JOURNAL OF VIROLOGY, July 1971, p. 56-61

Vol. 8, No. 1
Printed in U.S.A.

Preliminary Physiological Characterization of Temperature-Sensitive Mutants of Vesicular Stomatitis Virus

C. R. PRINGLE AND I. B. DUNCAN
Medical Research Council Virology Unit, Institute of Virology, Glasgow, Scotland

Received for publication 5 March 1971

Different temperature-sensitive mutants of vesicular stomatitis virus have been characterized in terms of their ability to induce synthesis of viral ribonucleic acid (RNA) in BHK-21 cells at 39 C (the restrictive temperature for these mutants). Mutants belonging to complementation groups I and IV (and probably II) did not induce actinomycin-resistant RNA synthesis in infected cells incubated at 39 C. All three mutants comprising complementation group III induced viral RNA synthesis at 39 C. The temperature sensitivity of the defective viral functions has also been studied by temperature-shift experiments. The functions associated with the mutants of groups I, II, and IV were required early, whereas the function associated with the group III mutants was not required until a late stage of the viral cycle. The heat sensitivity of extracellular virion was not correlated with complementation group.

The isolation and genetic characterization of temperature-sensitive (*ts*) mutants of vesicular stomatitis virus (VSV) type Indiana have been described (5, 6). A total of 210 *ts* mutants have now been classified into four mutually complementing groups. Complementation between mutants in different groups was efficient and unambiguous, whereas no complementation was observed with any combination of several mutants within the same group. Thus, the four groups of mutants are clearly demarcated genetically. It is unlikely, however, that the mutants in each group are all genetically identical, since they were isolated separately from a number of wild-type clones and some show phenotypic variation in respect to plaque morphology. The different mutants also differ in heat sensitivity of the extracellular virion, and this property will be considered in relation to complementation group.

The genetic data alone are not sufficient to decide whether these complementation groups correspond to individual cistrons or represent a different level of organization of the viral genome. We have begun, therefore, to characterize these *ts* mutations in physiological terms, examining first the temperature sensitivity of the defective functions and the ability of the mutants to induce actinomycin-resistant ribonucleic acid (RNA) synthesis at the restrictive temperature.

MATERIALS AND METHODS

Cells. BHK-21, clone 13 cells were used for virus propagation and assay according to established procedures (4).

Virus. The origin and genetic characteristics of the *ts* mutants used in these experiments have been described previously (5, 6). Mutants *ts* 11, *ts* 17, *ts* 18, *ts* 19, *ts* 115, *ts* 116, *ts* 117, *ts* 118 and *ts* 41, *ts* 42, *ts* 43, *ts* 44, *ts* 45, *ts* 46, and *ts* 48 were arbitrarily chosen as representative of complementation groups I and IV, respectively. Groups II and III were represented in toto by *ts* 21 and *ts* 22 and *ts* 31, *ts* 32, and *ts* 33, respectively. Mutants *ts* 32 and *ts* 33, which have not been described before, were obtained by the selective procedure described in this communication.

Estimation of viral RNA synthesis. Semiconfluent monolayers of BHK-21 cells in 30-ml bottles were prepared by incubating 2.5×10^6 cells at 37 C for 24 hr. Actinomycin D (2.5 μg/ml) was added to each bottle, and the bottles were incubated at 37 C for 1 hr. This reduced incorporation of ^{3}H-uridine at 4 hr to 1.1 to 1.3% of the value in untreated cells. The monolayers were then infected at an approximate multiplicity of 10 plaque-forming units (PFU)/cell and held at 4 C for 30 min. A 3-ml amount of chilled Eagle's medium containing 10% calf serum and 5 μCi of ^{3}H-uridine (20 to 31 Ci/mmole; Radiochemical Centre, Amersham, England) was added to each bottle, and the bottles were placed in a water bath at 39 C.

Bottles were removed at 0, 4, 8, and 12 hr to provide sequential samples for measurement of ^{3}H-uridine incorporation. A 0.3-ml amount of 20% (w/v) sodium dodecyl sulfate was added to each bottle, and the bottles were held at 37 C for 15 min.

An equal volume of 90% (v/v) redistilled phenol was added, and the bottles were shaken mechanically for 15 min at room temperature. The phases were separated by centrifugation, and a 50-μliter sample from the aqueous phase was pipetted onto a 2.5-cm paper filter disc (Whatman no. 1, qualitative grade). The filter discs were dried, washed twice in cold 20% (w/v) trichloroacetic acid and twice in ethanol, rinsed in ether, and air-dried. The discs were placed in 5 ml of scintillation fluid [0.4% (w/v) 2,5-diphenyloxazole, 0.02% (w/v) 1,4-di-2-(5-phenyloxazolyl) benzene in toluene], and the radioactivity was measured by using an ABAC SL 40 scintillation spectrometer (Intertechnique Ltd., Portslade, Sussex, England).

Temperature-shift experiments. Two mutants from each group were investigated to determine the temperature-sensitive period of the viral growth cycle. Replicate monolayers in 30-ml bottles were infected at a multiplicity of 1 and placed at 4 C for 30 min for adsorption. The inoculum (0.2 ml) was washed off with two changes of 4 ml of incubation medium. The bottles were then incubated totally immersed in a precision water bath (Lauda, $\pm$0.05 C) at 31 or 39 C. Pairs of bottles were transferred from 31 to 39 C (shift-up) or from 39 to 31 C (shift-down) at hourly intervals for as long as 8 hr, when all cultures were rapidly frozen. In addition at each hour pairs of bottles were removed from the water baths at 31 and 39 C and rapidly frozen to provide controls. Incubation was stopped at 8 hr, a period equivalent to twice the latent period, to avoid complications from a second cycle of infection.

Thermal inactivation. Crude virus preparations were diluted 10-fold into phosphate-buffered saline (PBS) containing 10% calf serum and exposed at 50 C in screw-capped bottles totally immersed in a water bath. Duplicate samples were transferred to iced water after periods of exposure at 50 C and diluted into cold PBS. Residual infectivity was assayed by plaque counting.

RESULTS

Ability to synthesize RNA at the restrictive temperature. Several mutants from each complementation group were examined for the ability to synthesize RNA at the restrictive temperature. Experiments showed that actinomycin-resistant RNA synthesis could be demonstrated adequately by infecting 5×10^6 cells with 5×10^7 PFU and phenol-extracting total labeled RNA. Figure 1 illustrates experiments with mutants from each of the four complementation groups. Only mutant *ts* 32 was capable of inducing RNA synthesis. In the case of the other three mutants (*ts* 11, *ts* 22, and *ts* 48), the rate of incorporation of ^{3}H-uridine in infected cells was no greater than in actinomycin-treated uninfected cells. The data presented in Table 1 were obtained in similar experiments and represent the level of ^{3}H-uridine incorporation at 12 hr or earlier, whichever was the greatest. In the case of groups I, III, and IV, all mutants within the same group have identical RNA phenotypes. Six of 7 mutants chosen at random from the 177 mutants in group I failed to induce any actinomycin-resistant RNA synthesis. The seventh (*ts* 17) showed a low level of RNA synthesis which may have been real or due to the presence of wild-type (ts^+) revertants. Similarly, 5 mutants from the 22 mutants in group IV showed no evidence of viral RNA synthesis.

The three mutants which comprise group III were each able to induce actinomycin-resistant synthesis. In two cases, the level of ^{3}H-uridine incorporation exceeded the wild-type level.

Group II contains only two mutants. One of these, mutant *ts* 22, was unable to induce viral RNA synthesis. The other, mutant *ts* 21, has given contradictory results. One subclone of this mutant showed no signs of viral RNA synthesis, whereas another subclone was able to induce RNA synthesis. Mutant *ts* 21 is genetically unstable, and the RNA synthesis observed could have been due to the presence of ts^+ revertants ($\sim 10^{-3}$). In another experiment, cells infected with *ts* 21, subclone *a*, were pulse-labeled with 8 μCi of ^{3}H-uridine per ml from 6 to 7.5 hr postinfection. The level of incorporation of ^{3}H-uridine at 7.5 hr was 1.4% of the ts^+ level, whereas incorporation in *ts* 33-infected cells in the same experiment was 111% of the ts^+ level. The group II mutants, therefore, have been classified tentatively as RNA-negative mutants. The discrepancy has not been entirely resolved, however, as mutant *ts* 22 is not appreciably more stable genetically than *ts* 21.

Temperature-shift experiments. Figures 2 to 5 contain results of temperature-shift experiments with mutants *ts* 11, *ts* 22, *ts* 32, and *ts* 42 representing complementation groups I, II, III, and IV, respectively. In each figure, the "shift-up" data are presented as the 8-hr yields from cultures transferred from 31 to 39 C at hourly intervals for as long as 8 hr. The "shift-down" data are presented likewise as 8-hr yields from cultures transferred at hourly intervals from 39 to 31 C. Each figure consists of data from a single experiment and includes plots of the titer at each transfer time in cultures maintained wholly at 31 or 39 C.

The "shift-down" profiles illustrate the effect of increasing periods of exposure at 39 C on the final yield. Mutants *ts* 11 (Fig. 2) and *ts* 22 (Fig. 3) were immediately affected, whereas *ts* 42 (Fig. 5) was unaffected by exposure for at least 1 hr and *ts* 32 (Fig. 4) for at least 5 hr at 39 C.

Mutants *ts* 11 and *ts* 22, however, are distin-

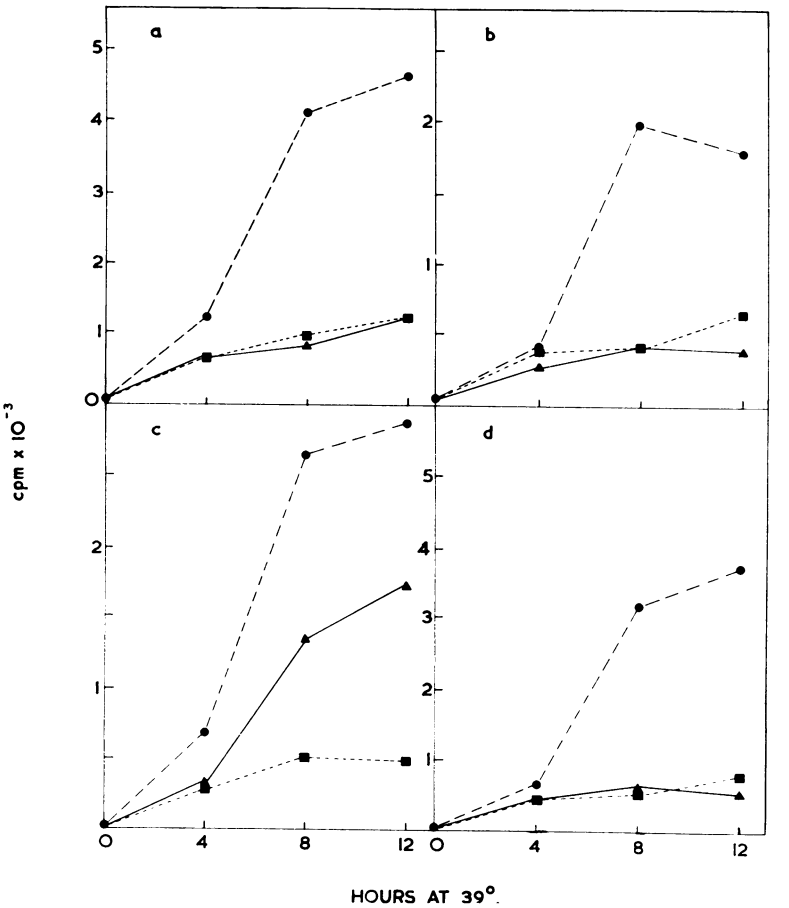

FIG. 1. *Actinomycin-resistant RNA synthesis in ts mutant-infected BHK-21 cells at 39 C. (a) Mutant ts 11; (b) mutant ts 22; (c) mutant ts 32; (d) mutant ts 48. Symbols: ■, uninfected cells; ●, wild-type infected cells; ▲, ts mutant infected cells.*

guishable by their "shift-up" profiles. The 8-hr yields from *ts* 11-infected cultures transferred to 39 C up to 4 hr after adsorption were no greater than the yields obtained from cultures harvested at the time of temperature shift. The 8-hr yields from *ts* 22-infected cells, on the other hand, exceeded the infectivity present at all times of transfer.

One other mutant from each complementation group (*ts* 16, *ts* 21, *ts* 31 and *ts* 41) has been investigated, and the results were similar to those obtained with their group counterpart. The profiles illustrated are probably characteristic for each of the four complementation groups.

Heat sensitivity of extracellular virus. The rate of thermal inactivation of these mutants at 39 C was not markedly different from that of the wild type. At 50 C, however, differences in heat sensitivity were apparent. Figures 6 and 7 illustrate that heat sensitivity is not correlated with complementation group. Six mutants from group IV (Fig. 6) showed two rates of thermal inactivation, whereas six mutants from group I (Fig. 7) exhibited a range of heat sensitivities. The rate of inactivation of wild-type virus is similar to that of *ts* 11 or *ts* 42. It should be noted that both heat-sensitive (*ts* 16 and *ts* 41) and heat-resistant mutants (*ts* 11 and *ts* 42) of groups I and IV were used in the temperature-shift experiments without affecting the characteristic profile obtained.

DISCUSSION

The RNA phenotype of these *ts* mutants of VSV agrees closely with their genetic classification by complementation test. Mutants belonging to groups I and IV are defective in RNA synthesis. The threshold of detection of RNA synthesis in these experiments was approximately 0.2 to 0.9%

TABLE 1. *Ability to synthesize RNA at 39 C*

Complementation group	Mutant	Clone	Incorporation of ^{3}H-uridine as percentage of *ts*$^+$ level[a]	RNA phenotype
I	*ts* 11	a	<0.8	−
	ts 11	a	<0.3	
	ts 17	a	3.6	(−)
	ts 18	a	<0.9	−
	ts 19	a	<0.9	−
	ts 115	a	<0.2	−
	ts 115	b	<0.8	
	ts 116	a	<0.8	−
	ts 117	a	<0.2	−
	ts 118	a	<0.3	−
	ts 118	b	<0.3	
II	*ts* 21	a	29.6	+
	ts 21	a	62.0	
	ts 21	a	21.2	
	ts 21	b	<0.3	−
	ts 21	b	<0.3	
	ts 22	a	<0.6	−
	ts 22	a	<0.6	
III	*ts* 31	a	113.6	+
	ts 31	a	29.0	
	ts 32	a	49.0	+
	ts 33	a	328.4	+
IV	*ts* 41	a	<0.8	−
	ts 41	b	<0.3	
	ts 42	a	<0.8	−
	ts 43	a	<0.2	−
	ts 44	a	<0.9	−
	ts 45	a	<0.8	−
	ts 46	a	<0.9	−
	ts 48	a	<0.3	−

[a] < indicates that ^{3}H-uridine incorporation was less than in uninfected actinomycin D-treated cells.

of wild-type synthesis. Increasing the actinomycin concentration to 4 μg/ml (continuously present) lowered the background of host cell RNA synthesis from 1.2 to 0.3% of normal synthesis without revealing any evidence of viral RNA synthesis. Higher concentrations of actinomycin D caused macroscopic cellular damage within 12 hr. Since these mutants complement efficiently, the temperature-sensitive step must be subsequent to adsorption and may be concerned with the initiation of infection, synthesis of messenger RNA, or replication of the viral genome. The "shift-down" experiments showed that the defective functions are required early but are not identical.

Any mutation involving the virion-associated transcriptase of VSV (1) should produce an abortive infection. Mutants of this type should be genetically stable because the probability of back-mutation would be minimal in the absence of replication. Mutant *ts* 11, for instance, produces an abortive infection at 39 C, and cultures can be "cured" of infection by incubation at 39 C for 96 hr (6). Work is in progress to examine the in vitro

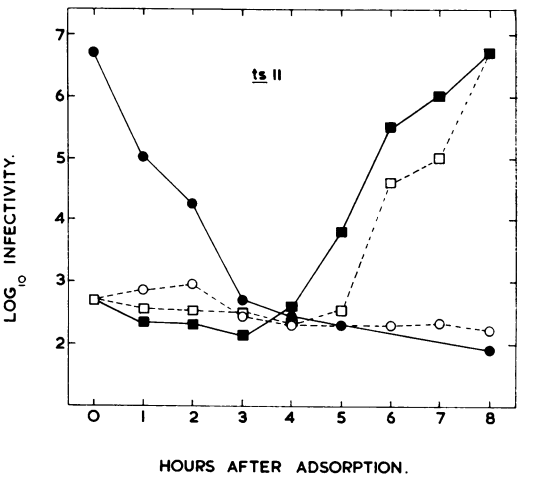

FIG. 2. *Mutant ts 11 (group I) temperature-shift experiment. The "shift-up" profile (■) represents the yield at 8 hr from duplicate cultures transferred from 31 to 39 C at hourly intervals for as long as 8 hr. The "shift-down" profile (●) represents the 8-hr yields from cultures transferred from 39 to 31 C at hourly intervals for as long as 8 hr. The titers in cultures maintained at 31 C (□) or 39 C (○) are superimposed to indicate the infectivity present at each time of transfer in the shift-up and shift-down cultures, respectively.*

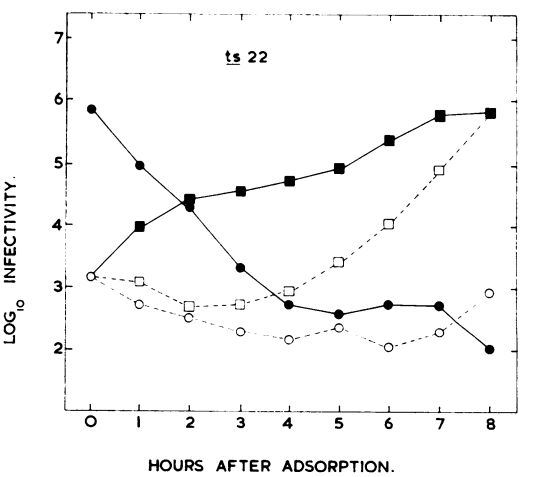

FIG. 3. *Mutant ts 22 (group II) temperature-shift experiment. Symbols as in Fig. 2.*

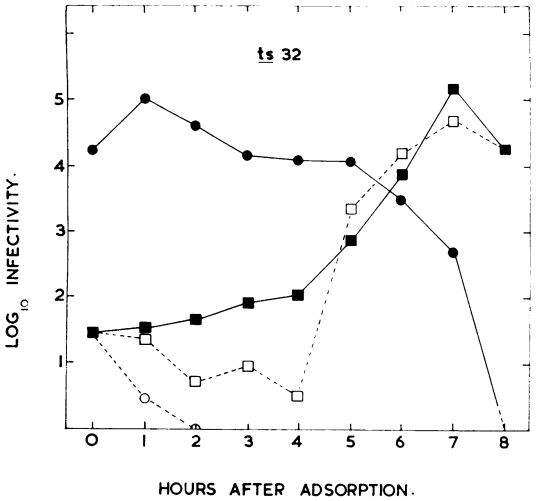

FIG. 4. *Mutant ts 32 (group III) temperature-shift experiment. Symbols as in Fig. 2.*

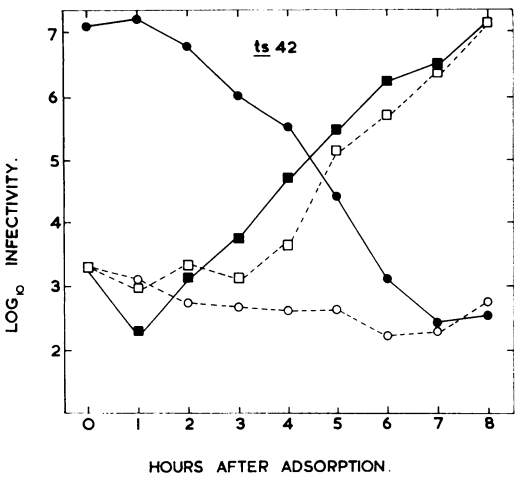

FIG. 5. *Mutant ts 42 (group IV) temperature-shift experiment. Symbols as in Fig. 2.*

temperature sensitivity of the virion transcriptase of different mutants.

Mutant *ts* 22 (complementation group II) was also deficient in RNA synthesis, and the "shift down" experiment showed that the defective function was required early. The "shift-up" profile, however, differentiates it from the functions associated with the mutants of groups I and IV. The other mutant in group II, *ts* 21, was able to induce RNA synthesis at 39 C under some circumstances. This difference in RNA phenotype within the same complementation group could represent a defect in nucleoprotein synthesis, such that in one case (*ts* 21) nascent RNA molecules were protected from endogenous nuclease activity, whereas in the other they were not.

Three groups of RNA-negative mutants have also been recognized in the heterotypic New Jersey M strain (Pringle, Duncan, and Stevenson, *in preparation*).

The mutants belonging to complementation group III all have RNA-positive phenotypes. This together with the temperature-shift data suggests that the temperature-sensitive defect involves a structural protein or maturation factor. The origin of these mutants (Table 2) supports this con-

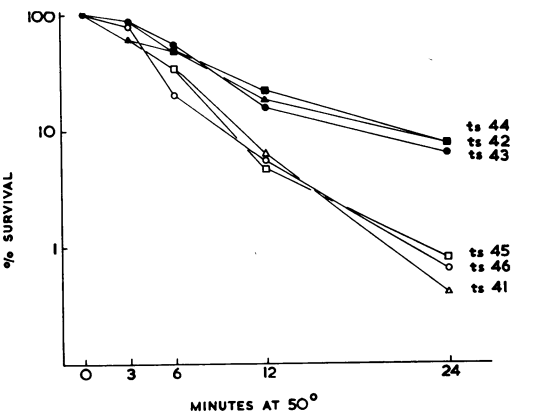

FIG. 6. *Thermal inactivation at 50 C of six group IV mutants.*

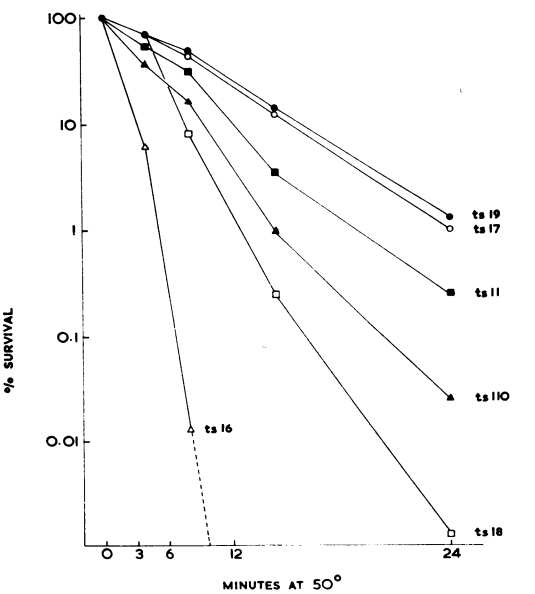

FIG. 7. *Thermal inactivation at 50 C of six group I mutants.*

TABLE 2. *Frequency of mutants of the four complementation groups among ts mutants of vesicular stomatitis virus isolated from mutagenized stock, with and without selection*

Isolation procedure	Complementation group					Total
	I	II	III	IV	Undetermined	
Unselected....	155	2	1	15	6	179
Late function selection.....	22	0	2	7	0	31
Total.....	177	2	3	22	6	210

clusion; the frequency of group III mutants among *ts* clones was $1/179$ in the absence of selection. If "late" function mutants were selected by incubating mutagenized virus at 39 C for 4 hr followed by 4 hr at 31 C, the frequency of isolation of group III mutants was increased more than 10-fold (2/31).

The heat sensitivity of the mutant virions does not correlate with complementation group. These *ts* clones may harbor additional mutations affecting structural components of the virion, although the isolation procedure would have ensured that the *ts* phenotype was determined by single-site mutations (5). It is possible, however, that all four groups of mutants affect structural components or even a single component such as the virion transcriptase. Work is now in progress to characterize further these mutants in terms of their ability to induce viral protein synthesis at 39 C.

Recently it has been shown that these induced *ts* mutants are genetically homologous with spontaneous *ts* mutants isolated in chick embryo cells from a different wild-type strain of VSV (2). The results of Lafay (3) indicate that the RNA phenotypes of the spontaneous mutants are similar to those of the corresponding induced mutants, except in the case of the group II mutants. This discrepancy may arise from the genetical instability of these particular mutants. We have also confirmed that *ts* 45 (Orsay), representing complementation group V (2), behaves as an RNA-positive mutant in BHK-21 cells.

ACKNOWLEDGMENTS

We thank Mary Stevenson for excellent technical assistance and J. H. Subak-Sharpe for valuable discussion.

One of us (I. B. Duncan) is in receipt of a Medical Research Council Studentship.

LITERATURE CITED

1. Baltimore, D., A. S. Huang, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Nat. Acad. Sci. U.S.A. 66:572–576.
2. Flamand, A., and C. R. Pringle. 1971. The homologies of spontaneous and induced temperature-sensitive mutants of vesicular stomatitis virus isolated in chick embryo and BHK-21 cells. J. Gen. Virol. 11:81–85.
3. Lafay, F. 1969. Etude des mutants thermosensibles du virus de la stomatite vésiculaire. Classification de quelques mutants d'après des critères de functionnement. C. R. Acad. Sci. 268:2385.
4. Macpherson, I. A., and M. G. P. Stoker. 1962. Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. Virology 16:147–151.
5. Pringle, C. R. 1970. The induction and genetic characterisation of conditional lethal mutants of vesicular stomatitis virus, p. 567–582. *In* The biology of large RNA viruses. Academic Press Inc., New York.
6. Pringle, C. R. 1970. Genetic characteristics of conditional lethal mutants of vesicular stomatitis virus induced by 5-fluorouracil, 5-azacytidine, and ethyl methane sulfonate. J. Virol. 5:559–567.