

Isolation of Temperature-Sensitive Conditional Lethal Mutants of "Fixed" Rabies Virus

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Received for publication 13 November 1970

In an attempt to induce temperature-sensitive (*ts*) conditional lethal mutants of rabies virus, stocks of a plaque-purified substrain of strain CVS fixed rabies virus were subjected to mutagenesis by HNO₂, 5-fluorouracil, or 5-azacytidine. It was necessary to prepare virus stocks from clones of mutagenized virus selected at random and to test subsequently each stock for possible *ts* characteristics by measuring its relative capacity for growth at permissive (33 C) and nonpermissive (40.5 C) temperatures. Five *ts* mutants were detected in tests of 161 clones of mutagenized virus. Each of the mutants exhibited a remarkably low incidence of reversion and little demonstrable "leakiness." One of the five *ts* mutants (*ts2*), which formed very small plaques, and another (*ts1*), which formed plaques of only slightly reduced size, were further characterized. Virus *ts1* was more thermostable at 40.5 C than the parental virus, but the *ts2* mutant was unchanged in this respect. The *ts1* virus exhibited normal pathogenicity for mice, but *ts2* virus caused a very irregular death pattern. Both deaths and survivors immune to rabies virus challenge were noted in all groups of mice inoculated with *ts2* virus, regardless of the virus dose.

Although the viral etiology of rabies has been recognized for nearly a century (13), application of modern virological techniques to the study of rabies virus has been possible only since its adaptation to growth in cell culture in 1958 by Kissling (6). Rabies virus does not consistently produce a cytopathic effect or plaques in monolayer cell culture. The practical study of genetically homogeneous clones of rabies virus became possible only after the development of an agarose-suspended cell plaquing technique (15). Unfortunately, this plaquing system tolerates only very narrow ranges of temperature and thus is not applicable to direct assay for temperature-sensitive (*ts*) conditional lethal mutants of rabies virus. Furthermore, plaque sizes vary for artifactual reasons in this system, so it has not been possible to select plaque-size variants from "wild" rabies populations.

The CVS "fixed" strain of rabies virus replicates in BHK monolayer cell cultures at temperatures ranging from 25 C to over 40 C. Plaque assay is performed at 35 C. Maximum yield of released virus is obtained at 33 C, but a temperature of 35 C is used for clone outgrowth, as that is the lowest temperature supporting cell multiplication. In an attempt to isolate *ts* mutants of rabies virus, we have selected at random clones of virus populations treated with chemical muta-

gens and determined their relative capacity for growth at 33 C (permissive temperature) and 40.5 C (nonpermissive temperature). The isolation by these techniques of five *ts* mutants and the preliminary characterization of two mutants are described in this report.

MATERIALS AND METHODS

Cell cultures. BHK subclone 21 (BHK-21) cells were propagated in Eagle's basal medium with double concentrations of vitamins and amino acids (BME) with 0.225% HCO₃⁻ and 10% fetal calf serum (FCS) added (BME FCS 10). BHK subclone 13S (BHK-13S) cells were propagated in BHK growth medium (10) with 0.17% HCO₃⁻ and 10% FCS.

Virus. CVS strain virus that had been passed 112 times in primary hamster kidney cell culture (7) was kindly supplied to us by P. E. Halonen. The virus was passed twice in BHK-21 cells, clone-purified three times in BHK-13S cells, and passed two or three more times on BHK-21 cells prior to use. Virus stocks were routinely prepared in BHK-21 cell cultures infected at a cell multiplicity of 0.1 to 10.0 and incubated for 72 to 96 hr at 33 C in a medium consisting of BME plus 0.1% bovine serum albumin (BME.1BA). Cell supernatant fluids clarified by centrifugation at 800 × *g* for 30 min at 4 C comprised the virus stock.

Virus cloning methods. All virus assays were performed by the agarose-suspended BHK-13S cell plaque technique (15). For virus cloning experiments, selected plaques were aspirated with a Pasteur pipette

and suspended in 1.0 ml of BME.1BA. To prepare cloned virus stocks, 0.5 ml of plaque suspension was added to 1.5×10^6 BHK-21 cells suspended in 0.3 ml of growth medium. The mixture was incubated in 30-ml plastic tissue culture flasks (Falcon Plastics Co.) standing on end for 30 min at 33 C. After this adsorption period, 5.0 ml of growth medium was added to each flask, and the flasks were placed flat and incubated at 35 C for 4 to 6 days, after which the supernatant fluids were harvested. Cloned CVS virus stocks prepared in this manner contained $10^{7.5}$ to $10^{8.7}$ plaque-forming units (PFU)/ml.

Chemical mutagenesis: nitrous acid. The method of Granoff (4) was employed for chemical mutagenesis. Two parts of virus suspension (undiluted virus stock) were mixed with one part of 4.0 M NaNO_2 in water and one part of acetate buffer, varying in pH from 4.0 to 4.8, at room temperature. At various intervals, samples of the reaction mixture were removed and the reaction was stopped by immediate 10- to 25-fold dilution in BME.1BA. Each sample was titrated immediately to yield plaques for selection as potential mutants.

5-AzaC and 5-FU. In experiments employing these mutagens, 5-azacytidine (5-AzaC; Calbiochem, B grade; reference 5) or 5-fluorouracil (5-FU; Calbiochem) was incorporated in the maintenance medium of BHK-21 cells infected with rabies as described above.

Assay of clones for *ts* mutants. BHK-21 cell cultures were inoculated with virus of each clone tested, at multiplicities of infection (MOI) of 10 to 100, and incubated at 40.5 C. Viral yields after 24 or 48 hr were measured. Virus clones giving yields at 40.5 C that were 10-fold (or more) less than normal control clones were subsequently retested by comparing their growth at both 40.5 and 33 C.

Mouse infectivity titrations. Groups of six 4- to 5-week-old female ICR mice were inoculated into the left cerebral hemisphere with 0.03 ml each of serial 10-fold dilutions of virus in BME.1BA. Inoculated animals were observed for at least 28 days after inoculation for the onset of paralysis or death.

RESULTS

Inactivation of rabies virus by HNO_2 . Rabies virus was rapidly inactivated by HNO_2 at rates that are markedly pH-dependent. For example, infectious virus titers were consistently reduced from 100-fold to 1,000-fold after incubation for 1 min in 1.0 M nitrous acid under conditions varying from pH 4.4 to 4.9. At pH 5.8 and 6.1, less than 10-fold reductions in virus titers were observed after incubation periods as long as 10 min. In control reaction mixtures of pH 4.4 in which NaNO_2 was replaced with NaCl, no reduction in virus titer was detected, thus establishing the specific role of the nitrous acid in the virus inactivation. The high degree of stability of rabies virus under acidic conditions of pH ≥ 4.5 has been previously reported by Turner and Kaplan (18) and by Kuwert and Liebenow (8).

Effect of 5-FU and 5-AzaC on the replication of

rabies virus. Incorporation of 5-FU in cell maintenance medium at a concentration of 2 mM led to a depression in rabies virus yield (after 48 hr incubation at 33 C) of from 65 to 85%. Under similar conditions, 5-AzaC at a concentration of 25 $\mu\text{g}/\text{ml}$ led to a 97 to $>99\%$ reduction in rabies virus yield.

Selection of rabies virus mutants. Initially, an attempt was made to select a heat-resistant CVS virus variant for production of a standard pool for chemical mutagenesis, to obviate the problem of the lability of rabies virus at high nonpermissive temperatures. Virus suspended in BME.1BA was heated at 60 C, and samples removed at various time intervals were titrated for residual infectivity. Several plaques were harvested from the titration of the last sample yielding surviving virus (5, 10, or 20 min) and used to prepare new virus stocks which were again heated at 60 C. After five such cycles of heating and regrowth of clones of surviving virus, no thermoresistant variants were obtained. The half-life at 60 C of the original CVS stock (suspended in BME.1BA) and of all the tested progeny clones (suspended in BME FCS 10) was approximately 36 sec. A clone designated CVS-HPP (CVS-heated and plaque-purified) was selected from the sixth heating experiment for use as a basic stock for mutagenesis because, although it was not demonstrably more heat-stable than the parental virus, it was assumed to be genetically homogeneous as a result of six consecutive clonings.

Five *ts* mutant clones were detected in studies of 161 clones of mutagen-treated CVS-HPP virus. The incidence of mutant clones was 1 (*ts1*) of 44 treated with HNO_2 , 2 (*ts2* and 3) of 91 grown in the presence of 5-AzaC, and 2 (*ts4* and 5) of 26 grown in the presence of 5-FU.

The relative capacity of each mutant virus (in the 2nd or 3rd BHK-21 passage after clone selection) for growth at 40.5 and 33 C is shown in Table 1. Although CVS stock virus gave yields at 40.5 C equal to 0.5 to 5.0% of those obtained at the permissive temperature, the mutant viruses, with a single exception, revealed no demonstrable replication at the nonpermissive temperature. The small amount of *ts1* progeny virus detected at 40.5 C was determined to be revertant non-*ts* virus. The results suggest that there is no measurable "leakiness" of these mutants at 40.5 C. An exact determination of reversion rates was not possible because of our technical inability to plaque rabies virus at the nonpermissive temperature. However, the total absence of detectable virus in 40.5-C cultures infected with four of the five mutants indicates that the incidence of revertants in those mutant stocks was less than one in 10^6 particles,

TABLE 1. Temperature sensitivity of CVS rabies virus and mutants^a

Virus	Virus yield (released virus, PFU/ml)						Fractional yield (40.5 C/33 C)	
	Permissive temp (33 C)			Nonpermissive temp (40.5 C)			24 hr	48 hr
	6 hr	24 hr	48 hr	6 hr	24 hr	48 hr		
Expt A								
CVS	$<5.0 \times 10^1$	9.0×10^5	2.0×10^8	10^1	1.6×10^4	2.2×10^6	0.018	0.011
<i>ts2</i>	1.6×10^2	9.0×10^5	8.5×10^7	2.0×10^2	$<5.0 \times 10^0$	$<5.0 \times 10^0$	<0.0000056	<0.00000059
Expt B								
CVS		2.2×10^4	1.2×10^6		10^2	6.0×10^4	0.0045	0.050
<i>ts1</i>		2.3×10^4	10^7		$<5.0 \times 10^0$	2.0×10^2	<0.00023	0.00002
<i>ts2</i>		10^4	1.4×10^7		$<5.0 \times 10^0$	$<5.0 \times 10^0$	<0.00050	<0.0000036
<i>ts3</i>		2.2×10^6	1.4×10^6		$<5.0 \times 10^0$	$<5.0 \times 10^0$	<0.0000023	<0.0000036
<i>ts4</i>		1.6×10^5	1.2×10^7		$<5.0 \times 10^0$	$<5.0 \times 10^0$	<0.000031	<0.0000042
<i>ts5</i>		5.5×10^5	7.5×10^6		$<5.0 \times 10^0$	$<5.0 \times 10^0$	<0.0000091	<0.0000067

^a BHK-21 monolayers in 30-ml plastic flasks were infected at a multiplicity of approximately 10. After adsorption for 30 min at 33 C, monolayers were washed twice with phosphate-buffered saline, treated with 1.0 ml of polyvalent rabies antiserum 1:1,000 for 10 min at 33 C, and again washed twice with phosphate-buffered saline prior to addition of cell maintenance medium. Times are calculated from the initiation of adsorption.

the minimum amount of virus in the inocula tested.

Of the *ts* mutant clones, one, *ts2*, produced unusually small plaques, whereas the size of plaques produced by the other four was normal or near-normal (Fig. 1). The small-plaque mutant *ts2* and the near-normal plaque-forming *ts* mutant, *ts1*, were selected for further study.

Preliminary characterization of mutants *ts1* and *ts2*. The effect of the cell MOI on the expression of the *ts* state of mutants *ts1* and *ts2* was tested in several experiments in which postinfection treatment of cells with antiserum was omitted, so that minute amounts of revertant virus might not be neutralized by antibody. In such experiments the *ts* state of mutant stocks was slightly obscured at 6 and 24 hr postinfection because of the release of unadsorbed inoculum virus observed both at permissive and nonpermissive temperatures. However, data obtained at 48 hr postinfection clearly revealed that no new infectious virus was produced at 40.5 C in culture inoculated with *ts1* or *ts2* virus stocks at MOI ranging from 0.13 to 8.0, whereas virus replication was readily demonstrated in cultures inoculated with wild-type virus at similar MOI.

As a further test of the stability of the *ts* state of these mutants, serial undiluted passages of second BHK-21 passage mutant stocks were performed in BHK-21 cells at the permissive temperature until the 10th passage level was attained. The results of temperature-sensitivity determinations of virus tested at each passage level are given in Table 2. Both *ts1* and *ts2* retained their *ts* mutant characteristics unaltered through nine successive passages at 33 C and then exhibited reversion at the 10th passage level. The conditions

leading to this abrupt conversion are not understood, but it is evident that both mutants are normally very stable when propagated at the permissive temperature.

The possibility that clones *ts1* and *ts2* may represent thermolabile mutants was tested by thermoinactivation studies at 40.5 C (Fig. 2). The results indicate that clone *ts2* virus is inactivated at a rate very similar to that of the parental virus (half-life, ca. 4.1 hr). Clone *ts1* virus is actually more thermoresistant than the parental stock (half-life, 7.5 hr).

Pathogenicity for mice. The response of weanling mice to intracerebral inoculation with serial dilutions of parental type CVS and *ts* mutant viruses is depicted in Fig. 3. Typical dose-response effects were observed with CVS, CVS-HPP, and mutant *ts1* viruses. The plaque-purified CVS virus appeared to be slightly less virulent for mice than the original CVS stock, causing greater than 50% mortality only at doses above 10 PFU, whereas the wild CVS stock was highly lethal even in doses of less than 1 PFU. Mutant *ts1* virus appeared slightly more pathogenic than the CVS-HPP stock from which it was derived, giving a dose-response curve similar to that of the CVS stock.

Mutant *ts2* virus exhibited an extremely aberrant dose-response relationship. Both rabies-specific deaths and survivors were observed in groups of mice receiving virus doses varying from ca. 1 to greater than 10^6 PFU.

The incubation period was prolonged in mice infected with *ts* mutants. Deaths of mice infected with *ts1* mutant virus occurred about 2 days later than in mice infected with comparable doses (in PFU) of CVS or CVS-HPP virus. Deaths of mice

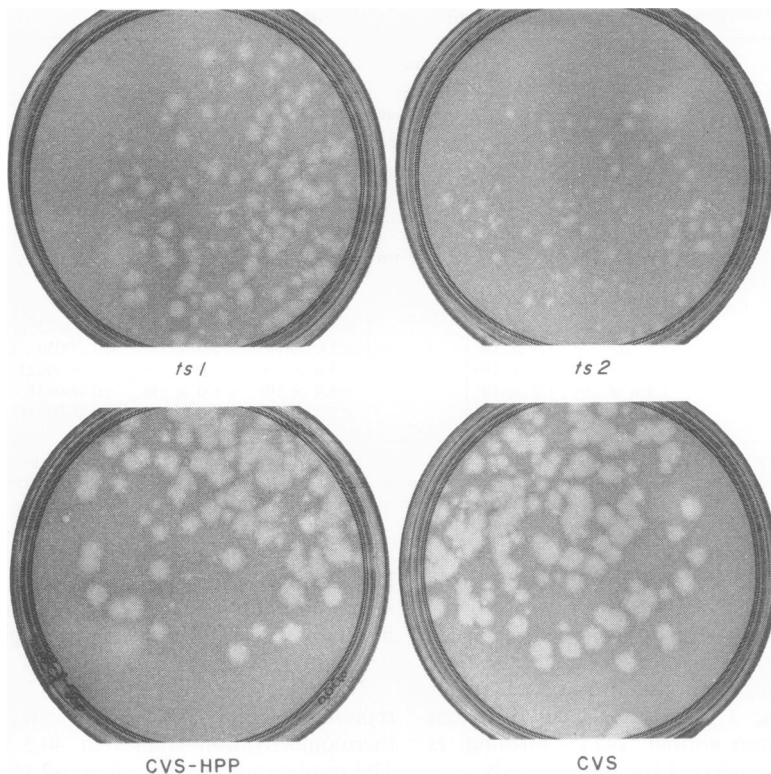


FIG. 1. Plaque morphology of mutant *ts1* and *ts2* rabies viruses, of the parental CVS-HPP rabies substrain, and of wild CVS virus.

TABLE 2. Temperature sensitivity of *ts1* and *ts2* virus after undiluted serial passage in BHK-21 cells at permissive temperature^a

Passage level	<i>ts1</i> Virus yield (PFU/ml)		<i>ts2</i> Virus yield	
	33 C	40.5 C	33 C	40.5 C
3	1.6×10^7	$<5.0 \times 10^0$	5.5×10^7	$<5.0 \times 10^0$
4	1.8×10^8	2.0×10^1	2.0×10^7	$<5.0 \times 10^0$
5	1.2×10^8	5.5×10^1	1.4×10^7	$<5.0 \times 10^0$
6	2.0×10^8	$<5.0 \times 10^0$	1.5×10^8	$<5.0 \times 10^0$
7	1.6×10^8	10^1	1.5×10^8	$<5.0 \times 10^0$
8	1.9×10^8	5.0×10^0	1.2×10^8	$<5.0 \times 10^0$
9	1.3×10^8	1.5×10^1	2.9×10^7	$<5.0 \times 10^0$
10	6.0×10^6	1.5×10^6	5.0×10^6	1.2×10^3

^a Techniques for the performance of temperature sensitivity determinations are described in Table 1. In the above experiments, only 48-hr virus yields were considered.

infected with *ts2* virus were delayed from 4 to 10 days. When mice surviving *ts2* infection were challenged with CVS virus (110 LD₅₀ inoculated intracerebrally, 28 days after the *ts2* inoculation), all animals receiving doses of *ts2* virus of 160 PFU or greater were protected. The 50% protective dose of *ts2* virus was 23 PFU.

To determine whether mouse deaths were the

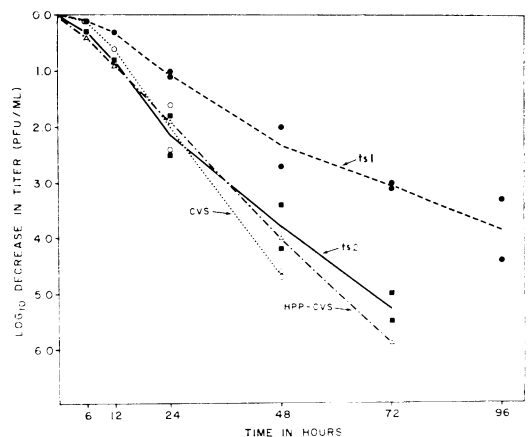


FIG. 2. Thermal inactivation of CVS rabies virus and of *ts* mutants *ts1* and *ts2* at 40.5 C. All viruses suspended in BME. IBA. Data from several experiments are included.

result of mutant or of revertant virus, virus was recovered from the brains of representative mice dying after infection with *ts1* or *ts2* virus. (Because of very low virus titers in mouse brains, brain isolate stocks had to be prepared in BHK-21

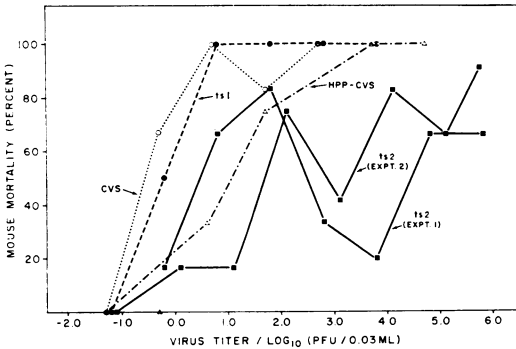


FIG. 3. Pathogenicity of CVS rabies virus and *ts* mutants for weanling mice. Groups of 6 to 12 mice were inoculated intracerebrally with 10-fold dilutions of virus and observed for 28 days after inoculation.

cells by the same methods used for preparation of stocks from plaques, prior to performance of temperature sensitivity determinations.) Virus from three of the six mice that died of *ts1* virus infection retained the *ts* mutant characteristic, whereas virus from three others was of revertant nature. Virus from three of the four mice that died of *ts2* virus infection was revertant with regard to both temperature sensitivity and plaque-size characteristics; virus from the fourth mouse retained both the *ts* and small-plaque characteristics of the inoculum.

DISCUSSION

We have described the selection of five *ts* mutants from a total of 161 clones of populations of CVS strain rabies subjected to mutagenesis by nitrous acid, 5-FU, or 5 AzaC. The importance of chemical mutagenesis for induction of the *ts* state was not determined.

Each of the five rabies *ts* mutants detected has exhibited no measurable "leakiness" at the non-permissive temperature. The incidence of reversion to the non-*ts* state has been low, and large stocks of *ts* mutant virus were prepared without difficulties. This experience with the rabies mutants differs from the general experience with *ts* mutants of other viruses (1) but parallels observations of *ts* mutants of the morphologically similar vesicular stomatitis virus described by Flamand (2, 3) and by Pringle (*In The biology of large RNA viruses*, Academic Press Inc., *in press*).

In addition to temperature sensitivity, mutant *ts1* exhibited increased thermostability at 40.5 C, whereas mutant *ts2* induced abnormally small plaques in BHK-13S cells and a sharply reduced incidence of lethal infections in mice. Mutants with known multiple markers should provide

valuable new tools for biological studies of the rabies virus. The only *in vitro* marker previously reported for a fixed rabies strain is the RCT 40-character found in a rabies strain adapted to growth at 26 to 28 C by Selimov and Nikitina (16). Whether the multiple markers of the *ts1* and *ts2* mutants represent multiple mutations or multiple manifestations of single-point mutations remains to be determined.

The behavior of small-plaque *ts* mutant *ts2* in mice is of particular interest. Although virus virulence *in vivo* has not usually been a primary concern of those studying *ts* mutants, reduced virulence has been described for chemically induced *ts* mutants of Semliki Forest disease virus (1) and influenza virus (9, 12). Reduced pathogenicity *in vivo* has also been described in the case of small-plaque mutants of foot-and-mouth disease virus induced by hydroxylamine (11), small-plaque mutants of Newcastle disease virus induced by nitrous acid (4), and a small-plaque mutant of vesicular stomatitis virus (VSV) induced by ultraviolet irradiation (17). However, to our knowledge, a pattern of pathogenicity such as that caused by *ts2* virus, in which virus-specific deaths and survivors both occur in animals inoculated with doses varying from 1 to 10⁶ PFU, has not previously been described. The explanation for this unusual death pattern is not clear; it is tempting to postulate a situation in which death occurs only if revertant virus appears in lethal amounts before multiplication of mutant virus induces protective immunity. Preliminary studies have revealed that the brains of three of the four mice dying after *ts2* infection contained revertant virus, whereas a fourth contained *ts* virus. The three mice yielding non-*ts* virus died after a near-normal incubation period of 10 days; the mouse yielding *ts* virus died only after a very prolonged incubation period of 28 days. The possibility that *ts2* virus may be mutant in a character critical for rabies pathogenesis will be further explored in the hope that information may be obtained concerning the unresolved question of how rabies virus causes death.

As noted above, rabies virus is the second virus of the rhabdovirus group from which *ts* mutants have been described. *ts* Mutants of VSV have been obtained in large numbers from virus stocks subjected to chemical mutagenesis and an incidence of 0.9 to 5.0% *ts* mutants in wild VSV preparations has been estimated (3, 4, 14). Complementation between appropriate *ts* mutants of VSV has been demonstrated to be unusually efficient, and recombination has been demonstrated. It has been possible to place the majority of VSV *ts* mutants within one of either four (14) or five (3) complementation groups. An adequate

comparison of the genetic properties of rabies virus with those of VSV will require: (i) determination that the known rabies mutants represent single-point mutations, (ii) performance of tests for complementation and recombination, and (iii) the isolation of many more *ts* rabies mutants, an accomplishment which will undoubtedly require the development of new and more efficient means for screening rabies virus clones for the presence of the *ts* condition.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Maria-Elvira Soriano.

This investigation was supported by Public Health Service Research Grants R01-AI 02954 and R22-AI 07988 from the National Institute of Allergy and Infectious Diseases and by funds from the World Health Organization.

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