

# PRODUCTION AND CHARACTERIZATION OF FLAVIVIRUS TEMPERATURE SENSITIVE MUTANTS

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## OBJECTIVES :

1. To obtain a battery of temperature sensitive (ts) flavivirus mutants for investigation of the biochemical functions of non-structural 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 :** In the AFRIMS Annual Report 1980-1981 we reported the mutagenesis of Japanese encephalitis virus (JEV) with N-methyl-N'-nitro-N-nitrosoguanidine (NG). Mutants derived in that study and those reported here are being compared to spontaneous ts mutants from persistently infected mosquito cells to determine the possible significance of these mutants in the epidemiology of flavivirus diseases.

**METHODS :** The virus strain used in these studies is the JEV strain H-32-74 HR1 described in the 1980-81 AFRIMS Annual Report. Recent oligonucleotide mapping kindly performed by Drs. Connie Schmaljohn and Joel Dalrymple at USAMRIID indicate that the parental strain H-32-74 is identical to the Nakayama strain of JEV. NG mutagenesis was performed as previously described.

**FLUOROURACIL MUTAGENESIS :** A 75 cm<sup>2</sup> T flask of *Aedes albopictus* C6/36 cells was infected with JEV-HR1 at an MOI of 1.0. After absorption, maintenance medium containing 2% FBS and 100 g/ml 5 fluorouracil (FU) was added. Extracellular virus was harvested after 48 hr at 28°C and plaqued on LLC-MK2 at 28 and 39°C. Plaques at 28°C were picked and suspended in 1.0 ml growth medium. These plaque isolates were used to inoculate 25cm<sup>2</sup> T flasks of C6/36 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.

## RESULTS :

**NG mutagenesis :** An additional 262 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 (Figure 1). Most of the clones expressed ratios that were distributed in a bell-shaped curve around a value of 1.0. Seven clones fell outside the distribution (six clones with ratios of 10/1 - 10 3/1 and one clone with a ratio of 10 4/1; Table 1).

One of these clones, VN, has been passed again in C6/36 cells to produce a large seed. The initial 28/39 ratio of VN was  $1.6 \times 10^2$  and that of the seed was 33. This number indicated a high degree of leakiness or reversion or a mixed population of virus. The population has been recloned and these clones are being analyzed to determine their ts phenotypes. Large seed cultures of clones LQ and EF-15 (from experiment NG-I, AFRIMS Annual Report 1980-81) showed little evidence of reversion or leakiness.

The plaque morphology of the clones was measured at 28 and 39°C. Differences in plaque morphology were more easily discerned at 39°C (Table 2). The JEV-HR1 strain, which was the parental strain used in these experiments, produced uniformly large plaques, most of the clones isolated after NG mutagenesis showed the large plaque phenotype. However, a number of small plaque mutants were cloned and are being examined for virulence and other markers.

**FU Mutagenesis :** Mutagenesis with one mutagen tends to produce mutants with a restricted genotype. FU was therefore used to induce mutations to increase the probability of getting mutants in all possible complementation groups. Thus far, 132 clones have been isolated from JEV-HR1 virus grown in the presence of FU. As described above the ratio of the virus titers at 28 and 39°C were used to select probable mutant clones (Figure 2).

Four ts clones were selected (Table 3). Stock seeds of these clones have been prepared in C6/36 cells and maintained their ts character. A number of plaque morphology mutants have also been isolated (Table 4) and the small plaque mutants have been selected for further analysis.

Thus far, 439 clones have been examined from JEV treated with NG. Of these 9 have been shown to be ts. Stability varies considerably from clone to clone, but at least three are stable enough for genetic studies. In addition 262 clones have been examined after growth of JEV in the presence of FU. At least four of these clones are ts, and three of the four are stable enough for genetic studies. Of the clones studied thus far, 16 from the NG experiments and 17 from the FU experiment are not ts but exhibit a stable small plaque morphology, in contrast to the large plaque exhibited by the parental JEV-HR1 strain. Experiments are in progress to produce more FU-induced mutants and to measure the virulence of the ts mutants and the plaque morphology mutants in mice and in monkeys.

Table 1. Candidate ts mutant clones from NG experiment II

<u>Clones</u> *	<u>Titer 28/Titer 39</u>	<u>Plaque morphology at 39°C</u>
LQ	2.8 x 10.2	L
OX	75	L
PJ	45	M
RR	12	L
SN	86	L
VC	4.5 x 10.7	-(S-M at 28°C)
VN	160	S

\* S = < 1.0 MM

M = 1.0 - 3.0 MM

L = > 3.0 MM

Table 2. Plaque morphology at 39°C of NG experiment II.

<u>Sizes of plaques</u>	<u># of clones</u>
L	117
M	50
S	16
S + M	31
S + M + L	9
S + L	5
M + L	21
No plaques at 39°C	1 (S + M at 28°C)

Table 3. Candidate ts mutant clones from FU experiment I

<u>Clone</u>	<u>Titer 28/Titer 39</u>	<u>Plaque morphology at 39°C</u>
HG	2.8 x 10.5	S
JB	1 x 10.4	- (S at 28°C)
JQ	33	L
JX	1.0 x 10.3	M + S

Table 4. Plaque morphology at 39°C of FU experiment I clones.

<u>Size of plaque</u>	<u># of clones</u>
L	58
M	14
S	17
S + M	8
S + M + L	3
S + L	4
M + L	9
0	1 (S at 28°C)



Fig. 1. Examination of clones from Nitrosoquinidine Experiment II for temperature sensitivity.

