

PRODUCTION OF FLAVIVIRUS TEMPERATURE SENSITIVE MUTANTS

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OBJECTIVE : (1) To obtain a battery of temperature sensitive (ts) flavivirus mutants for investigation of the biochemical functions of nonstructural virus-specified proteins. (2) To elucidate the relationship of the ts property to virulence (3) To determine the frequency and biochemical characteristics of naturally occurring flavivirus ts mutants isolated from field-caught mosquitoes.

BACKGROUND : The study of ts mutants has been instrumental in the elucidation of the biochemical and biological properties of most of the major virus groups. Much of the current information about the alphaviruses has come from investigation of the properties of ts mutants, although no successful isolation of a battery of flavivirus ts mutants has been reported. There is evidence to indicate that ts mutants arise spontaneously in infected mosquitoes. This observation has caused speculation about the possible significance of such natural mutants in the epidemiology of flavivirus diseases. The present study was designed to isolate ts mutants and test them for complementation and virulence characteristics.

METHODS : The virus strain used in these studies was H-32-74 isolated from a fatal human case in Vietnam by Dr. Thoa in 1974. It has been passed 5 times in suckling mouse brains. A heat resistant strain of this virus was prepared by cloning survivors of treatment at 60° for 2.5 minutes and is subsequently designated "JEV HRI."

Hydroxylamine mutagenesis : JEV HRI was treated for 20 min at 4°C with 1M hydroxylamine HCl. Virus was then dialyzed to remove excess hydroxylamine and plaqued on LLC-Mk2 cells at 28°C. Plaques were picked, suspended in 1.0 ml growth media, and used to inoculate bottles of C6/36 cells and LLC-Mk2 cells to produce viral seeds. These seeds were tested for plaque morphology and ts character by plaquing on LLC-Mk2 cells at 28° and 39°C. LLC-Mk2 cells were chosen because they will plaque JEV at both the permissive (28°C) and non-permissive (39°) temperatures. Although JEV plaques will form on C6/36 cells, these cells probably cannot survive 39°C incubation long enough to develop plaques.

Nitrosoguanidine mutagenesis : JEV HRI was treated for 15, 60, or 120 minutes at room temperature with 100 ug/ml N-methyl-N'-Nitro-N-Nitrosoguanidine (Nitrosoguanidine). Treated virus was dialyzed to remove excess mutagen and plaqued on LLC-Mk2 cells at 28°C. Plaque were picked and seeds prepared and examined as described for hydroxylamine mutagenesis.

RESULTS :

Hydroxylamine mutagenesis : Although only 12 plaque isolates have been examined thus far with the isolation of only one possible ts mutant, useful information has nonetheless been obtained from the study of these viruses. Growth curves were examined while growing stock seeds from plaque isolates in both LLC-Mk2 and C6/36 cells (Figure 1, plaque picks F-I). In all cases virus stocks grew to higher titer more quickly in mosquito cell cultures.

In many cases plaque picks were not recovered at all in the monkey kidney derived cell line, although they grew well in C6/36 cells. The reverse has not been observed to date. This difference may reflect a difference in the ability of the LLC-Mk2 cells to replicate virus at very low MOI, relatively poor growth of JEV in LLC-Mk2 at 28° or other factors. At any event subsequent stock seeds were grown in C6/36 and harvested at day 7.

Nitrosoguanidine mutagenesis : The low recovery of plaque picks on LLC-Mk2 cells at 28°C was again evident when nitrosoguanidine was used as the mutagen (Table 1, plaque picks R-Y).

Thus far 439 nitrosoguanidine plaque picks have been examined for ts character and plaque morphology. The ratio of the titer of the cloned virus at 28° and 39° has been used to select probable ts mutants. There is a certain amount of scatter around the ideal value of 1.0 for clones that are not ts (Figure 2). The distribution of values for the 160 clones of this experiment fits a bell-shaped curve with a 4 clones falling clearly outside the distribution as very likely ts mutants (3 $10^1/10^2/1$ and one $10^5/1$). The later has been passed a second time in C6/36 to make a large seed and has retained its ts character ($>10^4/1$). Thus far no reversion or leakiness has been detected in this virus, designed TS-1. TS-1 is small plaque virus at 28° and does not plaque at 39°C whereas the parent HRI virus causes a large plaque at both 28° as at 39°C. Another plaque pick, ppEF, which had 5.6 times as many plaques at 28° as at 39°C was cloned a second time by picking plaques (Table 2). Seeds were prepared from these clones in C6/36 clones examined 13 were not ts, 16 were stable ts mutant clones and 7 were ts but were leaky or had a high reversion frequency. One of the stable ts clones (EF8) was chosen to make a large seed stock, designated TS-2. The ts clones all evidenced small plaque morphology.

Thus, attempts have been made to isolate a battery of ts mutants from JEV. Thus far one possible ts mutants has been derived from JEV treated with hydroxylamine. At least 2 stable is mutants, TS-1 and TS-2, have been derived from nitrosoguanidine treated JEV as well as 3 more virus clones that are probably stable ts mutants that have not been analyzed to date. Further experiments are underway to produce more nitrosoguanidine-induced mutants and mutants induced by other methods. The virulence of these viruses will be examined by LD₅₀ experiments in thermally regulated mice and in monkeys.

Table 1. Comparison of LLC-Mk2 and C6/36 cells for growth and seeds from plaque picks at 28°C.

<u>Plaque Pick</u>	<u>Mutagen</u>	<u>LLc-Mk2</u>	<u>C6/36</u>
R	NG	> 1.7 x 10 ¹	1.8 x 10 ³
S	NG	> 1.7 x 10 ¹	1.3 x 10 ³
U	NG	4.0 x 10 ²	1.0 x 10 ⁴
W	NG	5.0 x 10 ¹	9.7 x 10 ⁶
X	NG	5.0 x 10 ¹	3.0 x 10 ⁶
Y	NG	> 1.7 x 10 ¹	1.6 x 10 ⁷

NG = Nitrosoguanidine; All titers given are PFU/ml on day 7 after infection.

Table 2. Temperature sensitive character of clones from ppEF.

<u>Plaque Pick</u>	<u>Titer 28°C</u> <u>Titer 39°C</u>	<u>Plaque Pick</u>	<u>Titer 28°C</u> <u>Titer 39°C</u>
EF (Parent)	5.55		
TS		NOT TS	
EF 4	3.6 x 10 ⁴	EF 3	0.53
EF 5	2.7 x 10 ⁴	EF 12	3.8
EF 7	7.0 x 10 ³	EF 16	0.46
EF 8	> 3.4 x 10 ⁶	EF 19	1.8
EF 9	5.3 x 10 ⁴	EF 23	1.5
EF 10	> 1.6 x 10 ⁶	EF 25	0.81
EF 15	> 6.1 x 10 ⁵	EF 26	4.4
EF 17	4.3 x 10 ⁵	EF 28	1.8
EF 18	4.4 x 10 ⁴	EF 29	0.75
EF 20	2.0 x 10 ⁴	EF 44	0.79
EF 24	2.6 x 10 ⁴	EF 45	4.7
EF 30	1.2 x 10 ⁴	EF 49	0.90
EF 31	1.8 x 10 ⁴		
EF 39	4.1 x 10 ⁴	TS BUT LEAKY OR HIGH REVERSION	
EF 40	2.1 x 10 ⁴	EF 14	22
EF 41	5.8 x 10 ³	EF 42	2.1 x 10 ²
		EF 43	10
		EF 46	13
		EF 47	1.2 x 10 ²
		EF 48	7.8
		EF 50	24

Fig. 1. Growth of Virus Clones in LLC-MK₂ and C6/36 cells at 28 C°.

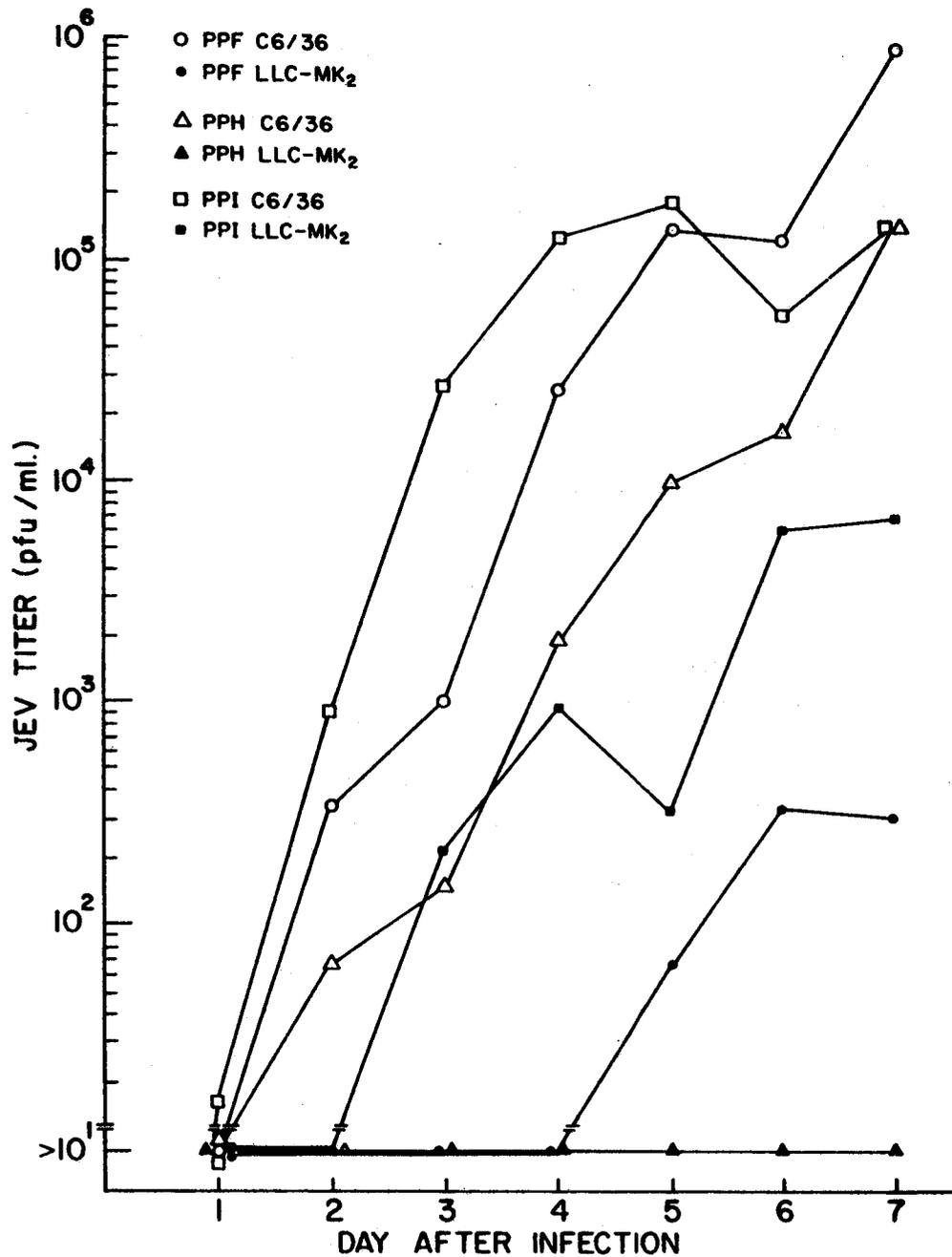


Fig. 2. Examination of Nitroguanidine Plaque Picks for temperature sensitivity.

