to initiate competitions; E to G, triplicate first-competition passages; H to J, triplicate second-competition passages; K, parent virus after four passages alone; L, alternately passaged virus after four passages alone; M, 100-bp ladder.

Overall, 27 of the 50 total nucleotide substitutions observed in the three genome regions were nonsynonymous. The BHK passages and C6/36 persistent infections resulted in the largest numbers of nucleotide substitutions, with 0.2 to 0.7% sequence change, while the clonal passages showed smaller amounts of change, 0 to 4 nucleotides (Table 2). The alternating cell passages also showed small amounts of change (one substitution each) compared with the single-host-cell, diluted passages (two to seven substitutions).

Analysis of individual nucleotide changes revealed that many were common to a particular host cell passage type. For example, both persistent infections of C6/36 cells resulted in four common, nonsynonymous substitutions in the nsP4 gene region, suggesting common, positive Darwinian selection for polymerase amino acid changes. It is possible that these mutations were present in the original parent population generated by a single BHK passage from the plaque clone pool. However, this seems unlikely, because these mutations did not appear in any of the BHK cell passage series. All four passage series exclusively involving C6/36 cells resulted in a change in the stop codon near the end of the nsP3 gene (Fig. 5); the serial diluted and undiluted C6/36 passages resulted in arginine (CGA) and cysteine (TGC) codons, while both persistent infection series also underwent the arginine substitution. Analysis of sequence electropherogram peaks for C6/36 cell passages 5 and 10 revealed a gradual increase in the proportion of the populations with sense codons, with the stop codons undetectable by passage 10. In contrast, all passage series involving BHK cells, either exclusively BHK or alternating C6/36-BHK, retained the stop codon with no evidence of mixed populations in these nucleotides.

Surprisingly, the untranslated genome regions we sequenced, the 26S junction region and 3′ UTR, contained the fewest consensus nucleotide sequence substitutions for most passage series. However, one of the diluted BHK cell passages (10^{-4}) underwent a 238-nucleotide deletion in the 3′ UTR, beginning 23 nucleotides downstream of the structural poly-

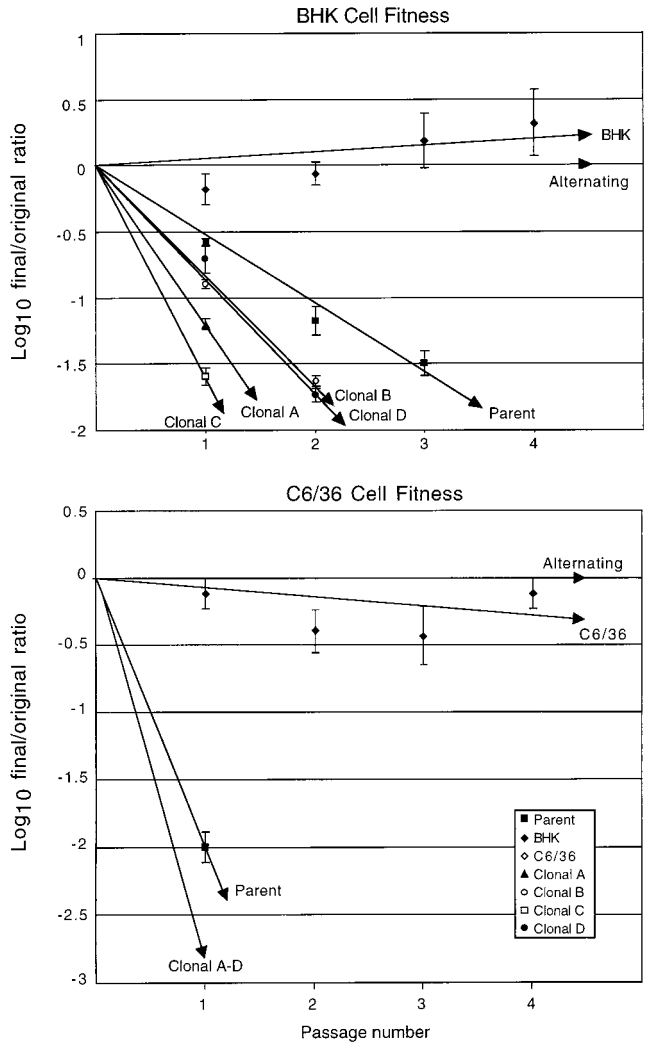


FIG. 4. Relative fitness plots for EEEV competition assays in BHK and C6/36 cells. Parent, BHK 10^{-4}-diluted, C6/36 10^{-2}-diluted (all 100 times), and clonally passaged (40 times, A to D) viruses were competed with the alternate diluted-passage virus that had lost the *Xho*I site in nsP4. Ratios of the competing viruses were estimated following *Xho*I digestion of RT-PCR amplicons, as shown in Fig. 3, by using densitometry and were plotted for up to four competition passages. Fitness vectors were plotted by regression. The alternating-passage virus served as the standard and thus has a fitness slope of 0 (horizontal line). The digested DNA bands corresponding to the clonally passaged viruses were not detected after the first C6/36 cell competition passage, so C6/36 ratios and vectors for the clonally passaged viruses represent the maximum possible values.

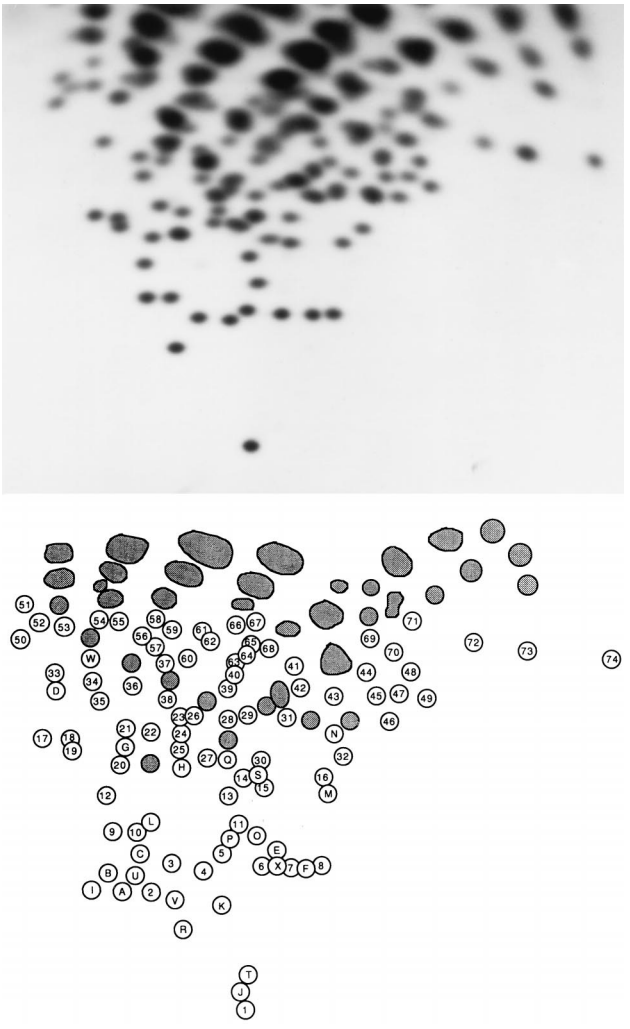


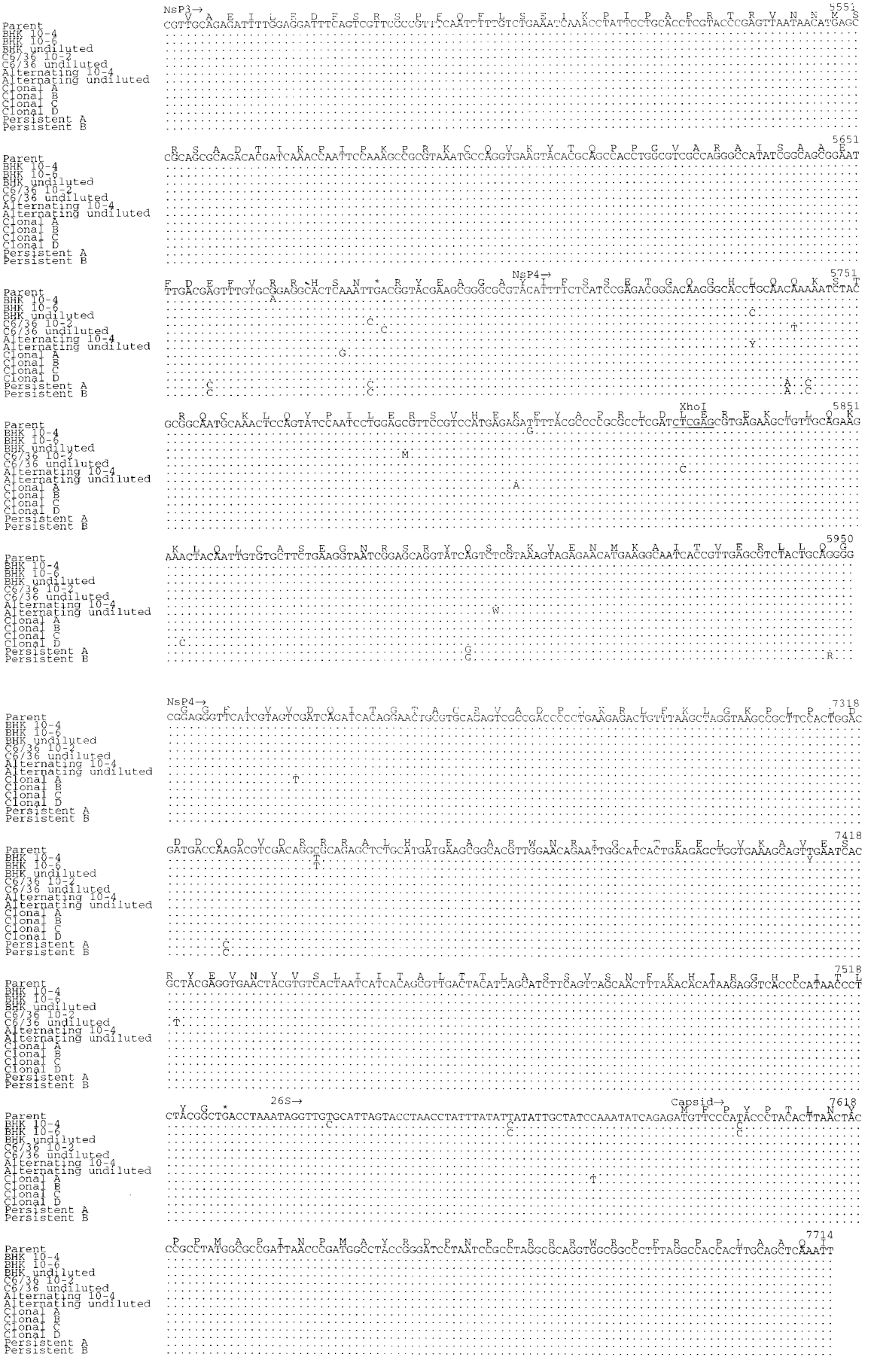
FIG. 5. T_1 oligonucleotide fingerprint of the clonal pool of parent EEEV used to initiate adaptation passages. Map below shows oligonucleotides analyzed for comparison of the parent virus to viruses generated after various passage histories (Table 1).

protein stop codon. This deletion region includes all but one of the repeated sequence elements believed to interact with cellular proteins and to possibly regulate translation of the alphavirus genome (32).

DISCUSSION

Evolutionary implications of an alternating host transmission cycle. In some respects, our results support the longstanding assumption that alphaviruses and other arboviruses must adopt a compromise fitness level for replication in both vertebrate and invertebrate cells. When EEEV was freed of the alternating host cell transmission cycle, dramatic fitness losses were observed in the bypassed cell environment. Another prediction of this hypothesis is that elimination of one host from an adaptation transmission cycle will result in the acquisition of higher fitness for the retained host than will occur in an alternating adaptation cycle. However, this was not observed in either BHK or C6/36 cell adaptation (Fig. 4). The ability of the alternating-host-cell-passaged viruses to acquire fitness gains comparable to those observed during single-host-cell adaptation indicates that two different kinds of genetic solutions to fitness gains occurred, both host-specific and more generalized adaptations. This remarkable ability of an RNA virus to adapt efficiently to two different host cell environments is probably reflected in the fact that nearly all arboviruses have RNA genomes.

The hypothesis that alternating host replication limits rates of arbovirus evolution is supported by our results. Using both RNA fingerprinting and limited genome sequencing, viruses subjected to alternating vertebrate-mosquito host cell passage exhibited consistently smaller amounts of genetic change than did viruses passaged in large populations in either cell type alone. However, the reasons for the relative genetic stasis in the alternating host cell adaptation may be more complex than


NsP3→
5551
V A E I L E D E S R S D F Q F L S E I K P I P A P R T R V N N K S
CGTTGCAGAGATTTTGGAGGATTTCAGTCGTTCGCCGTTCCAATTTTGTCTGAAATCAAACCTATTCCTGCACCTCGTACCCGAGTTAATAACATGAGC
5651
R S A D T I K P I P K P R K C Q V K Y T Q P P G V A R A I S A A E
CGCAGCGCAGACACGATCAAACCAATTCCAAAGCCGCGTAAATGCCAGGTGAAGTACACGCAGCCACCTGGCGTCGCCAGGGCCATATCGGCAGCGGAAT
NsP4→
5751
F D E F V R R H S N * R Y E A G A Y I F S S E T G Q G H L Q Q K S T
TTGACGAGTTTGTGCGGAGGCACTCAAATTGACGGTACGAAGCGGGCGCGTACATTTCTCATCCGAGACGGGACAAGGGCACCTGCAACAAAAATCTAC
XhoI
5851
R Q C K L Q Y P I L E R S V H E K F Y A P R L D L E R E K L L Q K
GCGGCAATGCAAACTCCAGTATCCAATCCTGGAGCGTTCCGTCCATGAGAGATTTTACGCCCCGCGCCTCGATCTCGAGCGTGAGAAGCTGTTGCAGAAG
5950
K L Q L C A S E G N R S R Y Q S R K V E N M K A I T V E R L L Q G
AAACTACAATTGTGTGCTTCTGAAGGTAATCGGAGCAGGTATCAGTCTCGTAAAGTAGAGAACATGAAGGCAATCACCGTTGAGCGTCTACTGCAGGGG
NsP4→
7318
G G F I V V D Q I T G T A C E V A D P K K R L F K L G K P L P I D
CGGAGGGTTCATCGTAGTCGATCAGATCACAGGAACTGCGTGCAGAGTCGCCGACCCCCTGAAGAGACTGTTTAAGCTAGGTAAGCCGCTTCCACTGGAC
7418
D D Q D V D R R R A L H D E A A R W N R I G I T E E L V K A V E S
GATGACCAAGACGTCGACAGGCGCAGAGCTCTGCATGATGAAGCGGCACGTTGGAACAGAATTGGCATCACTGAAGAGCTGGTGAAAGCAGTTGAATCAC
7518
R V E V N V V S L I I T A L T T L A S S V S N F K H I R G H P I T L
GCTACGAGGTGAACTACGTGTCACTAATCATCACAGCGTTGACTACATTAGCATCTTCAGTTAGCAACTTTAAACACATAAGAGGTCACCCCATAACCCT
26S→
Capsid→
7618
Y G * M P P Y P T L N V
CTACGGCTGACCTAAATAGGTTGTGCATTAGTACCTAACCTATTTATATTATATTGCTATCCAAATATCAGAGATGTTCCCATACCCTACACTTAACTAC
7714
P P M A P I N P M A Y R D P N P P R R R W R P F R P P L A A Q T
CCGCCTATGGCGCCGATTAACCCGATGGCCTACCGGGATCCTAATCCGCCTAGGCGCAGGTGGCGGCCCTTTAGGCCACCACTTGCAGCTCAAATT
Parent
BHK 10-4
BHK 10-6
BHK undiluted
C6/36 10-2
C6/36 undiluted
Alternating 10-4
Alternating undiluted
Clonal A
Clonal B
Clonal C
Clonal D
Persistent A
Persistent B

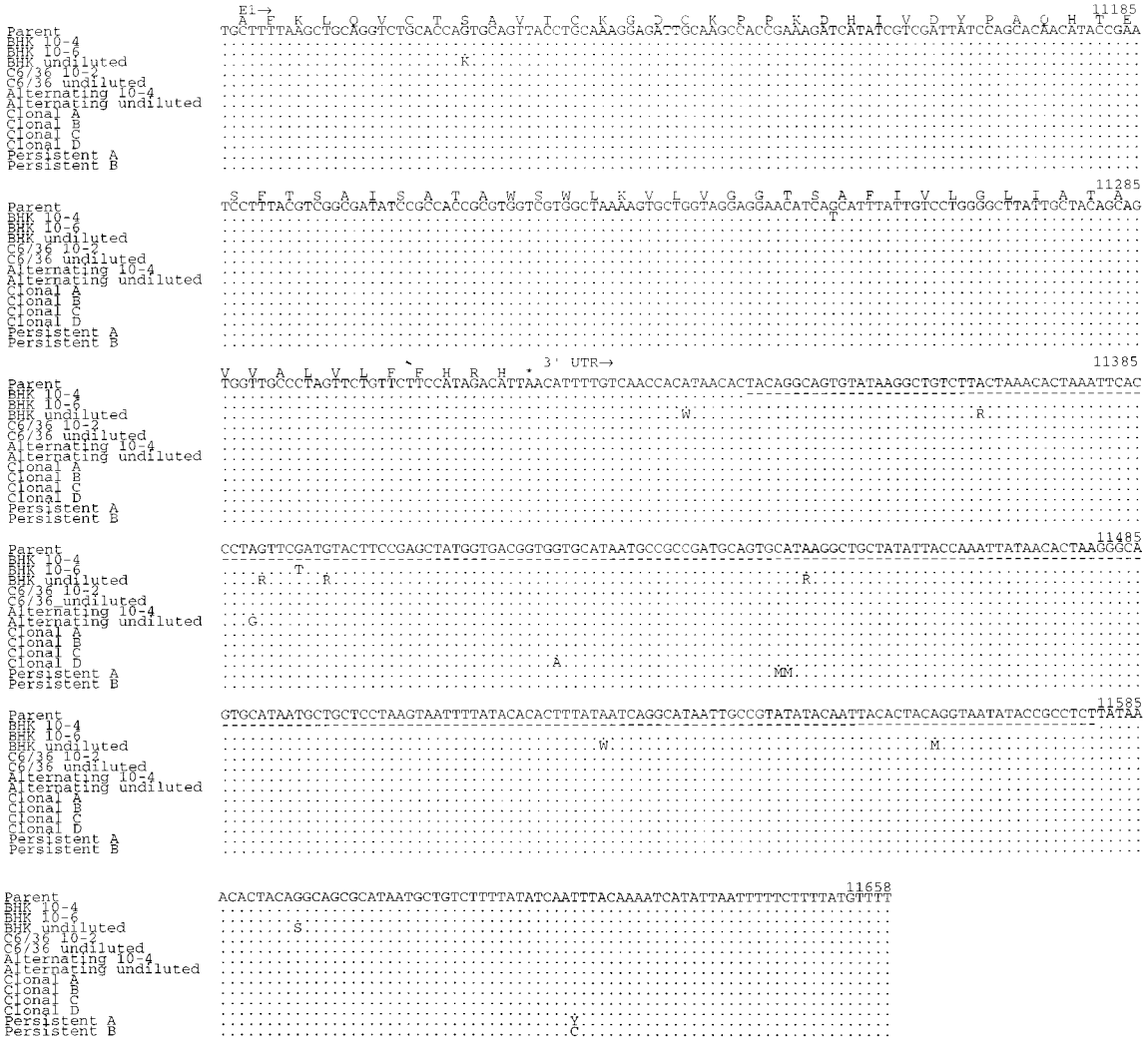


FIG. 6. Aligned nucleotide sequences for the three EEEV genome regions generated by RT-PCR. Deduced amino acid sequences of the parent strain are shown above second codon positions. Deletions are indicated by dashes, and dots indicate the same nucleotide as the parent virus. Ambiguous nucleotide symbols indicate mixed populations represented by multiple peaks in the electropherograms of both strand sequences. The *Xho*I site at positions 5825 to 5830, used as a genetic marker for competition fitness assays, is underlined. Numbers above nucleotides adjacent to righthand margins represent genomic EEEV numbering as previously published (36).

greater purifying selective constraints, since similar fitness gains occurred in alternating and single host cell adaptation.

Serial passage of EEEV in cell culture also demonstrated that this virus is capable of undergoing rapid sequence and fitness evolution under appropriate conditions. For example, the estimated 0.7 to 0.9% nucleotide sequence change that accompanied 100 passages in cell culture represents the equivalent of 44 to 56 years of natural EEEV evolution in North America (35). This provides further indirect evidence that the relatively low rates of alphavirus evolution in nature are not due to innate properties of their genome replication, such as high fidelity or proofreading by viral polymerases.

Two of the genetic changes accompanying single-host-cell replication are of particular interest. The replacement of the stop codon near the 3′ end of the nsP3 gene by arginine or cysteine codons occurred in all four C6/36 cell passage series, suggesting a selective advantage for the nsP1-nsP4 polyprotein open reading frame in mosquito cell replication. This may reflect more-efficient replication in mosquito cells when greater amounts of the nsP4 polymerase are present and a preference for lower polymerase levels (generated only by readthrough) in vertebrate cell replication. In alphaviruses, the nonstructural polyprotein precursor is cleaved by a virus-encoded protease that is part of nsP2 to produce four final products—nsP1, nsP2, nsP3 and nsP4—as well as partially digested polyproteins. In 8 of 10 alphaviruses (including the 82V2137 strain of EEEV previously sequenced [36]), there is an opal termination codon (UGA) between nsP3 and nsP4 that is read through with moderate efficiency (5 to 20%), whereas in two other alphaviruses, including o'nyong-nyong virus (ONNV), this codon has been replaced by a sense codon for arginine (CGA) (32). Although the first sequences of ONNV obtained indicated that it possesses a sense (arginine) codon rather than a termination codon (22), Lanciotti et al. (20) recently reported that consensus sequences of more recent ONNV isolates of lower passage history have both stop and sense codons at this position and exist in nature as quasispecies. All of the recent isolates with stop codons also have a C residue immediately downstream, which enhances readthrough in Sindbis virus (23). When four recent ONNV isolates with the opal termination

TABLE 2. Nucleotide sequence comparisons of serial passages to starting clone

Cell type	Dilution	No. of passages	No. of nucleotide substitutions in each genome region[a]			
			nsP3-nsP4	nsP4-C	E1-3′ UTR	Total
BHK	10^{-4}	100	2 (2)	5 (2)	1 (1)	8 (5)
	10^{-6}		0	2 (2)	1 (0)	3 (2)
	Undiluted		1 (1)	0	9 (1)	10 (2)
C6/36	10^{-4}	100	1 (1)	0	0	1 (1)
	Undiluted		2 (2)	1 (0)	0	3 (2)
Alternate	10^{-4}	100	1 (1)	0	0	1 (1)
	Undiluted		0	0	1 (0)	1 (0)
BHK	Clonal	40	2 (1)	2 (0)	0	4 (1)
			0	0	0	0
			0	0	0	0
			1 (1)	0	1 (0)	2 (1)
C6/36	Persistent	200 days	5 (5)	1 (1)	3 (0)	9 (6)
			6 (5)	1 (1)	1 (0)	8 (6)
Total			21 (19)	12 (6)	17 (2)	50 (27)

[a] Numbers in parentheses indicate nonsynonymous substitutions. Genome regions include nsP3-nsP4 (positions 5428 to 5969, including the C terminus of nsP3 and the N terminus of nsP4 and the termination codon), nsP4-C (genome positions 7196 to 7735, including the C terminus of the nsP4 gene, the promoter region and UTR of the 26S RNA 5′ region, and the N terminus of the capsid gene), and E1-3′ UTR (genome positions 11118 to 11678, including the C terminus of the E1 gene and the 3′ UTR).

codon were passaged in Vero cells (20), all were observed to acquire the arginine codon found in the original 1959 Gulu strain (22). These results suggest that the termination codon in ONNV is subject to different selective pressures than that of EEEV. Both results also suggest that previous passage in vertebrate or invertebrate cells may have influenced the sequences reported for some alphaviruses.

The effects of the opal termination codon versus sense codons on replication of Sindbis virus in both vertebrate and mosquito cells was studied experimentally by Li and Rice (24). Sense amino acids (serine, tryptophan, or arginine) or the other two translation termination codons (amber or ochre) were introduced into an infectious cDNA clone, and rescued viruses were analyzed for replication in chicken cells. The sense codon mutants overproduced nsP34 but not nsP4, indicating that the level of nsP4 is not regulated solely by read-through of the opal codon. NsP4 is rapidly degraded via an N end rule pathway (5), and Sindbis virus mutants that replace the termination codon with a sense codon do not accumulate excessive nsP4, presumably due to protein degradation (24). Temperature-sensitive Sindbis virus mutants that exhibit decreased levels of nsP34 and nsP4 production grow poorly in mosquito cells but normally in chicken cells at nonpermissive temperature (21), also suggesting that greater amounts of nsP4 are required for replication in mosquito cells. Li and Rice (24) also determined that the serine mutant of the Sindbis opal termination codon was gradually replaced by the opal virus during mixed infection of chick embryo fibroblasts. Taken together, these results suggest that the termination codon confers a fitness advantage for replication of some but not all alphaviruses in vertebrate cells.

The large deletion in the EEEV 3′ UTR following 100 diluted BHK cell passages also suggests that this genome region may function differently during replication in vertebrate than invertebrate cells. The alphavirus genomic RNA and 26S mRNA 3′ UTR include several repeated sequence elements believed to interact with cellular proteins (32). The previous finding that an engineered 3′ deletion of Sindbis virus repeated elements has a much greater effect on replication in C6/36 cells than in chicken cells (19) is consistent with our finding that the EEEV 3′ UTR can undergo extensive deletions accompanied by efficient replication in hamster cells. Both findings suggest that parts of the 3′ UTR may be more important for alphavirus replication in mosquitoes than in vertebrates.

Effects of clonal passages. Viruses undergoing clonal, plaque-to-plaque passages in BHK cells exhibited declines in replication in both BHK and C6/36 cells. The effect was most pronounced when replication was measured early (after 5 and 20 h, respectively) and when competition assays were used to measure relative fitness. Surprisingly, 20-h BHK cell yields of clonally passaged viruses were actually higher than those of the parent in one-step growth curves. A possible explanation for the apparent recovery of replication later in infection is that the reduced replication during early stages allowed the BHK cells to remain productive longer for virus replication. This hypothesis is supported by the observation that the clonally passaged viruses showed less CPE than the parent after 20 h of replication, and complete (no normal cells observed attached to the plastic) CPE occurred ca. 8 h later in the clonal passage group (data not shown). This difference in CPE of BHK cells was also noted in the C6/36-adapted viruses and the undiluted series adapted to both cell types. Another possible explanation for the apparent recovery of clonally passaged viruses later in BHK cell infection is that these viruses underwent reversion of deleterious mutations or compensatory mutations to regain fitness during hours 5 to 20 of replication. The small numbers of mutations detected in these clonally passaged viruses are consistent with this possibility and with previous studies of foot-and-mouth-disease virus, which showed that small numbers of mutations mediate fitness declines following clonal passage (13). To fully assess these hypotheses, an infectious cDNA clone of EEEV is needed to produce genetically defined viruses that can be used to test the effects of individual mutations or combinations.

Effects of DI particles on EEEV evolution. Our results indicate that the presence of DI particles, which accompany high multiplicity or persistent infection of cell cultures, can have a profound effect on EEEV fitness for acute replication in both vertebrate and mosquito cells. Whether DI particles exert a significant influence on arbovirus evolution in nature remains to be determined. DI particles have never been detected in infected mosquitoes, though extensive investigations have not been reported. Vertebrate host infections by alphaviruses generally are cleared after a few days, probably precluding the generation of large DI populations. Although mosquitoes become persistently infected with alphaviruses and can survive for several months, rates of EEEV saliva infection decline 1 to

3 weeks after an infectious blood meal (33, 40), and mosquitoes generally suffer high mortality rates in nature. These limitations on the time that infected mosquitoes can transmit suggest that DI particles may generally appear too late to influence the evolution of the virus population that is transmitted to the vertebrate host.

The greater amount of genetic change observed during serial, dilute passages of EEEV, relative to undiluted passages, was surprising considering previous findings with VSV. Spindler et al. (28) reported that high multiplicities of VSV infection favor rapid and random evolution. However, Sanchez-Palomino et al. (27) reported that dilute passages of human immunodeficiency virus type 1, with multiplicities of infection of 0.001, promote rapid genetic change in vitro. One possible explanation for the greater rate of EEEV change in the dilute passage series is that lower multiplicities resulted in more cycles of genome replication than in undiluted passages. Greater genome replication could augment evolution by either (i) increasing the possibility of adaptive change by generating more mutations, some of which might enhance fitness, or (ii) increasing the number of mutants available for stochastic change (genetic drift). A second possibility is that the reduction in EEEV population sizes, associated with dilution between passages, enhanced genetic drift. However, titers of virus in diluted passages indicated that, on average, inocula of the 10^{-6} passage series contained about 5×10^4 PFU of EEEV. If mutation frequencies are on the order of 10^{-4} (7, 8, 15, 29), most passages should have included a consensus genome in the inoculum. Although our results suggest that adaptive change, or natural selection, was responsible for much of the evolution we observed in vitro, genetic drift may also have played a role.

Another possible explanation for this apparent discrepancy between our results and those of Spindler et al. (28) is the nature of the viruses and the recent host passage history of the viruses used to initiate serial passages. Whereas Spindler et al. (28) used a VSV strain which had been passaged many times in BHK cell culture and was presumably "preadapted" to the BHK cells used, our experiments and those of Sanchez-Palomino et al. (27) used viruses of low passage history which may have undergone more adaptation during serial passages. The common nucleotide and oligonucleotide changes in our EEEV passage series suggest positive selection; dilute passages may have optimized selection by reducing the influence of DI particles in generating complex quasispecies populations that can suppress mutants of superior fitness (6). Sanchez-Palomino et al. (27) also speculated that their HIV mutants, which appeared only after dilute passage, may have been previously suppressed by DI genomes in complex quasispecies. Finally, DI particles of VSV may be better able to suppress high-fitness viruses and promote random change through selection for resistance to interference than DI particles of EEEV. Experiments such as those conducted by de la Torre and Holland (6), examining the ability of high-fitness EEEV to replicate in complex quasispecies populations, are needed to test this hypothesis. The possibility that transfers in vertebrate hosts at mammalian or avian core body temperatures might select additional genetic changes also should be addressed.

Host cell dependence of interference has been described previously for DI particles of other alphaviruses. Several authors have reported that DI particles of Semliki Forest and Sindbis viruses generated in vertebrate cells do not interfere with viral RNA synthesis in *A. albopictus* mosquito cells (11, 17, 31). Igarashi and Stollar (17) were unable to generate DI particles with serial undiluted passages in *A. albopictus* cells, although later work using longer incubation times for each serial passage resulted in DI particle generation (30). King et al. (18) also reported the generation of Sindbis virus DI particles in mosquito cells; these particles did not interfere with Sindbis virus replication in chicken cells, and others generated in chicken cells did not interfere in mosquito cells. Persistently infected mosquito cells have been shown to produce Sindbis virus DI particles that can be replicated in both vertebrate and mosquito cells (10, 12).

Our results also indicated some host cell dependence of interference; DI particles produced in C6/36 mosquito cells interfered more with EEEV replication in mosquito cells than in BHK cells, whereas DI particles produced in BHK cells interfered more with replication in BHK cells. However, in contrast to several studies with Sindbis and Semliki Forest viruses (11, 17, 31), DI particles of EEEV produced in vertebrate cells do appear to interfere with virus replication in mosquito cells, and vice versa. Further studies are needed to determine whether this reflects fundamental differences in the replication of EEEV versus Sindbis and Semliki Forest viruses and their corresponding DI particles.

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REFERENCES

1. **Chamberlain, R. W., and W. D. Sudia.** 1961. Mechanisms of transmission of viruses by mosquitoes. Annu. Rev. Entomol. **61:**371–390.
2. **Chao, L.** 1992. Evolution of sex in RNA viruses. Trends Ecol. Evol. **7:**147–151.
3. **Chao, L.** 1990. Fitness of RNA virus decreased by Muller's ratchet. Nature **348:**454–455.
4. **Cilnis, M. J., W. Kang, and S. C. Weaver.** 1996. Genetic conservation of Highlands J viruses. Virology **218:**343–351.
5. **de Groot, R. J., T. Rumenapf, R. J. Kuhn, E. G. Strauss, and J. H. Strauss.** 1991. Sindbis virus RNA polymerase is degraded by the N-end rule pathway. Proc. Natl. Acad. Sci. USA **88:**8967–8971.
6. **de la Torre, J. C., and J. J. Holland.** 1990. RNA virus quasispecies populations can suppress vastly superior mutant progeny. J. Virol. **64:**6278–6281.
7. **Domingo, E., and J. J. Holland.** 1994. Mutation rates and rapid evolution of RNA viruses, p. 161–184. *In* S. S. Morse (ed.), Evolutionary biology of viruses. Raven Press, New York, N.Y.
8. **Domingo, E., and J. J. Holland.** 1997. RNA virus mutations and fitness for survival. Annu. Rev. Microbiol. **51:**151–178.
9. **Duarte, E., D. Clarke, A. Moya, E. Domingo, and J. Holland.** 1992. Rapid fitness losses in mammalian RNA virus clones due to Muller's ratchet. Proc. Natl. Acad. Sci. USA **89:**6015–6019.
10. **Eaton, B. T.** 1978. Persistent togavirus infection of *Aedes albopictus* cells, p. 181–201. *In* E. Kurstak and K. Maramorosch (ed.), Viruses and environment. Academic Press, New York, N.Y.
11. **Eaton, B. T.** 1975. Defective interfering particles of Semliki Forest virus generated in BHK cells do not interfere with viral RNA synthesis in *Aedes albopictus* cells. Virology **68:**534–538.
12. **Eaton, B. T.** 1977. Evidence for the synthesis of defection interfering particles by *Aedes albopictus* cells persistently infected with Sindbis virus. Virology **77:**843–848.
13. **Escarmis, C., M. Davila, N. Charpentier, A. Bracho, A. Moya, and E. Domingo.** 1996. Genetic lesions associated with Muller's ratchet in an RNA virus. J. Mol. Biol. **264:**255–267.
14. **Holland, J. J., J. C. de la Torre, D. K. Clarke, and E. Duarte.** 1991. Quantitation of relative fitness and great adaptability of clonal populations of RNA viruses. J. Virol. **65:**2960–2967.
15. **Holland, J. J., J. C. de la Torre, and D. Steinhauer.** 1992. RNA virus populations as quasispecies. Curr. Top. Microbiol. Immunol. **176:**1–20.
16. **Holland, J. J., E. A. Grabau, C. L. Jones, and B. L. Semler.** 1979. Evolution of multiple genome mutations during long term persistent infection by vesicular stomatitis virus. Cell **16:**494–504.
17. **Igarashi, A., and V. Stollar.** 1976. Failure of defective interfering particles of Sindbis virus produced in BHK or chicken cells to affect viral replication in *Aedes albopictus* cells. J. Virol. **19:**398–408.
18. **King, C.-C., M. W. King, R. F. Garry, K. M.-M. Wan, E. T. Ulug, and M. R. F. Waite.** 1979. Effect of incubation time on the generation of defec-

tive-interfering particles during serial passage of Sindbis virus in *Aedes albopictus* and chick cells. Virology **96:**229–238.
19. **Kuhn, R. J., Z. Hong, and J. H. Strauss.** 1990. Mutagenesis of the 3′ nontranslated region of Sindbis virus RNA. J. Virol. **64:**1465–1476.
20. **Lanciotti, R. S., M. L. Ludwig, E. B. Rwaguma, J. J. Lutwama, T. M. Kram, N. Karabatsos, B. C. Cropp, and B. R. Miller.** 1998. Emergence of epidemic o'nyong-nyong fever in Uganda after a 35-year absence: genetic characterization of the virus. Virology **252:**258–268.
21. **Lemm, J. A., R. K. Durbin, V. Stollar, and C. M. Rice.** 1990. Mutations which alter the level or structure of nsP4 can affect the efficiency of Sindbis virus replication in a host-dependent manner. J. Virol. **64:**3001–3011.
22. **Levinson, R. S., J. H. Strauss, and E. G. Strauss.** 1990. Complete sequence of the genomic RNA of o'nyong-nyong virus and its use in the construction of alphavirus phylogenetic trees. Virology **175:**110–123.
23. **Li, G., and C. M. Rice.** 1993. The signal for translational readthrough of a UGA codon in Sindbis virus RNA involves a single cytidine residue immediately downstream of the termination codon. J. Virol. **67:**5062–5067.
24. **Li, G. P., and C. M. Rice.** 1989. Mutagenesis of the in-frame opal termination codon preceding nsP4 of Sindbis virus: studies of translational readthrough and its effect on virus replication. J. Virol. **63:**1326–1337.
25. **Muller, H. J.** 1964. The relation of recombination to mutational advance. Mutat. Res. **1:**2–9.
26. **Palma, E. L., and A. S. Huang.** 1974. Cyclic production of vesicular stomatitis virus caused by defective interfering particles. J. Infect. Dis. **129:**402–410.
27. **Sanchez-Palomino, S., J. M. Rojas, M. A. Martinez, E. M. Fenyo, R. Najera, E. Domingo, and C. Lopez-Galindez.** 1993. Dilute passage promotes expression of genetic and phenotypic variants of human immunodeficiency virus type 1 in cell culture. J. Virol. **67:**2938–2943.
28. **Spindler, K. R., F. M. Horodyski, and J. J. Holland.** 1982. High multiplicities of infection favor rapid and random evolution of vesicular stomatitis virus. Virology **119:**96–108.
29. **Steinhauer, D. A., and J. J. Holland.** 1987. Rapid evolution of RNA viruses. Annu. Rev. Microbiol. **41:**409–433.
30. **Stollar, V.** 1980. Defective interfering alphaviruses, p. 427–457. *In* R. W. Schlesinger (ed.), The togaviruses. Academic Press, New York, N.Y.
31. **Stollar, V., T. E. Shenk, R. Koo, A. Igarashi, and R. W. Schlesinger.** 1975. Observations of Aedes albopictus cell cultures persistently infected with Sindbis virus. Ann. N. Y. Acad. Sci. **266:**214–231.
32. **Strauss, J. H., and E. G. Strauss.** 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. **58:**491–562.
33. **Weaver, S. C.** 1997. Vector biology in viral pathogenesis, p. 329–352. *In* N. Nathanson (ed.), Viral pathogenesis. Lippincott-Raven, New York, N.Y.
34. **Weaver, S. C., L. A. Bellew, L. A. Gousset, P. A. Repik, T. W. Scott, and J. J. Holland.** 1993. Diversity within natural populations of eastern equine encephalomyelitis virus. Virology **195:**700–709.
35. **Weaver, S. C., L. A. Bellew, A. Hagenbaugh, V. Mallampalli, J. J. Holland, and T. W. Scott.** 1994. Evolution of alphaviruses in the eastern equine encephalomyelitis complex. J. Virol. **68:**158–169.
36. **Weaver, S. C., A. Hagenbaugh, L. A. Bellew, S. V. Netesov, V. E. Volchkov, G.-J. J. Chang, D. K. Clarke, L. Gousset, T. W. Scott, D. W. Trent, and J. J. Holland.** 1993. A comparison of the nucleotide sequences of eastern and western equine encephalomyelitis viruses with those of other alphaviruses and related RNA viruses. Virology **197:**375–390.
37. **Weaver, S. C., W. Kang, Y. Shirako, T. Rumenapf, E. G. Strauss, and J. H. Strauss.** 1997. Recombinational history and molecular evolution of western equine encephalomyelitis complex alphaviruses. J. Virol. **71:**613–623.
38. **Weaver, S. C., R. Rico-Hesse, and T. W. Scott.** 1992. Genetic diversity and slow rates of evolution in New World alphaviruses. Curr. Top. Microbiol. Immunol. **176:**99–117.
39. **Weaver, S. C., T. W. Scott, and L. H. Lorenz.** 1991. Detection of eastern equine encephalomyelitis virus deposition in *Culiseta melanura* following ingestion of radiolabeled virus in blood meals. Am. J. Trop. Med. Hyg. **44:** 250–259.
40. **Weaver, S. C., T. W. Scott, and L. H. Lorenz.** 1990. Patterns of eastern equine encephalomyelitis virus infection in *Culiseta melanura*. J. Med. Entomol. **27:**878–891.