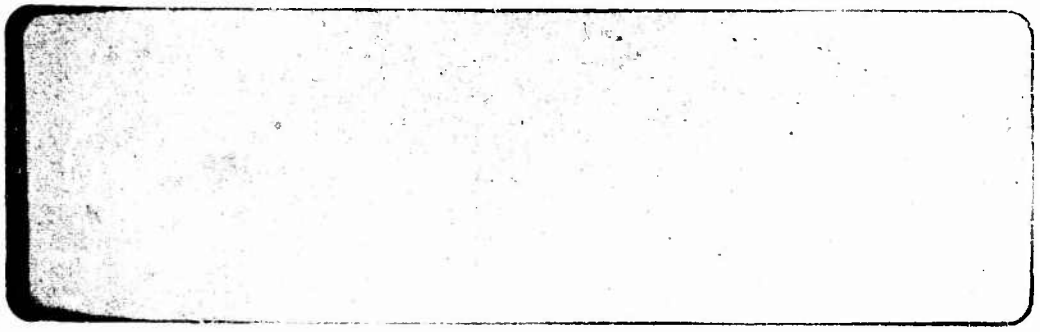

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Temperature-sensitive Steps in the Biosynthesis of Venezuelan Equine Encephalitis Virus

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In contrast to Eastern equine encephalitis virus, the replication of Venezuelan equine encephalitis (VEE) virus was strongly inhibited at 44 C in chick embryo cells. The inhibited steps were analyzed by shifting the incubating temperatures up or down, and by determining during the shifts the rate and extent of infectious ribonucleic acid (RNA) synthesis, intact virus synthesis, and formation of complement-fixing antigen or of antigen detectable by a direct fluorescent-antibody technique. The inhibition appeared to be due to two temperature-sensitive steps involved in the synthesis of VEE virus in chick embryo cells. The first step of inhibition at 44 C occurred early in virus replication and could be completely reversed simply by transferring cultures to 37 C. The inhibition appeared to take place at some point between the time when the virus entered the cell and was uncoated and the beginning of viral RNA synthesis. The second temperature-sensitive step in VEE virus synthesis was irreversible; it occurred at a point after the synthesis of viral RNA, and before the formation of virus protein measured as complement-fixing antigen or as antigen that could be stained with fluorescent antibody.

The arboviruses in group A are so classified because they share common antigens (11). They also share many other properties; it has been demonstrated, in fact, that those that have been examined can take part in phenotypic mixing (9). The relatedness of the viruses within the group is so close that several investigators of arboviruses have suggested that many of them may be mutants of each other or may have been derived from a common ancestor (11, 18, 24). For these reasons, in reviewing the literature and concepts related to the present paper, we have found it useful to adopt the following. Within the group, a temperature-insensitive virus compared with a sensitive virus in a common host cell is analogous (but not equal) to a wild-type parent and mutants derived from it. Only the latter, however, are properly called conditional lethal mutants (temperature-sensitive type). Such mutants can infect cells, reproduce, and form complete, infectious virus under one set of (permissive) conditions, but under another set of (restrictive) conditions they are blocked in one or more steps in virus reproduction. The genetic lesions responsible for the abortive infections which are observed under restrictive conditions have been found to be widely distributed over the viral genome (4, 10, 14). Thus, gene function and the biosynthesis and morphological development of the virus may be studied by the principles similar to those established by Beadle and Tatum (3), who used differentially blocked biochemical mutants of *Neurospora* for elucidating biosynthetic pathways (2). The potential of conditional lethal mutants for the study of animal viral genetics and physiology has been recently pointed out by Fenner and Sambrook (15). Like their bacterial viral counterparts, two subclasses of conditional lethal animal virus mutants have been isolated and studied: (i) the temperature-sensitive mutants mentioned earlier (6, 8, 13, 17, 19) and (ii) host-dependent viruses (1, 16, 23).

After we observed that some of our group A arboviruses were temperature-sensitive in chick embryo (CE) cells (4), it was of interest to locate and define the temperature-sensitive steps in their biosynthesis. Of two viruses examined thus far, Eastern equine encephalitis (EEE) and Venezuelan equine encephalitis (VEE), an apparent double block was found in the latter.

MATERIALS AND METHODS

Viruses. Two group A arboviruses were used: the Trinidad strain of VEE virus and the Louisiana strain of EEE virus. The origin, passage histories, properties of the strains, their preparation as seeds, and the suspended-cell plaque method for their assays have been described by Brown (4).

Cell cultures and media. CE cell monolayers were prepared from minced, trypsinized, 10-day-old chick

embryos. A 5-ml amount of growth medium containing 20 to 30 million cells was added to 60-mm plastic petri dishes. The growth medium consisted of 0.5% lactalbumin hydrolysate, 0.1% yeast extract, Hanks balanced salts solution, 10% calf serum, and 0.14% sodium bicarbonate. Confluent CE monolayers were obtained after 24 hr of incubation at 37 C in an incubator supplied with a mixture of 5% carbon dioxide-95% air. All experiments were performed on CE monolayers 24 hr old.

Growth studies. For studying the growth response of the viruses at different temperatures, the monolayers were infected at an input multiplicity of about 100 plaque-forming units (PFU) per cell. The virus was allowed to adsorb to the CE monolayers for 15 min at room temperature. The infected monolayers were washed twice with phosphate-buffered saline (*p*H 7.4) to reduce the residual virus titer in the supernatant medium, and were overlaid with lactalbumin hydrolysate medium containing 10% calf serum. The cultures were placed at the appropriate incubation temperature, and the viral growth was followed at 2-hr intervals.

Some of the early studies done at several different temperatures were performed in the temperature gradient plate described by Brown (4). Later studies done at 37 or 44 C were carried out in water-jacketed incubators. All cultures were incubated in the presence of a mixture of 5% carbon dioxide-95% air.

Extraction and assay of infectious ribonucleic acid (RNA). Infected CE monolayers were removed with a rubber policeman and were suspended in 0.02 M phosphate-0.001 M ethylenediaminetetraacetic acid (EDTA) buffer, *p*H 7.4. These suspensions were extracted twice with cold phenol, and viral RNA was precipitated from the aqueous phase with ethyl alcohol containing 2% potassium acetate. The precipitate was dissolved in phosphate-buffered saline and then was assayed on CE monolayers treated with 1 M NaCl by the method described by Colon and Idoine (12).

Complement-fixation test for antigen. The complement-fixation test was performed according to the standardized diagnostic complement-fixation method of the U.S. Public Health Service (26). The antiserum was obtained from guinea pigs immunized with infectious virus that was purified by zonal centrifugation in a sucrose gradient. This antiserum did not react with host-cell components, and specifically detected the presence of virus antigen in infected cell extracts.

RESULTS

CE cells have unusual heat tolerance and can survive temperatures up to 46.5 C for at least 48 hr. The growth of EEE and VEE viruses in CE monolayer cultures incubated at various temperatures is shown in Fig. 1. Maximal growth rate of EEE virus was obtained up to 44 C. Data not shown indicated that the rate of EEE virus multiplication was changed only slightly at 47 C, and that a significant virus titer (about 3 $\log_{10}$ PFU/ml) was maintained between 10 and 20 hr at this temperature. During the same

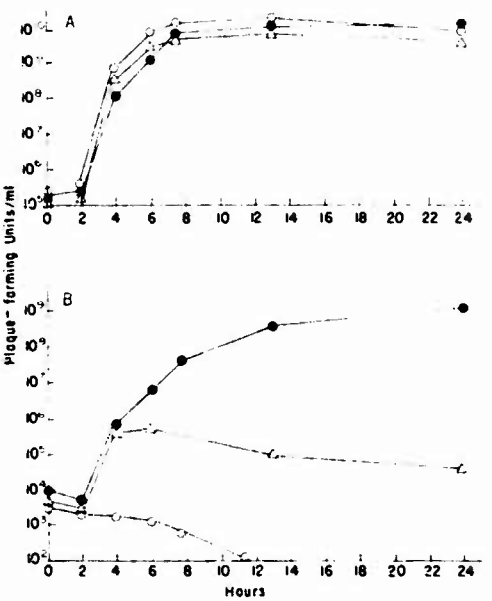

FIG. 1. *Curves showing the effect of different temperatures upon the growth of EEE (A) and VEE (B) viruses. Infected cell cultures were kept at 37 C (●), 42 C (△), and 44 C (○).*

experiment, in marked contrast to EEE virus, VEE virus showed a significant reduction in yield at 42 C, and multiplied very little, if at all, at 44 C (Fig. 1B). Data not shown indicated that the VEE virus titer at 46 C dropped at the rate of 1 $\log_{10}$ PFU/ml every 6 hr. The data in Fig. 1 show that (i) CE cells were able to support viral multiplication at 46 C, and that (ii) at 42 C and above, the multiplication of VEE virus was blocked at some temperature-sensitive step(s). The inability of VEE virus to multiply at elevated temperatures was not due to its inherent heat lability, since VEE virus held at 50 C in a cell-free medium was significantly more stable than EEE virus (20). The data suggested that one or more virus-induced products required for the synthesis of VEE virus were inactive or not made at temperatures above 40 C.

The remainder of the experiments were designed to identify and describe the temperature-sensitive steps in the biosynthesis of VEE virus.

Temperature-shift experiments. A large number of CE monolayers infected with VEE virus were prepared and treated as follows. A control group of plates was placed in a 44 C incubator, and the remainder were incubated at 37 C. At 1-hr intervals, one set of plates was transferred from 37 to 44 C, and, at the same time, one plate from each temperature group was assayed for virus

titer. With this system, it was possible to determine the effect on the growth cycle of transferring cultures from 37 to 44 C at different but sequential stages after infection. The results of a typical experiment are shown in Fig. 2.

As cultures were transferred from 37 to 44 C, most of the virus replication was halted almost immediately. When the plates were transferred before 2 hr, the growth curve followed that of the 44 C control culture. Transferring the cultures after 2 hr resulted in an immediate arrest in the biosynthesis of infectious virus. When infection of cultures was carried out with infectious RNA (IRNA) instead of intact virus and incubation was done immediately at 44 C, again no virus replication was detected. This suggested that sensitivity to high temperature occurred after uncoating of virus.

The effect of transferring VEE virus-infected CE cultures in a shift-downward experiment from 44 to 37 C is shown in Fig. 3. The procedure used for sampling and transferring cultures was the same as that just described, except that the infected monolayers were placed initially at 44 C for varying intervals of time before transfer. Virus growth was initiated within 4 hr after transferring the cultures to the 37 C incubator. If the transferred cultures were incubated at 37 C long enough, the virus titer would eventually reach that of the 37 C control culture. A similar

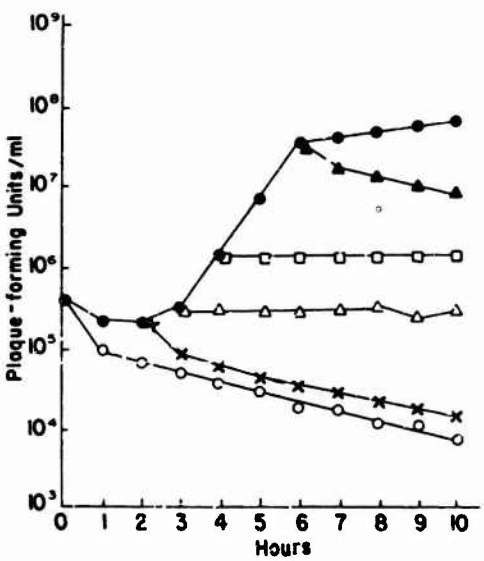

FIG. 2. *Curves showing the effect of temperature shift-up, from 37 to 44 C at different times, upon the growth of VEE virus in CE cells. Cultures were transferred at 0 hr (○), 2 hr (×), 3 hr (△), 4 hr (□), and 6 hr (▲). The control culture (●) was held at 37 C.*

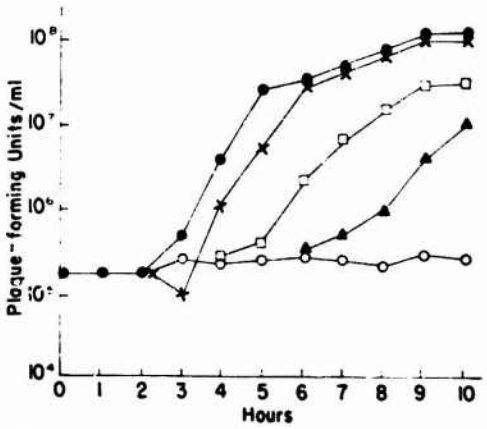

FIG. 3. *Curves showing the effect of temperature shift-down, from 44 to 37 C, upon the growth response of VEE virus in CE cells. Cultures were transferred at 0 hr (●), 2 hr (×), 4 hr (□), and 6 hr (▲). The control culture (○) was held at 44 C.*

experiment was performed in which the cultures were transferred from 44 to 37 C at 28 hr; virus growth was initiated as usual and attained the usual peak titer.

These data suggested that, although the virus-induced system appeared to be inhibited by temperatures of 44 C, it was not irreversibly damaged, because transferring the cultures to 37 C re-established viral synthesis after approximately the same latent period as controls. The latter result suggested that the inhibition was reversible with good efficiency. To estimate the efficiency of reversibility of the inhibition at 44 C, an additional experiment was carried out. CE monolayers were infected with approximately 70 PFU of VEE virus per petri dish and were allowed to adsorb the virus for 15 min. After washing, 5 ml of liquid medium was added to each plate. Five plates were placed at 44 C for 24 hr, then placed at 37 C for 0.5 hr, washed once, and overlaid with nutrient agar of the same composition as was used for the suspended-cell plaque technique. Two sets of controls were included. The first set was used to test whether preincubation of uninfected cells at 44 C in itself reduced the efficiency of plaque formation compared with the normal plaque technique. Thus, uninfected cultures incubated at 44 C for the same periods of time as the test cultures were brought back to 37 C for 0.5 hr. They were then inoculated with 70 PFU of VEE virus, allowed to adsorb virus for the same time as the test cultures, and overlaid with nutrient agar. The second set of controls was a typical plaque assay in which the monolayers inoculated with 70

PFU were overlaid immediately after adsorption and incubated at 37 C; these therefore served as controls for the first set of controls. The results of this experiment (Table 1) showed that inhibition of plaque formation by virus incubated at 44 C for 24 hr can be reversed with essentially 100% efficiency. These results and those of previous experiments indicated that, after uncoating, the virus RNA is stabilized in a form that resists inactivation at 44 C for at least 24 hr.

Preliminary evidence for a second block. Figure 4 illustrates the effect of two sequential shifts in temperatures upon the growth response of VEE virus. One set of infected CE cell cultures was placed at 44 C, held for 24 hr, and then transferred to 37 C. A second set of cultures was placed at 37 C for 2 hr, transferred to 44 C for 22 hr, and then returned to 37 C. The virus growth response at 37 C was followed for an additional 9 hr. Figure 4 shows that virus growth in infected cultures held only at 44 C was not inhibited and rose to maximal titer when placed at 37 C. However, when similar cultures were first incubated for 2 hr at 37 C prior to being transferred to 44 C, virus titers did not increase upon being returned to 37 C the next day.

The information obtained in this experiment, and that obtained in the two previous experiments, suggested that there are at least two temperature-sensitive steps involved in the synthesis of VEE virus. The first step occurred early in the replication sequence, as indicated by the completely reversible inhibition of virus growth of cultures placed initially at 44 C. The second step occurred later in the replication cycle, as demonstrated by the irreversible inhibition of virus growth at 44 C after preincubation at 37 C. If the early step of virus synthesis were the only temperature-sensitive step involved, then preincubation at 37 C would by-pass the critical event and virus could replicate at 44 C. Evidence will be presented later to demonstrate that preincubation at 37 C did by-pass the first temperature-sensitive step, but that virus growth was still strongly inhibited at the high temperature.

TABLE 1. *Reversibility of inhibition of VEE virus plaque formation at 44 C*

Sample	Treatment	Plaque count (avg of five plates)[a]
Control 1[b]	Normal plaque technique, 37 C[c]	64
Control 2[b]	Uninfected cells incubated at 44 C for 24 hr, followed by normal plaque technique at 37 C	70
Test[b]	Infected cells incubated at 44 C for 24 hr, followed by normal plaque technique at 37 C	73

[a] Each plate was inoculated with 70 PFU.
[b] See text for details.
[c] All adsorptions were done at room temperature (about 22 C) for 15 min.

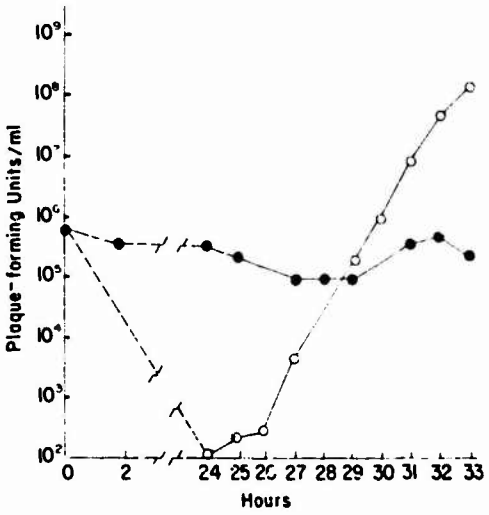

FIG. 4. *Effect of preincubation at 37 and 44 C upon VEE virus growth response at 37 C. Symbols: (●), infected cultures held for 2 hr at 37 C, then transferred to 44 C; (○), infected cultures held at 44 C; solid line, incubated at 37 C; dashed line, incubated at 44 C.*

Synthesis of IRNA and mature virus. The next series of experiments were designed to obtain further information on the early and late temperature-sensitive steps in the replication of VEE virus by following the synthesis of both IRNA and mature virus. It was shown by Wecker (27) and confirmed by Colon and Idoine (Bacteriol. Proc., p. 159, 1963) that by extracting with cold phenol IRNA can be obtained from arbovirus-infected cells that is not associated with mature virus. This permits a simple differentiation between IRNA in some form(s) of virus precursor(s) and the mature virus.

The results of a representative experiment are shown in Fig. 5. In cultures infected with virus and maintained at 37 C, IRNA synthesis increased without an appreciable lag period and reached maximal titer at 8 hr (curve D). Virus synthesis, detected after a 2-hr lag period, increased for approximately 8 hr (curve A). Cultures incubated at 44 C exhibited synthesis of neither IRNA (curve F) nor mature virus (curve

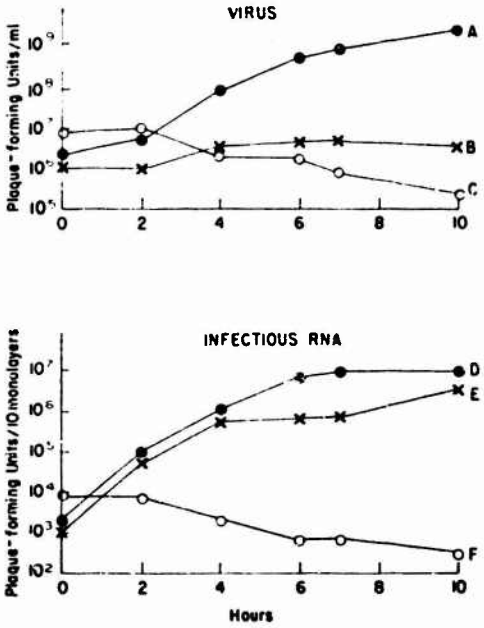

FIG. 5. *Curves showing the rate of virus synthesis and IRNA formation at 37 C (●), 44 C (○), and during preincubation at 37 C for 2 hr and at 44 C (×).*

C). Furthermore, it was shown in previous experiments that, when the cultures were infected with IRNA instead of intact virus and incubated at 44 C, there was no increase in either mature virus or IRNA synthesis, both of which increased in controls incubated at 37 C. This suggested that the first block at 44 C occurred at a point between uncoating of virus and IRNA synthesis.

The early temperature block was by-passed by incubating infected cultures for 2 hr at 37 C before shifting them to 44 C for an additional 8 hr. The results in Fig. 5 (curve E) show that the IRNA titer began to increase during the first 2 hr at 37 C and that it continued to increase even after the cultures were placed at 44 C, at about the same rate as cultures held only at 37 C. Later, the rate of IRNA synthesis decreased, and the final yield was lower than that of cultures held only at 37 C (curve D). In several experiments, the final yield at 10 hr at 44 C varied between 30 and 50% of that at 37 C. It appeared that preincubation at 37 C by-passed the early temperature-sensitive step and permitted significant synthesis of viral RNA (compare curves D and E) without a corresponding synthesis of mature virus (compare curves A and B). The lower terminal rates and yield of IRNA synthesis after shifting to 44 C may be due to the inhibition of further polymerase synthesis, which began during the 37 C preincubation, or perhaps to the labile nature of the IRNA made at 44 C which was not incorporated into infectious virus particles, or to both.

Synthesis of virus protein. Since synthesis of mature infectious virus was strongly inhibited, it was of interest to test whether virus-specific protein measured as complement-fixing antigen could be detected by antibody made against infectious virus. Monolayers of CE cells in petri plates were infected with VEE virus and subjected to different temperatures of incubation. At various times during the incubation periods, in groups of five plates each, the monolayers were washed twice, suspended in phosphate-buffered saline, and disrupted in a sonic oscillator. The cell debris was removed by centrifugation, and the cell extract was analyzed for complement-fixing antigen.

Virus complement-fixing antigen was not detectable between 0 and 24 hr at 44 C before or after preincubation for 2 hr at 37 C, although it was detectable in controls (at 37 C, the reciprocal of titer showing complete fixation of complement was 2, 4, 16, 128, and 128 at 4, 6, 8, 10, and 24 hr, respectively; no VEE virus antigen was formed at 0 or 2 hr). These results suggested that virus-specific protein, either in a structurally intact virus particle (25) or in an incomplete form [e.g., the core (21)], was not made in significant amounts at 44 C.

Julius E. Officer, of our laboratories, used a direct fluorescent-antibody technique to examine infected cells under conditions similar to those described for detecting complement-fixation antigen. He found specific antigen only in the infected cells which were incubated continuously at 37 C. The results support those mentioned above, which indicate that, even in the presence of continued IRNA synthesis at 44 C, no virus-specific protein was detected.

DISCUSSION

The results of the present study indicate that VEE virus, Trinidad strain, is apparently a doubly blocked temperature-sensitive virus. The characteristics of the two distinct blocks are given as follows. In temperature-sensitive step I, (i) virus multiplication was reversibly inhibited at 44 C; (ii) no IRNA synthesis occurred; and (iii) no virus complement fixation or antigen detected by the direct fluorescent-antibody technique was observed. In temperature-sensitive step II, (i) virus multiplication was irreversibly inhibited at 44 C; (ii) IRNA was synthesized; and (iii) no virus complement fixation or antigen

detected by the direct fluorescent-antibody technique was observed. The result of experiments in which IRNA synthesis was inhibited at high temperature after cells were infected by either intact virus or its IRNA indicate that the early block occurs after uncoating and before IRNA synthesis. The fact that the early temperature-sensitive step was reversible with approximately 100% efficiency suggests that the IRNA assumes a stabilized form in the cell, which we expect will lend itself to isolation and further analysis.

Since IRNA synthesis at 44 C continues once it begins during preincubation at 37 C, it appears that enzyme (polymerase?) activity may be stable at high temperature and that some step preceding the formation of the active enzyme may be inhibited in the early temperature-sensitive step.

When the first step was by-passed by a short preincubation period at 37 C before shifting to 44 C, it was found that considerably more than 99% of the viral synthesis was inhibited. IRNA synthesis, however, continued at a rate comparable to 37 C controls; the rate then decreased until a 50 to 70% lower final yield was reached. The lower rates may be due to the inhibition of new polymerase synthesis or to the greater heat lability of newly synthesized viral RNA that was not incorporated into intact virus particles, or to both. In contrast to controls at 37 C, significant amounts of complement-fixation antigen (or antigen detected by the direct fluorescent-antibody technique) were not detected at 44 C with or without a preincubation period at 37 C. Thus, complement-fixation antigen in mature (25) or incomplete virus particles (21, 28) was not made in significant amounts at 44 C. To determine which, if any, virus-specific proteins are made at high temperature will require other analytical techniques.

By use of the same reasoning used by Burge and Pfefferkorn (7), who made use of differentially blocked temperature-sensitive mutants of Sindbis virus, it may be suggested that, physiologically, two cistrons have been demonstrated in the doubly blocked VEE virus. It remains to be seen whether the cistrons can be demonstrated and mapped genetically.

One alternative explanation to account for the apparent double block in VEE virus replication at elevated temperature is that a regulatory gene for translation control has mutated in this virus so that it prevents *all* virus-directed protein synthesis at high temperature. Presumably, EEE virus possesses the functioning gene. Such a mutation in VEE virus could be expressed at the molecular level in a number of ways, some of which are subject to experimental verification. For example, the IRNA may not be able to bind to ribosomes at high temperature, or, if bound to ribosomes, it may not be able to function. Although there is no proof for the idea of a single regulatory gene in RNA viruses, hypotheses or mechanisms have been proposed for translational control of protein synthesis in a polycistronic RNA virus genome (22). If a point mutation in VEE were involved, one would predict that a mutant could be isolated that was capable of multiplication at a temperature above 44 C. None has been isolated thus far in a number of attempts to do so. On the other hand, tests for a deletion cannot be carried out in VEE virus at present because of insufficient knowledge and technology in the genetics of arboviruses.

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