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ISOLATION AND BASIC PROPERTIES OF TEMPERATURE—SENSITIVE MUTANTS OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS

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Summary. — Fifteen temperature-sensitive (ts) mutants were isolated from 4 Venezuelan equine encephalomyelitis virus clones by nitrous acid treatment or by growing the virus in the presence of 5-fluoruracil. Three of them were classified as RNA^- mutants by their inability to synthesize RNA at nonpermissive temperature (1.5—3.3 % in respect to the wild type). The remaining 11 mutants showed a slight decrease of RNA synthesis at the nonpermissive temperature (32—73 %) and were referred to the RNA^+ phenotype. One mutant possessed $RNA^\pm$ phenotype (18 %). Five complementation groups were determined by complementation analysis of the mutants.

Key words: *alphaviruses*; *ts mutants*

Introduction

Temperature-sensitive (ts- mutants have become one of the main objects of genetic investigations in virology. Determination of the number of the complementation groups and of the nature of the biochemical defect of ts mutants yielded a great body of data on the genome structure and on reproduction stages of viruses belonging to different taxonomic groups. Ts-mutants have been successfully used in genetic and biochemical studies on Sindbis and Semliki forest alphaviruses (Burge and Pfefferkon, 1966*a*, *b*, 1967, 1968; Tan *et al.*, 1969; Atkins *et al.*, 1974; Keränen and Kääriäinen, 1974; Strauss *et al.*, 1976, 1977). But so far only little is known about ts mutants of Venezuelan equine encephalomyelitis (VEE) virus (Pancake *et al.*, 1976).

The aim of the present study was to obtain VEE virus ts mutants and to investigate their basic properties, the nature of the biochemical defect and their distribution into complementation groups.

Materials and Methods

Cell cultures. Primary trypsinized chicken embryo cell (CEC) cultures were used.

Virus. Four clones (Nos 15, 16, 25, 31) isolated from a laboratory strain of VEE virus and characterized by a ts^+ phenotype served as wild type virus.

Table 1. Basic properties of VEE virus ts mutants

Mutant	EOP 40 °C/35 °C		Level of leakage*
	1st passage	3rd passage	
15/1	2.0×10^{-2}	1.7×10^{-2}	4.0×10^{-3}
15/2	6.0×10^{-2}	5.0×10^{-2}	4.0×10^{-3}
15/6	9.0×10^{-3}	6.0×10^{-3}	4.0×10^{-3}
15/7	2.0×10^{-2}	2.0×10^{-2}	1.0×10^{-2}
15/10	7.0×10^{-3}	4.0×10^{-3}	2.6×10^{-2}
15/14	1.0×10^{-6}	1.0×10^{-6}	3.0×10^{-4}
25/9	4.4×10^{-2}	4.0×10^{-2}	8.0×10^{-3}
25/17	5.0×10^{-2}	5.0×10^{-2}	4.4×10^{-3}
25/19	9.0×10^{-2}	6.0×10^{-2}	1.0×10^{-2}
25/20	8.0×10^{-6}	2.6×10^{-2}	Not done
25/21	2.6×10^{-2}	1.6×10^{-2}	8.3×10^{-3}
31/9	2.0×10^{-2}	2.1×10^{-2}	4.0×10^{-3}
31/17	5.0×10^{-4}	8.0×10^{-3}	1.0×10^{-3}
31/14	3.0×10^{-3}	3.0×10^{-2}	1.0×10^{-4}
16/6	5.0×10^{-3}	1.1×10^{-2}	9.0×10^{-3}

*The level of leakage was determined as the ratio of the virus yield at 40 °C to that at 35 °C; titration was at 35 °C.

Induction of ts mutants. (*1*) CEC cultures were infected at a multiplicity of 5—10 PFU/cell and during one reproduction cycle (6.5 hr) the virus was grown in the presence of 0.25 μg/ml 5-fluorouracil. (*2*) The virus suspension was treated with nitrous acid according to Burge and Pfefferkorn (1966*a*).

Isolation of ts mutants. The mutagen-treated virus was titrated in CEC cultures by the plaque method. Infected cultures were incubated for 24 hr at nonpermissive (40 °C) or permissive (35 °C) temperature and then shifted to 35 and 40 °C, respectively. On the fourth day, the presumed ts mutants were isolated from plaques occurring after transfer of the infected cultures to permissive conditions or from plaques preserving their size after transfer to nonpermissive temperature. The isolates obtained were tested for the presence of a ts-defect by paralelel titration at 35 and 40 °C and the efficiency of plaquing (EOP) was determined by the ratio of the virus yield at 40 °C to the yield at 35 °C (EOP 40 °C/35 °C).

The results were evaluated statistically by the method of Fisher (Urbakh, 1964).

Determination of RNA synthesis. The ability of ts mutant to synthesize RNA under n op ermissive conditions was estimated by incorporation of ^{3}H-uridine into the acid-insoluble frnction of infected cells. CEC cultures were treated with actinomycin D (1 μg/ml for 2 hr), and infected either with mutant or wild type virus at a multiplicity of 10 PFU/cell. One hour later maintenance medium containing ^{3}H-uridine (5 μCi/ml) was added. Immediately after inoculation, one part of cultures was incubated at 35 °C and the other at 40 °C; uninfected cells served as controls. Six hours post infection (p. i.) the cell monolayer was washed, fixed with 5 % trichloroacetic acid, washed twice with 5 % trichloroacetic acid, then treated with an ethanol-ether mixture, dissolved in 0.3 N NaOH and placed on paper filters. Radioactivity was determined in a liquid scintillator. RNA synthesis was determined as the ratio of ^{3}H-uridine incorporation to the incorporation of the wild type virus label under nonpermissive conditions and expressed in %. Simultaneously, ^{3}H-uridine incorporation into cells infected with ts mutants under permissive and nonpermissive conditions was compared.

Temperature-shift experiments. CEC cultures were infected at a multiplicity of 2.5 — 5.0 PFU/cell. After adsorption, the cell monolayers were washed, medium 199 was added and the incubation was initiated at 35 °C. Then after 2, 4 and 6 hr the cultures were shifted to 40 °C. Control infected cells were incubated in parallel at 35 and 40 °C for 8 hr. The culture fluids were harvested after 8 hr and titrated at 35 °C.

The complementation test was done according to Burge and Pfefferkorn (1966*b*). The level of complementation was determined as the ratio of the virus yield at 40 °C (mixed infection) to the sum of the 2 mutant yields in single infections at 40 °C. Complementation was considered positive if the complementation level was 2 or higher and regularly reproduced in further tests.

Results

Primary isolation of ts mutants was done from small plaques formed on titration of mutagen-treated virus. The proportion of small plaque variants in the population of mutagen-treated viruses (2836 of 14817) exceeded that (420 of 5397) in the wild type virus population ($P < 0.01$). From small plaques specifically occurring on titration of mutagen-treated virus, we isolated 2287 clones, 210 of which were characterized by the ts$^-$ phenotype (9.2 % of the total number of plaques examined. Two ts mutants were detected among 382 isolates obtained from small plaques formed by the wild type virus. The difference in the content of ts variants in the small plaque fraction of the mutagen-treated virus and that of the wild type virus was statistically significant ($P < 0.01$).

The mutant ts$^-$ phenotype was expressed to a different degree. For further study we selected 15 isolates characterized by maximal decrease in the virus yield at nonpermissive temperature.

One of the essential features of ts mutants is the EOP because it reflects the reproduction level of the ts$^-$ virus under nonpermissive conditions as well as the extent of ts$^-$ phenotype expression. Table 1 presents the results of EOP 40 °C/35 °C determination for all 15 ts mutants studied. The EOP va-

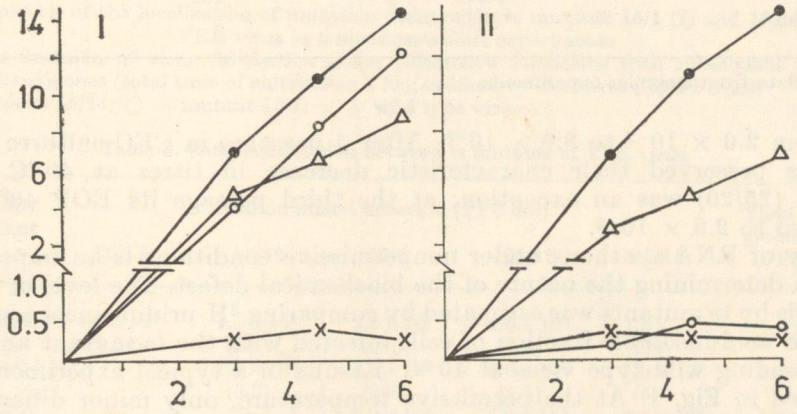

Fig. 1.

^{3}H-Uridine incorporation into acid-insoluble fraction of cells infected with the wild type virus or ts mutants of VEE virus at permissive (I) and nonpermissive (II) temperature

Abscissa: time in hr; ordinate: ^{3}H-uridine incorporation (count/min × 10^{-3})

● — wild type; △ — mutant 31/14; ○ — mutant 31/9; × — uninfected cells

Table 2. Ability of ts mutants of VEE virus to synthesize RNA under nonpermissive conditions

Mutant	Incorporation of ^{3}H-uridine into acid-insoluble fraction of infected cells at 40 °C		RNA phenotype
	count/min	% of wild type	
15/1	8 436	65	+
15/2	8 490	67	+
15/6	7 983	63	+
15/7	7 349	58	+
15/10	8 363	66	+
15/14	418	3.3	−
Clone 15	12 672	100	
Cell control	284		
31/14	10 504	54	+
31/9	311	1.5	−
31/17	508	2.6	−
Clone 31	19 543	100	
Cell control	208		
25/9	3 873	64	+
25/17	2 541	42	+
25/19	1 089	18	±
25/20	1 936	32	+
25/21	3 147	52	+
Clone 25	6 052	100	
Cell control	414		
16/6	6 223	73	+
Clone 16	8 526	100	
Cell control	383		

Average data from 3 similar experiments.

ried from 2.0×10^{-2} to 8.0×10^{-6}. After 3 passages in CEC cultures most mutants preserved their characteristic decrease in titres at 40 °C. One mutant (25/20) was an exception: at the third passage its EOP 40°/35°C increased to 2.6×10^{-2}.

Study of RNA synthesis under nonpermissive conditions is an important stage in determining the nature of the biochemical defect. The level of RNA synthesis by ts mutants was estimated by comparing ^{3}H-uridine incorporation into the acid-insoluble fraction of cells infected with the ts mutant and the corresponding wild type virus at 40 °C. Results of a typical experiment are presented in Fig. 1. At the permissive temperature, only minor differences in the incorporation of the label were observed between the wild type virus and ts mutants 31/14 and 31/9. At nonpermissive temperature, label incorporation by mutant 31/9 was dramatically decreased as compared to the incorporation at permissive temperature or with wild type virus.

Data on RNA synthesis by ts mutants at nonpermissive temperature are summarized in Table 2. Most mutants showed a moderate decline in

^{3}H-uridine incorporation into the acid-insoluble fraction of the infected cells. With these mutants, the level of label incorporation was 32—73 % of that with the wild type virus. Three mutants (15/14, 31/9 and 31/17) had a low (1.5—3.3 %) ability to ^{3}H-uridine incorporation. With mutant 25/19, the RNA synthesis was 18 % of that with the wild type.

Localization of the ts defect in the virus reproduction cycle was also studied by temperature-shift experiments (Fig 2). Initially, the infected cultu-

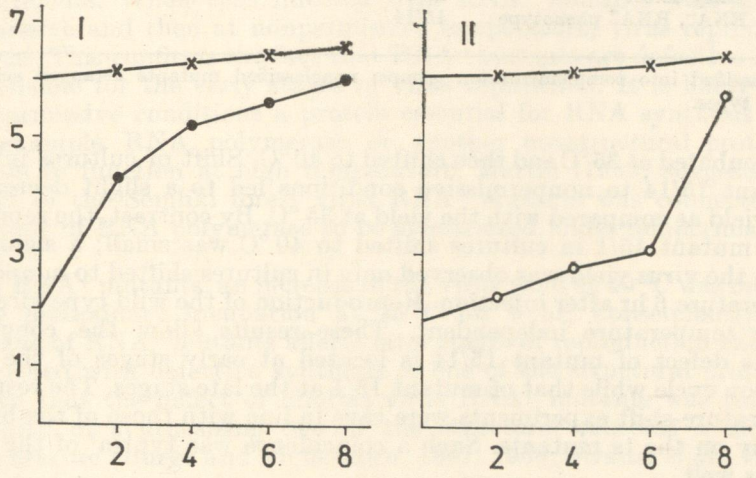

Fig. 2.
Determination of the localization of mutation damages in ts mutants 15/1 (I) and 15/14 (II) of VEE virus in temperature-shift experiments
Abscissa: duration of virus cultivation under permissive conditions with subsequent shift to nonpermissive ones (total time of cultivation 8 hr); ordinate: virus titres (log PFU/ml)
● — mutant 15/14; ○ — mutant 15/I; × — wild type virus

Table 3. Complementation between ts mutants of VEE virus

Complementation group	Mutant	Yield on mixed infection (PFU/ml)					Yield in single infection (PFU/ml)
		25/17	15/16	15/2	25/19	15/14	
I	25/9	4.0×10^6 (1.99)	1.5×10^7 (15.4)	3.5×10^7 (343)	1.0×10^5 (3.5)	1.5×10^6 (19)	7.5×10^4
	25/17	—	1.4×10^7 (3.3)	8.0×10^8 (380)	5.0×10^6 (2.1)	1.0×10^7 (5)	2.0×10^6
II	15/6	—	—	5.0×10^7 (50)	5.0×10^6 (5)	2.0×10^8 (200)	9.0×10^5
					1.5×10^7 (200)	5.0×10^6 (150)	3.0×10^4
III	15/2	—	—	—	—	2.0×10^6 (47)	4.0×10^4
IV	25/19	—	—	—	—	—	2.5×10^3
V	15/14	—	—	—	—	—	

Table 4. Distribution of ts mutants of VEE virus according to complementation groups

Complementation group	Physiological groups (RNA phenotype)	Mutants* Classified	Nonclassified
I	Late defect	25/9, 25/17	15/10, 16/6, 15/1
II	RNA^+ phenotype	15/6	15/7, 25/21
III		15/2	31/14, 25/20
IV	Early defect	25/19	31/9, 31/17
V	RNA^-, $RNA^{\pm}$ phenotype	15/14	

*Mutants classified into complementation groups; nonclassified mutants arranged according physiological types.

res were incubated at 35 °C and then shifted to 40 °C. Shift of cultures infected with mutant 15/14 to nonpermissive conditions led to a slight decrease in the virus yield as compared with the yield at 35 °C. By contrast, the reproduction of ts mutant 15/1 in cultures shifted to 40 °C was small; a significant increase in the virus yield was observed only in cultures shifted to nonpermissive temperature 6 hr after infection. Reproduction of the wild type virus was practically temperature independent. These results allow the conclusion that the ts defect of mutant 15/14 is located at early stages of the virus reproduction cycle while that of mutant 15/1 at the late stages. The results of the temperature-shift experiments were thus in line with those of the biochemical study on the ts mutants. Such a coincidence was typica' of the other mutants as well.

All the isolated ts mutants were studied in the complementation test. Complementation was clear-cut among 5 ts mutants (Table 3). Five complementation groups could be detected. Mutant 25/17, which did not react with mutant 25/9 in the complementation test bud did so with mutants of the other groups was included into group I. Groups I-III included mutants with an RNA^+ phenotype, while groups IV-V contained mutants with an RNA^- or $RNA^{\pm}$ phenotype (Table 4).

Discussion

Fifteen ts mutants of VEE virus were isolated and their basic properties studied. None of them was fully deprived of the capacity to multiply under nonpermissive conditions, but the virus yield declined considerably. The preserved ability of the virus to form plaques under nonpermissive conditions could depend on the presence of ts^+ revertants in the population as well as on leakage of ts mutants. As follows from our experiments, after 2—3 passages in CEC cultures the isolated ts mutants retained their specific low titres at 40 °C. It can be assumed, therefore, that the formation of plaques at nonpermissive temperature was not due to the presence of ts^+ revertants in the population. Thus the given EOP 40 °C/35 °C values reflect the level of reproduction of the ts mutant under nonpermissive conditions. Mutant 25/20 was an exception: after 3 passages the difference in its titres at 40 °C and

35 °C was greatly reduced. The increased capacity for plaque formation at 40 °C after passaging should evidently be explained by a process of reversion to ts$^+$ phenotype.

Based on the results concerning RNA synthesis by ts mutants under nonpermissive conditions, the mutants can be divided into two physiological groups: those with defects in RNA synthesis (RNA$^-$ phenotype) and those with a block at later stages of virus reproduction (RNA$^+$ phenotype).

The biochemical data obtained were confirmed in temperature-shift experiments. When cells infected with RNA$^-$ mutants were incubated at permissive and then at nonpermissive temperature, virus reproduction was normal. This confirms the fact that RNA$^-$ mutants are defective in functions responsible for the early stages of virus replication. It is likely that under nonpermissive conditions a protein essential for RNA synthesis is blocked for example RNA polymerase or another nonstructural protein which is unable to function at high temperature. Martin (1969) suggested that the defect of the Semliki forest virus RNA$^-$ mutants was connected with the inability of RNA polymerase to be synthesized under nonpermissive conditions.

In RNA$^+$ mutants, an increase in the virus yield at 40 °C was only observed after preliminary incubation at 35 °C for 6 hr. Consequently, mutation damage of RNA$^+$ mutants affects later stages of reproduction and is possibly connected with defective synthesis of one of the structural proteins. It was shown that the defect of Sindbis virus RNA$^+$ mutants was caused by the inability of synthesizing one of the structural proteins a nonpermissive temperature (Burge and Pfefferkorn, 1967, 1968; Strauss *et al.*, 1976).

The complementation test proved that VEE virus ts mutants can be complemented. The establishment of 5 complementation groups was based on the clear-cut complementation among 5 mutants included into the given groups. Genetic analysis also revealed a group of nonclassified mutants which did not complement with mutants of two or more groups. This phenomenon might be due to the character and localization of mutation damages, particularly, double and multiple damages.

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