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BEHAVIOUR OF TS MUTANTS OF PSEUDORABIES VIRUS IN THE RANGE BETWEEN PERMISSIVE AND NONPERMISSIVE TEMPERATURE[1])

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Summary. — The growth properties of pseudorabies virus (PRV) strain BUK, wild type and its four ts mutants, were examined using highly accurate temperature measurements in the incubator and on the cell sheet. The transition from permissive to nonpermissive temperature occurred within an interval of 1° C. The midpoint of this interval was termed the semipermissive temperature since at this temperature the reproduction and consequently the plaquing of mutant viruses was not completely restricted. The "leakiness" of ts mutants might be due to their incubation at semipermissive temperature. Under semipermissive conditions and at high virus inputs, three mutants exhibited nearly complete inhibition of plaquing. In an experimental mixture of to mutants and PRV-BUK wild type, the latter was not affected by the inhibition. This might be due to an enhanced sensitivity of the mutants, but not of the wild type, to interferon under these conditions.

Key words: pseudorabies virus; ts mutants; growth characteristics

Introduction

Vo Duong Huy *et al.* (1977) reported the selection of ts mutant pseudorabies virus (PRV) strain BUK via shift-down from nonpermissive (npT°) to permissive temperature (pT°). The isolated mutants belong to four complementation groups and between pT° show stable burst size ratios of three to four orders of magnitude. At variance with this is the fact that, on plating these mutants under nonpermissive conditions, plaques were usually formed, indicating that these mutants might be "leaky". However, Vo Duong Huy *et al.* (1977) pointed out that because of evaporation the temperature in the open system (Petri dish) should be about one degree lower than in the surrounding air environment. A further temperature increase in an open system

[1]) Dedicated to Prof. K. Spies on the occasion of his sixtieth birthday.

Table 1. Growth behaviour of PRV-BUK wild type and its ts mutants between 36 and 42° C

Temp. (° C)	PRV-BUK	ts1	ts5	ts8	ts9
36	6.9	6.3	6.8	6.0	6.5
38	7.2	5.3	6.3	5.7	5.6
38.5	7.1	5.5	6.2	5.9	6.1
39	6.3	5.3/3.9/4.2	4.7/3.0/6.5	6.0/4.2/5.0	6.4/3.0/3.9
39.5	6.1	2.0	2.5	0.5	0
40	6.3	2.0	0.8	2.1	0
40.5	5.7	0	0	0	0.4
41	4.6	0.3	0	0.1	0
42	2.4	0	0.5	0	0.4
Background	1.9	2.0	2.0	1.4	1.8

The data are average log PFU/0.1 ml values from at least three independent experiments. Incubation time 17 hr.

is not tolerated by chick embryo cells (CEC) under agar (Vo Duong Huy *et al.*, 1972).

This necessitated the use of highly precise temperature control to test the possible causes of "leakiness". In addition, the properties of 4 ts mutants, whose relative positions on a linkage map were confirmed by two-factor crosses (Hegenscheid *et al.*, in preparation), were determined under exactly reproducible temperature and incubation conditions.

Materials and Methods

Pseudorabies virus strain BUK (PRV-BUK) (Škoda, 1962) was a gift from the Institute of Virology, Slovak Academy of Sciences, Bratislava. For the isolation and preliminary characterization of ts mutants representing 4 complementation groups see Vo Duong Huy *et al.* (1977). The ts mutants were plaque purified three times before complementation experiments, and the mutants ts1, ts5, ts8 and ts9 were subsequently re-cloned by three single-plaque passages.

The efficiency of replication (EOR) (Schaffer, 1975) was determined in CEC monolayers under one-step conditions [multiplicity of infection (MOI) > 1; removal of non-adsorbed virus by addition of antiserum followed by washing of the cells; the incubation period approximately corresponded to the duration of one viral replication cycle in the employed cells]. The cells were grown in cylindrical, tightly covered culture vessels, then infected, treated with antiserum and incubated for 17 hr at the desired temperature (accuracy of $\pm$ 0.02° C). Virus was harvested by three cycles of rapid freezing and thawing and pelleting the cell debris at $1{,}500 \times g$.

The efficiency of plating (EOP) was determined by plating the viruses at 36° C (permissive temperature, pT°), 39° C (semipermissive temperature, spT°) and 40° C (nonpermissive temperature, npT°).

The temperature in the incubator was measured with a Universal Electronic Multipoint Recorder (Honeywell). The temperature distribution in the incubator, the equilibration time after placing the plates in the incubator and the effect of the heating periods on the internal temperature were monitored with sensors in Petri dishes at 14 locations. All data were expressed relative to the plaque count at pT°, i. e. an EOP of 1 indicated a plaque count not significantly different from that at pT°.

Results

Efficiency of replication of PRV-BUK and its ts mutants

The growth of PRV-BUK and the mutants ts1, ts5, ts8 and ts9 was studied under defined one-step conditions of cultivation in the temperature

range from 36 to 42° C. From 40.5° C upwards a slight restriction of the growth of the wild-type virus became evident; the restriction was fully expressed at 41° C (yield: 4.6 PFU/0.1 ml; Table 1). At 42° C no replication of PRV-BUK wild-type virus was demonstrable during the 17 hr incubation period. These results proved that the cell-virus system was intact under the conditions applied.

All investigated mutants exhibited an almost uniform behaviour regarding their temperature sensitivity. Up to 38.5° C their replication was normal. At 39° C several independent experiments revealed yields typical of permissive conditions but sometimes also reduced yields indicative of a beginning temperature effect. At 39.5° C the yields were indistinguishable from background virus remaining after antiserum treatment and washing, i. e. from this temperature on the mutants no longer replicated under one-step conditions.

The transition from permissive to nonpermissive temperature occurred within an interval of only 1° C (Fig. 1), the so-called spT° being approximately the middle of this interval.

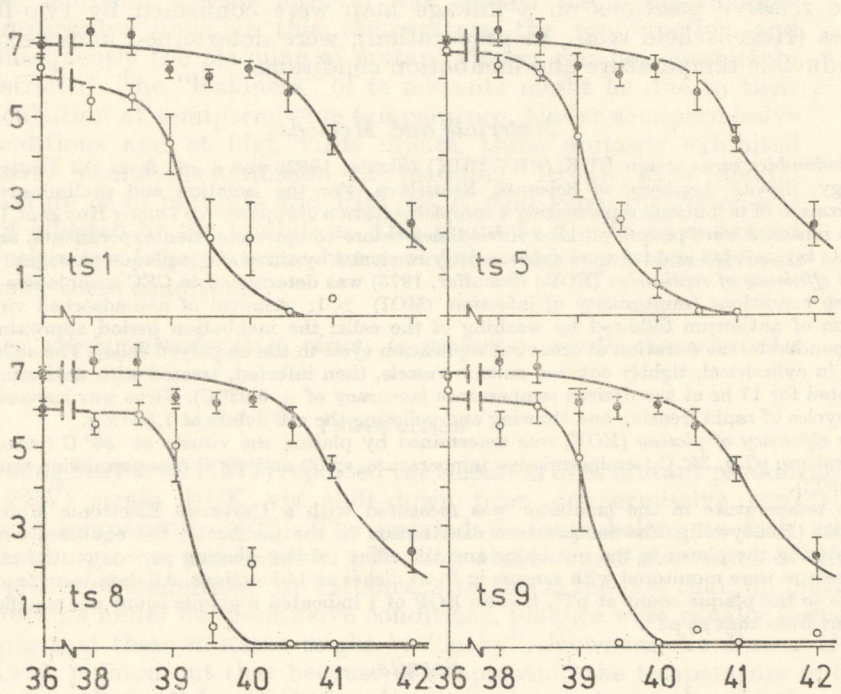

Fig. 1.
Temperature profiles of PRV-BUK (●) and its ts mutants (○)
Abscissae: temperature of incubation in °C; ordinates: virus yield in log PFU/0.1 ml

Table 2. Replication of PRV-BUK and its ts mutants at 40° C

Virus	log PFU/0.1 ml after incubation at 40° C for 17 hr	24 hr	41 hr	48 hr	36° C
ts1	2.2(2.1)	2.3(2.1)	2.8(2.1)	2.2(2.1)	6.3
ts5	0.6(1.6)	0.5(2.2)	— (1.6)	0.7(2.2)	7.1
ts8	2.0(1.0)	2.3(1.5)	2.7(1.0)	2.6(1.5)	6.0
ts9	— (1.5)	— (1.6)	— (1.5)	0.7(1.6)	6.7
PRV-BUK	6.3(2.3)	6.3(2.2)	6.1(2.3)	6.4(2.2)	7.3

In parantheses: values for bacground virus.

Measurements of the temperature distribution in the incubator showed that, due to the inertia of the temperature regulation system (the "on" and "off" periods of the heater), the positions of the Petri dishes in the incubator and especially the constant evaporation from the open dishes, the required npT° of 40° C was never attained. The temperature of the cell sheet was always in the semipermissive range between 39 and 39.5f C. This means that the previous studies (Vo Duong Huy *et al.*, 1972, 1977) were actually performed not in the npT° but in the spT° region and, therefore, a new interpretation of these data is required.

Prolonged incubation at the npT°

We further examined whether the arrested virus growth would be resumed during prolonged incubation periods at 40° C. CEC monolayers were infected as described and incubated for 17, 24, 41 or 48 hr at 40° C. Parallel incubations at the pT° served as controls. The data summarized in Table 2 show that prolonged incubation at 40° C did not raise the titre of any of the mutants, so that a slowed down replication at 40° C can definitely be excluded.

Table 3. Thermal inactivation of PRV-BUK wild type and its ts mutants at 40° C

Virus	log PFU/0.1 ml after 0 hr	4 hr	8 hr	24 hr	$\bar{m}$ (0-8)	Titre diference	(24/$\bar{m}$)
ts1	6.3	5.7	5.7	3.7	5.9	−2.2	
ts5	7.0	7.0	6.6	5.5	6.9	−1.4	
ts8	6.1	6.0	5.8	4.4	6.0	−1.6	
ts9	6.4	6.2	6.6	4.6	6.4	−1.8	
PRV-BUK	7.0	6.6	6.5	4.3	6.7	−2.4	

Table 4. Comparison of EOP's at nonpermissive and permissive temperatures

Virus	40° C (npT°)	36 ° C (pT°)
ts1	$< 10^1$	1.6×10^4
ts5	$< 10^1$	3×10^5
ts8	$< 10^1$	2×10^5
ts9	$< 10^1$	6.3×10^4
PRV-BUK	4.8×10^5	5×10^5

Thermal inactivation of PRV-BUK wild type and ts mutants at npT°

The thermostabilities of the wild type and ts mutants were determined at npT°. Following 4, 8 or 24 hr incubation of virus stocks, 0.1 ml portions were withdrawn and assayed for free infectious virus at pT°. The results (Table 3) showed that the mutants as well as the wild-type virus were stable for up to 8 hr at 40° C and inactivated during the next 16 hr to the same degree i. e. by one to two orders of magnitude. Therefore, none of the mutants was more thermolabile than the PRV-BUK wild-type.

Efficiency of plating of PRV-BUK and the ts mutants

EOP at 40° C (npT°). The EOPs of the PRV-BUK wild-type and the mutants ts1, ts5, ts8 and ts9 on CEC monolayers were tested at 40 $\pm$ 0.02° C. The infected cell monolayers were incubated for 48 hr in tightly covered

Table 5. Comparison of EOPs of PRV-BUK wild-type and its ts mutants at 36 and 39° C

Virus	Plaque counts at 36° C			Plaque counts at 39° C			Limits of significance* for differences in plaque number (p = 1%)
ts1	2	2	3	2	0	5	0— 9
	11	7	3	4	5	7	1—11
	13	7	4	7	2	—	1—12
ts5	16	21	19	22	12	10	8—25
	36	25	27	30	14	27	16—37
	10	24	15	10	6	6	5—19
ts8	23	15	24	15	14	7	8—25
ts9	5	24	17	6	9	8	4—19
	8	6	12	2	6	—	2—14
PRV-BUK	4	6	6	1	0	1	0— 6
	3	1	4	5	1	—	0— 6

* According to Lorenz (1963) tables.
EOP was 1 throughout.

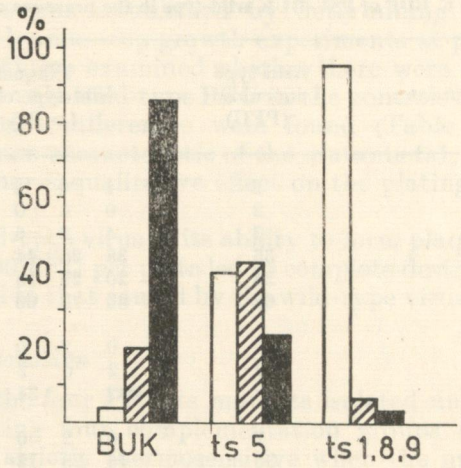

Fig. 2.
Plaque size distribution of PRV-BUK wild type and its ts mutants
Plaque diameter:
Empty columms — < 0.5 mm
Shaded columms — $0.5 < 1$ mm
Black columms — $\geqq 1$ mm

culture vessels. Simultaneously, the EOPs at pT° (36° C) were determined as control values. None of the mutants was able to form plaques under these conditions at 40° C, whereas the PRV-BUK wild type exhibited an EOP of 1 even at the npT° (Table 4). The average EOP (npT°/pT°) of the mutants was 10^{-4} to 10^{-5}.

EOP at 39° C (spT°). Pools of known titre of the ts mutants and the PRV-BUK wild type were plated in parallel at pT° (36° C) and spT° (39° C) in Petri dishes (open system). The plaque counts in parallel dilutions were compared and the significance of the differences at a given probability of error of 1% was estimated with the help of published tables (Lorenz, 1963). No statistically significant differences were demonstrable (Table 5). The EOP ratios (spT°/pT°) were 1 for all the mutants as well as for the wild type.

Plaque formation of PRV-BUK and ts mutants at spT°

Single plaque isolates of PRV-BUK and the ts mutants were diluted so that an inoculum of 0.1 ml contained about 50 PFU. Viruses from one plaque were plated on three to six Petri dishes. An adsorption period of 1 hr at 39° C was followed by the addition of overlay agar and further incubation at the spT°.

In all cases the ts mutants were able to form visible plaques after 48 hr under these conditions. Using a jig with holes of 0.5 and 1 mm it was easy to sort the plaques into three categories of size, namely with diameters < 0.5 mm, $0.5-1$ mm and > 1 mm. In this way a total of 55 isolated wild-type and mutant plaques were studied. The distribution of plaque sizes was estimated after an assignment of around 13000 plaques to the categories mentioned (Fig. 2). On the basis of a probability of error of $p = 0.005$, the frequency of "large" ($\geqq 1$ mm) and "small" ($\leqq 0.5$ mm) plaques was

Table 6. EOP of PSV-BUK wild-type in the presence of 10^4 PFU of its ts mutants after 48 hr at spT°

Mutant	Wild-type inoculum (PFU)	Plaques of wild-type character (> 0.5 mm)	Limits of significance* for differences in plaque numbers (p = 1%)
ts1	2	1 0 2 — —	—
	2	0 4 0 — —	0— 6
	3	4 2 6 — —	0— 9
	36	38 22 44 24 —	22—46
	28	20 26 16 32 —	15—36
	62	86 70 66 80 —	53—85
ts8	2	0 2 0 0 2	—
	2	2 0 2 4 —	0— 6
	36	32 — 34 — —	23—46
ts9	2	0 2 0 2 —	—
	36	16 28 18 — —	18—39

* According to Lorenz (1963) tables.
EOP was 1 throughout.

determined from the plaque size distribution measured at spT°. The mutants ts1, ts8 and ts9 produced not less than 85% small and no more than 3% large plaques. The results with the wild type were inverse, since no more than 2.5% small and no less than 80% large plaques appeared. The mutant ts5 occupied an intermediate position with respect to plaque size distribution (Fig. 2).

If an inoculum contained more than 1000 PFU wild-type virus, confluent plaques were formed also at spT°, i. e. the cell monolayer was practically killed due to a cytopathic effect (CPE) which was fully expressed on inoculation of 10^4 or more PFU (Lorenz, 1963). Surprisingly, this effect failed to appear when 10^4 PFU/0.1 ml of the mutant ts1 were inoculated. On the contrary, at these high input MOIs plaque formation was completely inhibited, despite normal plaque formation at low inocula. At the end of the incubation period, the CEC monolayer was still able. In the case of the mutants ts8 and ts9 plaque formation was also severely restricted; there appeared only pin-point palques, the size of which ($\leq$ 0.1 mm) was at the border of macroscopic visibility.

We then tested whether the observed inhibition of plaquing would also extend to the wild-type virus if this were inoculated in a mixture with a ts mutant. Of each ts mutant, a pool with about 10^4 PFU/0.1 ml was prepared and supplemented with different concentrations of wild-type virus. As controls, the pure wild-type and mutant viruses were plated in parallel at pT° and spT°. After 48 hr of incubation at spT°, wild-type plaques appeared in all mixed platings, the number of which corresponded to average wild-type input. The PFU distribution in parallel platings was of the Pisson type. The

wild-type identity of these plaques was established by determining the plaque size distribution at spT° and by one-step growth experiments at pT°. With the help of tables (Lorenz, 1963) we examined whether there were significant differences ($p < 1\%$) between the wild-type PFU in the controls and in the mixed platings. No significant differences were found (Table 6). Apparently, the inhibition phenomenon characteristic of the mutants ts1, ts8 and ts9 had neither a quantitative nor a qualitative effect on the plating of admixed wild-type PRV-BUK.

The mutant ts5 resembled the wild-type virus in its ability to form plaques at high inocula. Even an input of 1000 PFU per plate led to complete destruction of the cell layer at spT°, identical to that caused by the wild-type virus.

Discussion

The present results showed that the four PRV ts mutants isolated under shif-down conditions and representing four complementation groups (Vo Duong Huy *et al.*, 1972, 1977), are strictly thermosensitive when the npT° of 40° C is exactly maintained. In fact, under stable conditions of cultivation and temperature, inhibition of virus reproduction already begins at 39.5f C.

It was striking that all four mutants were restricted at the npTf and exhibited almost identical transition characteristica (Fig. 1). The transition range between unimpaired virus growth and absolute inhibition was from 39 to 39.5° C, i. e. $< 1°$ C. Within this transition interval all ts mutants displayed reduced virus replication and an incompletely inhibited ability to form plaques. Schaffer (1975) discussed two possible explanations four such behaviour: "1. Reversion of ts mutants to the ts^+ phenotype; 2. the so-called "leaky" phenomenon in which the mutant, at the nonpermissive temperature, manifests the function of wild-type virus". The problem of reversion was relatively easy to exclude by characterizing single plaques and by stability testing (Hegenscheid, 1977).

Explanations of the "leaky" phenomenon have rarely been considered in the literature. In the PRV-BUK ts mutants, the EOR was lowered in the transition interval pT° npT° but definitely above that at npT°, i.e. there was a partial inhibition of virus replication. In addition, the virus yields varied in spite of identical external conditions in repeated experiments. Thus, the yields at 39° C (as measured in the CEC monolayer) varied as follows: 5.3, 3.9 and 4.2 log PFU/0.1 ml for ts1; 4.7, 3.0 and 6.5 log PFU/0.1 ml for ts5; 6.0, 4.2 and 5.0 for ts8 and 6.4, 3.0 and 3.9 log PFU/0.1 ml for ts9. The EOP in the temperature transition range was 1 for all the mutants (Table 5). However, the resulting mutant plaques were much smaller than the wild-type plaques (Fig. 2).

In contrast to the variability of the EOR in this temperature interval, the phenotypic capacity to form plaques remained constant. We therefore propose to call the midpoint between pT° and npT° the semipermissive temperature (spT°).

The spT° was identical for the four studied ts mutants of PRV-BUK, despite their assignment to different complementation groups. One of the

reasons for this identity in the switch from pT° to npT° is the selection procedure (shift-down) which led to a "synchronization" of the isolates (Vo Duong Huy *et al.*, 1977). At a temperature of 39° C on the monolayer before the shift-down only such mutant virus candidates were recognized which displayed a partially inhibited replication (EOR) and reduced plaque size characteristic of this temperature. Mutants with spT° above 39° C would have and had the appearance of wild-type plaques after the shift-down. On the other hand, mutants with spT° below 39° C should have also escaped detection because their infective centres would have been inactivated at their npT° (39° C or less).

We would like to emphasize, however, that this "synchronization" could only be stabilized by repeated cloning of phenotypically small ts mutant plaques. After 3 cloning cycles EOP values (npT°/pT°) still varied from 10^{-3} to 1 (Vo Duong Huy *et al.*, 1977) and at least 3 further single-plaque passages were necessary to achieve constant EOP ratios of 1 (Hegenscheid, 1977).

Our results indicate that the so-called leaky phenomenon is a general property of ts mutant viruses as a phenotypic sign of their respective spT°. In cases where ts mutants were selected randomly (Pringle *et al.*, 1973; Brown *et al.*, 1973; Schaffer *et al.*, 1973; Subak-Sharpe *et al.*, 1974) or by the shift-up technique (Simpson and Hirst, 1968; Flamand, 1970; Lake and Mackenzie, 1973), no synchronization at a preselected spT° takes place. The apparent leakiness of some of the mutants simply reflects the fact that the arbitrarily selected npT° was actually the spT° for this group of mutants. Due to the general variability of an only partially inhibited virus replication, such mutants vary in their behaviour during repeated experiments, sometimes resembling a ts mutant, sometimes the wild-type virus. If an increase in temperature were tolerable by the employed virus-host system, such leaky mutants should exhibit a stable ts character.

The significant difference between the selection method of Vo Duong Huy *et al.* (1972, 1977) and those of other authors does not only consist in the shift-down but in the fact that the selection was not performed at the npT° but at the spT°, a fact of which the authors were not aware at the time. Another consequence of this selection technique is an identical EOP for all ts mutants of PRV-BUK with concomitant reduction of plaque diameter at spT°. These significant differences in plaque morphology are the basis for the discrimination between the PRV-BUK wild type and the ts mutants. By evaluating 13,000 individual plaques the ability of the wild type and the consistent disability of the ts mutants to form large plaques was established. The easy discrimination between the latter and the former is expressed in a probability of error $<0.5\%$ when only 50 plaques are subjectively evaluated and assigned. This procedure requires less work and material than an assignment based on EOR differences. Its reliability was confirmed in 95 independent tests giving reproducible plaque size distributions. Systematic errors are excluded by running standard wild-type and standard ts mutant virus controls in each experiment. The procedure is thus as specific as the determination of EOP differences since in both cases the CPE is used as

a measure of viral reproductive capacity. In our case the measure refers to the quality (size) of the plaques while the measure for EOP determinations is the quantity of visible plaques. The ultimate criterion of assignment on both cases is the ability of the viruses to replicate under one-step conditions (Vo Duong Huy *et al.*, 1977) which adequately reflect biological conditions of growth at npT°.

In contrast to the behaviour of ts5 and the wild-type virus the plating of ts1, ts8 and ts9 at high inocula (10^4 PFU) at spT° did not result in cell killing by a CPE. The inhibition of plaque formation by these mutants had no influence whatsoever on the plaquing of the wild type, when it was mixed with the mutants. This observation can be used to determine the titre of wild-type PFU in such ts mutant stocks (Hegenscheid, 1977).

The reason for the inhibitory effect could be an enhanced interferon sensitivity of ts1, ts8 and ts9 as compared with ts5 and the wild type. Lomnici (1974*a*, *b*) described such a coupling of mutant character and interferon sensitivity for PRV; the mutants were five to ten times more sensitive to interferon than the wild type.

A decision whether the plaquing of ts5 at spT° should be considered exceptional or not awaits the isolation and examination of more PRV ts mutants.

In summary, we have shown that a number of simple, economical, reliable and reproducible methods valid for all plaque-forming viruses allows a characterization of phenotypic properties of PRV-BUK ts mutants.

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