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Genetic Characteristics of Conditional Lethal Mutants of Vesicular Stomatitis Virus Induced by 5-Fluorouracil, 5-Azacytidine, and Ethyl Methane Sulfonate

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One hundred and seventy-five temperature-sensitive (*ts*) mutants of vesicular stomatitis virus (type Indiana-C) induced by 5-fluorouracil (FU), 5-azacytidine (ACR), and ethyl methane sulfonate (EMS) have been assigned to four complementation groups by a qualitative test. Group I contains 151 mutants; group II, 2 mutants; group III, 1 mutant; and group IV, 15 mutants; 6 are unclassified. FU was much more effective as a mutagen than either ACR or EMS. The proportion of the mutants belonging to groups I and IV, however, was similar in the case of all three mutagens. Fifteen mutants from groups I and IV have been used to obtain quantitative complementation data. Both groups appear to be homogeneous. Complementation yields increase with increasing multiplicity, but the number of particles per cell required to elicit maximal complementation is small. The pattern of genetic recombination parallels that of complementation. No recombination could be detected in crosses within group I (<0.001%) or group IV (<0.07%), whereas recombination (0.31 to 3.4%) was observed in crosses between groups I and IV. Recombination frequency did not increase with multiplicity above an input of 0.6 plaque-forming units per cell. Many group I mutants have very low reversion rates, and BHK 21 clone 13 cells infected with one of these mutants have been "cured" of infection by prolonged exposure at the restrictive temperature.

Temperature-sensitive (*ts*) mutants of vesicular stomatitis virus (VSV) can be obtained readily (1; C. R. Pringle, *In* The Biology of Large RNA Viruses, Academic Press Inc., *in press*; Howatson *personal communication*; Wagner, *personal communication*), and the isolation of 105 mutants induced by the mutagen 5-fluorouracil (FU) has been reported from this laboratory (Pringle, *in press*). Efficient complementation was observed between certain mutants, and the 105 mutants were assigned to 4 groups on the basis of a qualitative complementation test. Mutants from different complementation groups were found to undergo genetic recombination, whereas the limited data available suggested that mutants in the same group do not recombine, or do so only with very low frequency.

A total of 175 induced mutants has now been isolated and characterized. In the majority of cases, the mutagen employed was FU; in the remaining cases, the mutagen was the nucleoside analogue, 5-azacytidine (ACR), or the alkylating agent, ethyl methane sulfonate (EMS). The specificity of the three mutagens has been compared in terms of the frequency and complementation grouping of the mutants induced by them. In addition, several mutants from the two largest complementation groups have been studied in detail to obtain quantitative data concerning complementation and recombination. The results fully substantiate the preliminary classification deduced from qualitative tests.

MATERIALS AND METHODS

Cells. All virus propagation took place in BHK 21, clone 13 cells (4), by standard procedures.

Virus. The initial virus stock was an avirulent derivative of the Indiana-C strain, obtained from J. B. Brooksby of the Animal Virus Research Institute, Pirbright, Surrey. This wild-type (ts^+) virus was cloned by four successive isolations from monolayer cultures bearing single plaques.

Mutagenesis by EMS. A 0.1-ml amount of EMS (Koch-Light Ltd.) was added to 10 ml of 0.5 M sodium acetate and shaken for 2 min at 31 C. An equal volume of the virus sample was added, and the mixture was incubated at 31 C. Samples were withdrawn at intervals, and the reaction was stopped by 10-fold dilution into 1% (w/v) sodium thiosulphite in phosphate-buffered saline. The samples were assayed for infectivity after 2 hr at 4 C. Calf serum was added after 6 hr, before further storage at 4 C.

Mutagenesis by ACR and FU. Both ACR (Calbiochem, Los Angeles, Calif.) and FU (Koch-Light Ltd.) were dissolved in distilled water and diluted into Eagle's medium to give the appropriate concentration. Monolayers were infected at low multiplicity, and, after adsorption of virus, the inoculum was removed and Eagle's medium containing the analogue was added. The cultures were incubated at 31 C until maximum cytopathic effect was observed in control cultures incubated in parallel without mutagen. The treated cultures were then harvested, and calf serum was added to the culture fluids before storage at −20 C.

Isolation of mutants. Temperature-sensitive mutants were mainly isolated from monolayers showing single plaques. This procedure obviated the need for further cloning and, at the same time, provided an accurate estimate of mutant frequency, since no mutants were lost by contamination from adjacent wild-type plaques. For convenience, the mutants have been renumbered by the following convention. The first digit indicates the complementation group to which the mutant belongs, and the second identifies the individual mutant within the group. For instance, *ts* 11 is the initially isolated mutant of complementation group I. The four complementation groups are designated by Roman numerals.

Complementation. Ability to complement was measured quantitatively by infecting monolayer cultures of 3×10^6 to 5×10^6 cells in screw-cap bottles [30 ml (1 oz)] with pairs of mutants to give a combined multiplicity of approximately 10 (except where stated otherwise). The singly infected control cultures received half of this multiplicity. In all these experiments, the multiplicity of infection is given as the ratio of plaque-forming units (PFU) per cell, not as the adsorbed multiplicity. After 20 min of adsorption at 31 C, the cultures were washed twice with 4 ml of medium and then 4 ml of Eagle's medium containing 1% calf serum was added. The infected monolayers were subsequently incubated for 7.5 hr (a period equivalent to twice the length of latent period) and completely immersed in a water bath at 39 ± 0.05 C. The cultures were then rapidly frozen by immersion in an ethanol-solid carbon dioxide bath and stored at −20 C. Complementation was measured as the ratio [yield of $(x + y)$ titrated at 31 C − yield of $(x + y)$ titrated at 39 C]/[yield of (x) titrated at 31 C + yield of (y) titrated at 31 C] where x and y are any pair of mutants. This value is referred to as the *complementation index* in this paper. All mixed and single infections were duplicated, and the data in Tables 4–6 and Fig. 5 were obtained in single experiments. Statistical significance was tested by a Student t test with one degree of freedom.

Qualitative complementation tests were carried out by infecting monolayers in petri dishes with a 10^{-3} dilution of the sample and standard dilutions of representative mutants of the four groups, with the appropriate controls. The cultures were scored for presence or absence of cytopathic effect after 48 hr of incubation at 39 C.

Recombination. Experiments to detect genetic recombination were set up in the same way as the complementation experiments, except that the multiplicity in the singly infected controls was approximately 10. After adsorption and washing as described above, the infected monolayers were incubated at 31 C for 8 hr (twice the length of the latent period). The cultures were then rapidly frozen and stored at −20 C. Recombination frequency between any two mutants x and y was measured as the ratio [yield of $(x \times y)$ titrated at 39 C − 1/2 yield of (x) titrated at 39 C + yield of (y) titrated at 39 C)]/[yield of $(x \times y)$ titrated at 31 C] × 100.

All crosses were duplicated, and the data in Tables 8–10 and Fig. 6 were obtained in single experiments.

RESULTS

Properties of the mutants. *ts* Mutants obtained by chemical mutagenesis show considerable variation in such properties as plaque morphology, stability, penetrance (leakiness), and thermal inactivation rate. These properties have not yet been investigated for all 175 mutants; however, Table 1 presents comparative data on the origin, plaque morphology, leakiness, and reversion rate of the 15 mutants used in the present series of experiments. Monolayer cultures of BHK cells were infected at a multiplicity of 1. After 20 min at 31 C for adsorption, the cultures were washed twice and then incubated for 8 hr at 39 C (the restrictive temperature). The *total yield* was calculated as the ratio [yield (x), titrated at 31 C]/[yield of ts^+, titrated at 31 C] × 100, where x is any mutant and ts^+ is the wild type. The *revertant yield* was calculated as the ratio [yield (x), titrated at 39 C]/[yield ts^+, titrated at 39 C] × 100. The *leak yield* was calculated as *total yield* minus the *revertant yield.*

The value presented as the leak yield includes a component contributed by uneclipsed virus, which will depend on the rate of eclipse for each mutant.

Table 1 shows that mutants belonging to group I tended to be very stable. Mutant *ts* 11 indeed apparently did not revert and cultures maintained at 39 C gradually lost the capacity to yield infectious virus (Fig. 1). Extrapolation of the line in Fig. 1 suggests that the yield would be 10^{-10} of the initial yield after 60 hr. Therefore, monolayers of 3×10^6 cells were infected with 10^9 PFU of mutant *ts* 11 and held at 39 C for 72 hr. No cytopathic effect was observed initially on transfer to 31 C, but virus with the mutant phenotype

TABLE 1. *Origin and characteristics of 15 representative ts mutants*

Complementation group	Mutant	Mutagen	Concn of mutagen (μg/ml)	Wild-type clone of origin	Plaque morphology	Eight-hour yields at 39 C as percentage of ts^+	
						Revertant yield[a]	Leak yield[b]
I	*ts* 11	FU	50	8/2	Minute	<0.001	0.001
	ts 16	FU	50	8/4	Small	<0.001	0.03
	ts 17	FU	50	8/4	Normal	<0.001	0.20
	ts 18	FU	50	8/21	Normal	<0.001	0.10
	ts 19	FU	5	13/157	Normal	0.03	0.18
	ts 110	FU	50	8/21	Minute	0.03	0.27
II	*ts* 21	FU	50	8/1	Small	0.002	0.73
III	*ts* 31	FU	50	8/4	Small	0.001	0.24
IV	*ts* 41	FU	50	8/2	Small	0.004	0.23
	ts 42	FU	50	8/4	Normal	0.013	0.15
	ts 43	FU	50	8/4	Normal	0.191	0.04
	ts 44	FU	50	8/4	Normal	0.060	0.49
	ts 45	FU	50	8/21	Normal	0.020	0.13
	ts 46	FU	50	8/4	Normal	0.025	0.10
	ts 47	ACR	5	16/5	Normal	ND[c]	
Wild type	ts^+			8/2	Normal		

[a] Plague-forming units at 39 C as a percentage of the wild-type yield at 39 C.
[b] Plague-forming units at 31 C as a percentage of the wild-type yield minus the revertant yield.
[c] Not done.

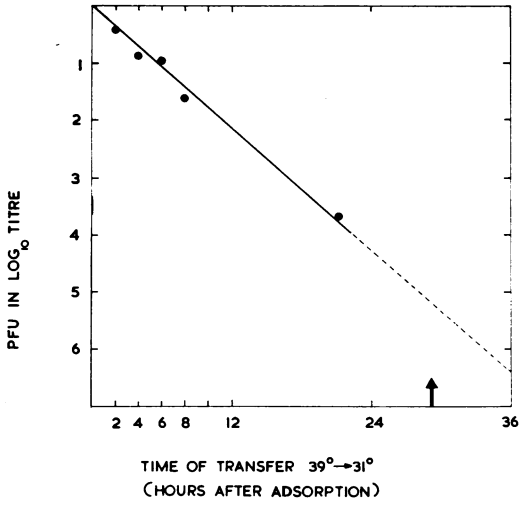

FIG. 1. *Decay of plaque-forming ability of ts 11-infected cells during incubation at 39 C. The arrow at 29 hr indicates cytopathic effect, but no plaque formation. The plates were stained and counted 48 hr after transfer to 31 C.*

subsequently reappeared at the second transfer and destroyed the cells. The experiment was repeated, increasing the period at 39 C to 120 hr, with medium (1% serum) changes every 48 hr. The mean number of cells obtained from the infected cultures at this time was 4.25×10^6, compared with 4.5×10^6 from the uninfected controls. No cytopathic effect was observed during five subsequent transfers, equivalent to approximately 20 cell generations, and no infectious virus was detected in the medium. The cultures were apparently "cured" of infection. Whether this "curing" represented a completely abortive infection, or was brought about by differential cell death, could not be determined directly because of the low plating efficiency of normal BHK cells at 39 C.

Mutagenesis by FU. Figure 2 shows the yield of infectious virus and the percentage of *ts* mutants isolated, relative to the concentration of the analogue. FU is a very efficient mutagen for induction of *ts* mutants of VSV. The curve in Fig. 2 suggests that the spontaneous frequency of *ts* mutants would be approximately 1%. However, isolation and direct screening of clones from untreated virus indicated that the frequency of spontaneous mutants was less than 0.9% (Pringle, *in press*). The curves obtained by assay at the permissive and restrictive temperatures are different, due in part to the high frequency of *ts* clones.

Mutagenesis by ACR. Figure 3 shows similar data for ACR. In this case, the curves obtained

by titration at permissive and restrictive temperatures did not differ and only the 31 C assay is shown. The mutagenicity of ACR is low in relation to its inactivating effect. From these data, the frequency of spontaneous mutants again could be estimated as approximately 1%.

Mutagenesis by EMS. Figure 4 shows that the mutagenic effect of EMS was also low relative to inactivation. The curves obtained by assay at 31 and 39 C did not differ, and only the 31 C data are shown. An estimate of the spontaneous mutant frequency cannot be made because of the inflexion in the inactivation curve, which is possibly due to the time required for penetration of the agent to the nucleoprotein core of the virus particle.

Classification of the mutants by qualitative complementation test. In Tables 2 and 3, the 175 mutants were classified by qualitative complementation test. Four complementation groups have previously been identified, with the majority of mutants falling into group I (Pringle, *in press*). In the case of EMS, which was a relatively poor mutagen, an attempt was made to obtain a larger sample of mutants by selection. Monolayers infected with mutagenized virus were incubated for 48 hr at 31 C, followed by 24 hr at 39 C. Small plaques were then isolated and examined. Table 2 shows that this procedure increased the isolation rate of *ts* mutants approximately threefold. This rather meager enrichment was not unexpected since the small plaques, representing presumptive mutants, had to be isolated from a background of normal plaque-forming ts^+ virus. Nevertheless, sufficient mutants were obtained to show that the spectrum of mutants induced by the three muta-

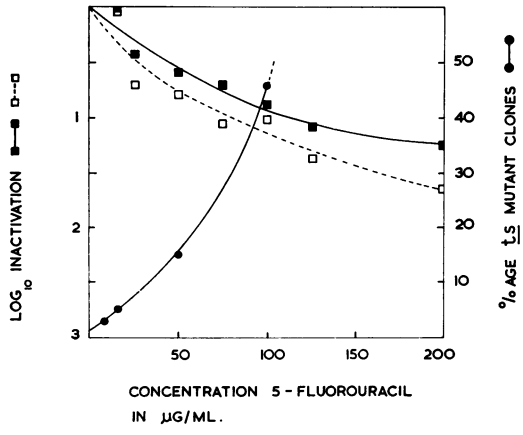

FIG. 2. *Induction of ts mutants by FU. Symbols:* ■, *titer (PFU/0.2 ml) at 31 C;* □, *titer at 39 C; and* ●, *percentage of ts mutant clones isolated.*

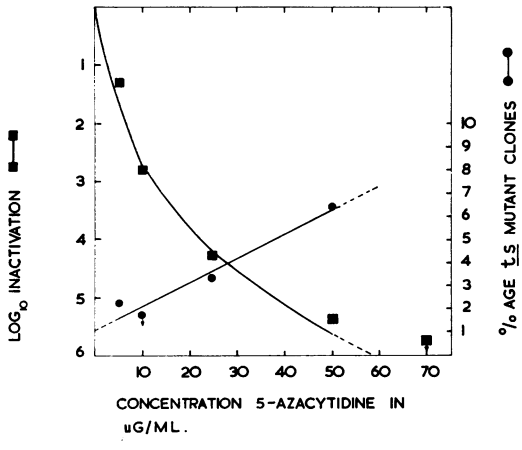

FIG. 3. *Induction of ts mutants by ACR. Symbols:* ■, *titer (PFU/0.2 ml) at 31 C; and* ●, *percentage of ts mutant clones isolated.*

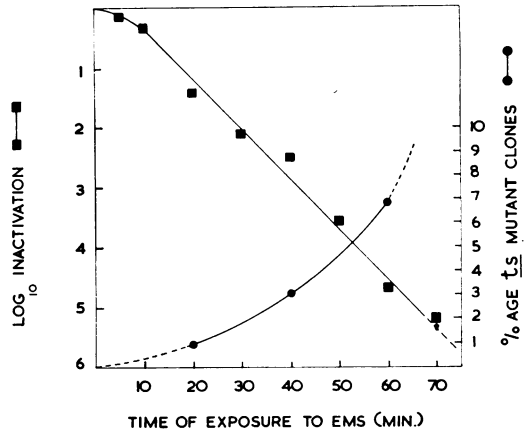

FIG. 4. *Induction of ts mutants by EMS. Symbols:* ■, *titer (PFU/0.2 ml) at 31 C; and* ●, *percentage of ts mutant clones isolated.*

TABLE 2. *Classification of EMS-induced mutants into complementation groups*

EMS[a] treatment	Total tested	Complementation group				Total per cent of *ts*
		I (no.)	II (no.)	III (no.)	IV (no.)	
Selected[b] 60 min	143	24	0	0	5	20.3
Unselected						
60 min	62	4	0	0	0	6.5
40 min	68	2	0	0	0	2.9
20 min	69	1	0	0	0	1.5

[a] Ethyl methane sulfonate.
[b] Selection of small plaques.

gens is qualitatively similar (Table 3). The majority of the mutants fall into group I or group IV. Six mutants did not complement and will be examined at another time. They probably represent multiple mutants, since they were all obtained after treatment with FU, the most potent of the three mutagens.

Complementation between mutants of groups I and IV. Table 4 contains the results of a complementation test carried out with six mutants of group I. There is no evidence of complementation (i.e., indices significantly greater than 1) in any of the 15 possible pairwise combinations of these mutants.

Table 5 contains the results of a complementation test carried out with six group IV mutants. The complementation indices observed do not differ significantly from unity. The complementation indices for mixed infections of any of these mutants and mutant *ts* 11 of group I (bottom line of Table 5) are significant ($P < 0.01$) and at least an order of magnitude greater.

Table 6 contains the results of complementation tests between six group I and six group IV mutants. Significant complementation ($P < 0.01$) was uniformly observed. The complementation indices are at least an order of magnitude greater than those observed in the control series of tests

TABLE 3. *Classification of ts mutants of vesicular stomatitis virus induced by 5-fluorouracil (FU), 5-azacytidine (ACR), and ethyl methane sulfonate (EMS) into complementation groups*

Mutagen	Complementation group								Unclassified	
	I		II		III		IV			
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
ACR	10	90.0	0	0	0	0	1	10.0	0	0
FU	109	85.2	2	1.6	1	0.8	10	7.8	6	4.7
EMS	32	88.9	0	0	0	0	4	11.1	0	0

TABLE 4. *Complementation within group I*[a]

Mutants	*ts* 11	*ts* 16	*ts* 17	*ts* 18	*ts* 19	*ts* 110
ts 11	(1.5×10^4)					
ts 16	1.02	($<10^2$)				
ts 17	0.30	<1.41	(10^3)			
ts 18	0.78	<0.10	1.41	(1.1×10^3)		
ts 19	0.78	<1.00	0.24	0.20	(1.7×10^2)	
ts 110	0.66	<1.00	<0.20	<0.20	<1.10	($<10^{-2}$)

[a] Values are mean complementation indices, calculated as described in Materials and Methods. The figures in parentheses are the yields in the single infections.

TABLE 5. *Complementation within group IV*[a]

Mutants	Group IV						Group I, *ts* 11
	ts 41	*ts* 43	*ts* 44	*ts* 45	*ts* 46	*ts* 47	
Group IV							
ts 41	(2.5×10^4)						
ts 43	0.89	(2.6×10^3)					
ts 44	1.98	0.83	(1.7×10^5)				
ts 45	1.63	1.26	2.30	(4.4×10^4)			
ts 46	0.59	0.59	0.49	0.34	(4.6×10^3)		
ts 47	2.70	0.52	4.97	4.37	0.45	(3.6×10^4)	
Group I							
ts 11	409	873	137	196	219	465	(1.2×10^4)

[a] Values are mean complementation indices calculated as described in Materials and Methods. The figures in parentheses are the yields in the single infections.

TABLE 6. *Complementation between mutants of groups I and IV*[a]

Mutants	Group I						
	ts 11	*ts* 16	*ts* 17	*ts* 18	*ts* 19	*ts* 110	Single infection yield
Group IV							
ts 41	389	252	547	396	176	150	4.8×10^3
ts 42	619	ND[b]	ND	ND	ND	ND	1.2×10^4
ts 43	295	35	89	321	131	28	1.7×10^3
ts 44	661	93	564	463	674	111	3.6×10^3
ts 45	240	48	287	290	376	166	5.1×10^3
ts 46	912	85	817	145	89	183	2.1×10^3
Group I							
ts 11	0.99	<0.10	1.07	0.40	0.45	0.51	
Single infection yield	10^2	2.5×10^2	3.2×10^3	3.2×10^3	3.2×10^3	6.0×10^2	

[a] Values are mean complementation indices, calculated as described in Materials and Methods.
[b] Not done.

included in this experiment involving the six group I mutants and *ts* 11.

Complementation and multiplicity of infection. Figure 5 illustrates the relation between total multiplicity of the parental complementing mutants (*ts* 11 and *ts* 21) and the yield under restrictive conditions. The yield in the mixed infection increased until the input multiplicity reached 6 PFU/cell, whereafter it levelled off. Only a small number of particles per cell, therefore, is required to elicit maximal complementation, and the increase in yield with multiplicity was probably due to the increasing number of cells infected by both mutants.

The high efficiency of complementation could be demonstrated by infecting cells at high multiplicity (>5) with *ts* 11, which forms no plaques at the restrictive temperature, and simultaneously superinfecting with any complementing mutant. Plaque formation always resulted after incubation at the restrictive temperature. Under these conditions, the plaque count often exceeded the control count of the superinfecting mutant at 31 C, indicating some rescue of genetic information in addition to normal complementation. Table 7 shows the results of titration of the same sample of mutant *ts* 21 on *ts* 11-infected monolayers at intervals after storage at −20 C. Rescue of genetic information was not observed in samples which had lost infectivity by prolonged storage at −20 C.

Recombination between mutants of groups I and IV. Table 8 contains the results of pairwise crosses of six mutants of group I in all combinations. The percentages represent the frequency of the wild-type class of recombinant. There is no evidence of recombination.

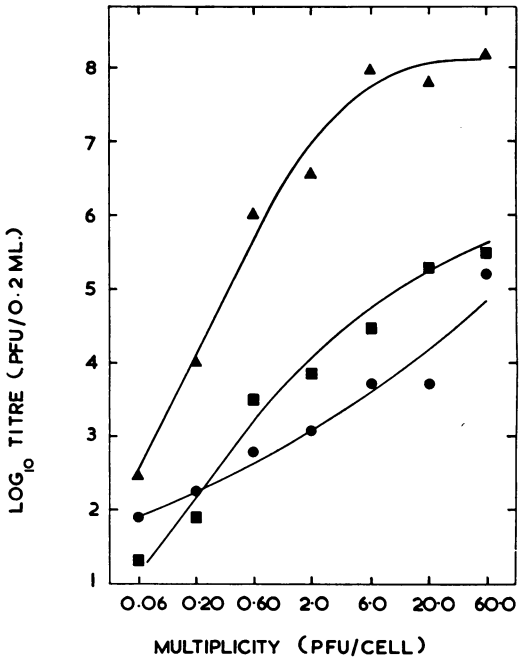

FIG. 5. *Relation between complementation yield and multiplicity of infection. Symbols:* ■, *yield of mutant ts 21 alone;* ●, *yield of mutant ts 11 alone; and* ▲, *yield from mixed infection of ts 11 + ts 21.*

Table 9 shows the results of pairwise crosses of five mutants of group IV in all possible combinations. Again there is no evidence of recombination. In this case, the higher reversion rate of the mutants reduces the resolution.

Table 10 contains the results of crosses of six mutants of group I and five mutants of group IV

TABLE 7. *Comparison of plaque count of ts 21 at 39 C in normal cells and cells infected with 40 plaque-forming units (PFU)/cell of ts 11, after periods of storage at −20 C*

Virus sample	Time of storage (months)	PFU at 31 C	PFU at 39 C	PFU at 39 C in presence of 40 PFU/cell of *ts* 11
ts 21	0	2.3×10^7	7.0×10^4	5.3×10^7
	1.5	4.0×10^6	5.4×10^4	1.2×10^7
	5.5	5.0×10^4	5.0×10^2	8.5×10^4
	7	1.2×10^5	3×10^2	6.0×10^4
	13	2.0×10^3	10^1	5.5×10^2
ts 11	0	1.1×10^8	$<10^{-1}$	$<10^{-1}$

TABLE 8. *Group I mutants; percentage of wild type at 31 C in mixed infection yields*

Mutants	Mutants					
	ts 11	*ts* 16	*ts* 17	*ts* 18	*ts* 19	*ts* 110
ts 11	<.0003					
ts 16	<.0004	<.030				
ts 17	<.0003	<.0001	.0029			
ts 18	<.0001	<.0005	.0004	<.0001		
ts 19	<.0011	<.0008	<.0010	<.0002	.0013	
ts 110	.013	.840	.054	.043	.028	.666

TABLE 9. *Group IV mutants; percentage of wild type in mixed infection yields at 31 C*

Mutants	Mutants				
	ts 41	*ts* 42	*ts* 43	*ts* 45	*ts* 46
ts 41	.003				
ts 42	.003	.014			
ts 43	.003	.019	<.200		
ts 45	.003	.012	.005	.020	
ts 46	.002	.026	.069	.004	.018

in all possible combinations. Statistically significant ($P < 0.01$) evidence of recombination was obtained in every cross. The data do not suggest any obvious genetic linearity.

In view of the efficiency of complementation, it was possible that a proportion of the "recombinant" plaques observed at 39 C resulted from complementation in cells infected with virions of both parental genotypes. Reconstruction experiments were carried out in which monolayers were infected with a 1:1 mixture of *ts* 11 and *ts* 41. No plaques appeared (as a result of complementation) until the input multiplicity exceeded 10^4 PFU/10^6 cells. Consequently, recombination could be unambiguously resolved from complementation down to a frequency of 0.01%, at least, and the proportion of plaques due to complementation in these recombination experiments between group I and IV mutants was negligible.

Recombination and multiplicity of infection. Figure 6 illustrates the relation between total multiplicity of infection and frequency of the wild-type recombinant class in the progeny of a cross of mutants *ts* 11 and *ts* 21. The frequency of the recombinants was almost constant above an input multiplicity of 0.6 PFU/cell, in contrast to previous findings with foot-and-mouth disease virus in BHK cells (5), where frequency increased with multiplicity. The frequency of the wild-type recombinant observed in this experiment was approximately 0.48%, which is similar to the previous estimate of 0.58% (Pringle, *in press*).

DISCUSSION

The quantitative complementation results confirm the grouping arrived at by the earlier qualitative test. Groups I and IV which contain the majority of the mutants are apparently homogeneous. The homogeneity of group II, containing two mutants, and group III, containing one mutant, cannot yet be assessed.

In general, the results of the recombination experiments agree with the complementation data. There is no evidence of close linkage between the group I and IV mutants tested, a finding which could have been expected in view of the small size of the genome of VSV. The absence of linkage between mutants in different complementation groups suggests that the functional units of the viral genome (cistrons) may be physically discrete.

TABLE 10. *Percentage of wild-type recombinant virus in yields from mixed infection of mutants of groups I and IV at 31 C*

Mutants	Mutants						
	ts 11	*ts* 16	*ts* 17	*ts* 18	*ts* 19	*ts* 110	Single infection yield
ts 41	3.40	0.82	1.16	1.49	1.10	0.77	<0.01
ts 42	0.63	0.70	0.77	0.82	0.33	0.32	0.04
ts 44	0.49	0.31	0.62	0.81	0.71	1.41	0.02
ts 45	1.75	1.19	0.93	1.37	1.83	1.64	0.35
ts 46	0.84	0.94	1.23	1.31	1.39	0.94	0.15
Single infection yield	<0.01	<0.01	<0.01	<0.01	<0.01	0.16	

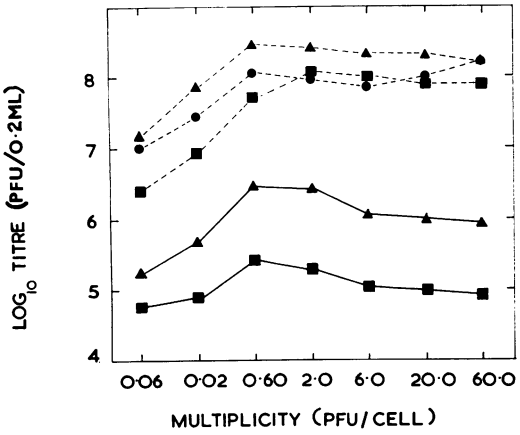

FIG. 6. *Relation between yield of wild-type recombinants and multiplicity of infection in the cross ts 11 × ts 21. Titer (PFU/0.2 ml) of ts 11 × ts 21 at 39 C (solid line, ▲) and at 31 C (broken line, ▲). Titer of mutant ts 21 alone at 39 C (solid line, ■) and at 31 C (broken line, ■). Titer of mutant ts 11 alone at 31 C (●); no plaques were observed at 39 C.*

A physical basis for subdivision of the viral genome is suggested by the existence of two particle forms of VSV, the infective virion and the interfering T particle, which contains approximately one third of the ribonucleic acid (RNA) content of the virion (3). However, it has not been possible so far to demonstrate complementation between mutant virions and T particles obtained from wild-type virus or from complementing mutants (Pringle, *in press*). Consequently, if the RNA of the T particle is a functional part of the viral genome, none of the four cistrons identified so far is located in this portion of the genome.

The time course of production of recombinants (Pringle, *in press*) and their apparent independence of multiplicity suggest reassortment of subunits rather than true genetic recombination from crossover. Only detailed analysis of progeny virus will resolve this question. Preliminary experiments have shown that the genotype of progeny clones can be determined by complementation test (Pringle, *in press*), and it has been confirmed that the frequency of the wild-type recombinant in the progeny is at least twofold more than that estimated indirectly by differential titration at 31 and 39 C (*unpublished observations*). This discrepancy can probably be accounted for in terms of differences in plating efficiencies at 31 and 39 C. Isolation of the double-mutant recombinant, however, has not been unequivocally confirmed.

Mutants belonging to groups I and IV were obtained in comparable proportions with all three mutagens. FU was strikingly more effective as a mutagen than either ACR or EMS. Since the coding changes induced by FU and EMS would be different, it is unlikely that the unequal frequency of isolation of the four types of mutant was a result of inherent mutability of particular single nucleotide sites. More likely, the preferential recovery of mutants belonging to groups I and IV reflects a difference in the number of codons in each cistron potentially competent to yield by mutation a temperature-sensitive product. The mode of action of ACR is not known precisely, but it is believed to be incorporated into viral nucleic acid (2).

In the majority of the mutants, the temperature-sensitive phenotype is due to a single-step mutational change. Nevertheless, in view of the effectiveness of FU as a mutagen, it is possible that individual *ts* mutants may carry additional mutational lesions which do not affect the temperature-sensitive phenotype. This question cannot be resolved until the defective *ts* functions have been identified. Experiments are in progress to characterize these mutants in physiological terms.

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