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Isolation and Characterization of Temperature-Sensitive Mutants of Vesicular Stomatitis Virus, New Jersey Serotype

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Forty-eight temperature-sensitive (*ts*) mutants have been isolated from a wild-type strain of the New Jersey serotype of vesicular stomatitis virus (VSV) after exposure to the base analogue mutagen 5-fluorouracil. Of these mutants, 47 have been classified into 6 nonoverlapping complementation groups containing 21, 17, 4, 3, 2, and 1 mutant, respectively (1 mutant remaining unallocated). The ribonucleic acid (RNA) phenotype of 23 of these mutants has been established. Four of the six groups contain one or more mutants unable to synthesize detectable amounts of viral RNA under restrictive conditions (39 C). No complementation was observed in mixed infection with *ts* mutants from the five established complementation groups of the Indiana serotype of VSV.

There are two immunologically distinct types of vesicular stomatitis virus (VSV) known as the Indiana and New Jersey serotypes (8). Most isolates of VSV resemble one or the other of these serotypes unequivocally, although considerable antigenic variation can be recognized within a single serotype (4). Kang and Prevec (9) have demonstrated that the surface proteins of the two serotypes are immunologically distinct, whereas the internal nucleoproteins show cross-reactions. These considerations suggest that the two serotypes are only distantly related. This communication is concerned with a comparative study of temperature-sensitive (*ts*) mutants of the two serotypes to assess genetic relatedness by the ability to complement in mixed infection.

ts Mutants of wild-type strains of the Indiana serotype have been characterized in some detail in genetic (5, 6, 12, 13) and biochemical terms (10, 14-16; Szilágyi and Pringle and Wunner and Pringle, *in preparation*).

Furthermore, Indiana *ts* mutants isolated in different laboratories, from different wild-type strains multiplying in different host cells, have been shown by complementation test to be genetically homologous (7).

We now describe the isolation and genetic characterization of *ts* mutants of a wild-type strain of the New Jersey serotype, which allows a comparison of the genetic structure of the two types of VSV. It has not been possible, however, to establish precise genetic homologies because of the failure to obtain complementation in mixed infections of the two serotypes.

MATERIALS AND METHODS

Cells. All virus propagation was carried out in BHK-21, clone 13 cells (11) by standard procedures.

Virus. The initial virus stock was a BHK-21-passaged derivative of the New Jersey M strain obtained from J. B. Brooksby of the Animal Virus Research Institute, Pirbright, Surrey. This strain largely retains its initial virulence for cattle. The wild-type (ts^+) virus was cloned by three successive isolations from monolayer cultures bearing single plaques.

The origin and genetic characteristics of the *ts* mutants of the avirulent Indiana C strain used in the interstrain complementation have been described previously (12, 13).

Mutagenesis by 5-flourouracil. 5-Fluorouracil (5-FU; Koch-Light Laboratories Ltd., Colnbrook) was dissolved in distilled water and diluted to the required concentration with Eagle's medium. Monolayers were infected with wild-type virus at low multiplicity [1 plaque-forming unit (PFU)/cell]. The inoculum was removed by two washes of 5 ml of Eagle's medium after a 30-min adsorption period at 31 C. Fresh Eagle's medium containing 10% calf serum and the analogue was added, and the cultures were incubated for 24 hr at 31 C, at which time cytopathic effect was maximal in control cultures. The supernatant fluids from these cultures were stored at −20 C until required.

Isolation of mutants. The mutagenized virus was plated at limiting dilution, and clones were isolated by picking plaques from monolayers bearing single or a few well isolated plaques into 2 ml of Eagle's

medium containing 10% calf serum. These clones were screened for growth at the permissive (31 C) and the restrictive (39 C) temperature by inoculating duplicate monolayers with 0.2 ml of the primary isolate and incubating at the two temperatures Clones which failed to produce plaques at 39 C were passaged to establish mutant stocks. Further cloning was not necessary in the majority of cases.

Complementation tests. The pattern of complementation between mutants was established initially by presence or absence of cytopathic effect in pair-wise mixed infections. Complementation was quantitated as follows. Monolayers containing 3×10^6 to 5×10^6 cells in 30-ml bottles were infected in duplicate with pairs of mutants to give a multiplicity of 10 to 20 PFU/cell. Singly infected control cultures were infected at approximately half this multiplicity. The infected monolayers were adsorbed at 4 C for 25 min and then were washed twice with two changes of 5 ml of cold Eagle's medium containing 1% calf serum to remove the inoculum. A final 5-ml volume of the same medium was added, and the culture bottles were incubated totally immersed in a water bath at 39 ± 0.05 C for 8 hr. Then the cultures were rapidly frozen and stored at −20 C. Infectivity was assayed at 31 C, and the results are expressed as complementation indexes. The complementation index is defined as the ratio yield of $(x + y)$, assayed at 31 C, minus yield of $(x + y)$, assayed at 39 C, divided by yield of x + yield of y, assayed at 31 C, where x and y are any pair of mutants: i.e., $Y(x + y)^{31\,C} - Y(x + y)^{39\,C} / Yx^{31\,C} + Yy^{31\,C}$. Ratios greater than unity indicate complementation, and the significance of the ratios can be tested by Student's t test with 1 degree of freedom by using the duplicate results as independent estimates.

Estimation of viral RNA synthesis. Monolayers of 5×10^6 cells in 30-ml bottles were infected with 0.2 ml of the appropriate mutant stock at an approximate multiplicity of 10 PFU/cell. The inoculum was adsorbed for 30 min at 4 C before addition of 3 ml of ice-cold Eagle's medium containing 10% calf serum, 2.5 μg of actinomycin D per ml (Merck, Sharp & Dohme, Rahway, N.J.), and 5 μCi of ^{3}H-uridine (20 to 31 Ci/mmole; Radiochemical Centre, Amersham). The culture bottles were then incubated totally immersed in a water bath at 39 ± 0.05 C. Cultures were removed at 0, 4, 8, and 12 hr to provide sequential samples for measurement of incorporation of ^{3}H-uridine as previously described (14).

RESULTS

Induction of ts mutants by 5-FU. A total of 48 *ts* mutants has been found among 874 clones isolated from wild-type VSV, New Jersey serotype. Table 1 gives the percentages of *ts* mutants recovered at different mutagen concentrations. The percentage yield increased with mutagen concentration. An estimate of the spontaneous mutation frequency can be obtained from these data by extrapolation, as done previously for *ts* mutants of the Indiana wild-type strain (12). The reliability of this extrapolation method has been confirmed recently by direct isolation of spontaneous Indiana *ts* mutants (*unpublished data*). The estimated frequency of spontaneous *ts* mutants in the New Jersey wild-type stock is 0.75%, which is similar to the estimate for the Indiana wild type (0.7%).

TABLE 1. *Induction of ts mutants of VSV (New Jersey, M strain) by 5-FU*[a]

5-FU concn (μg/ml)	No. of clones isolated	No. of *ts* mutants obtained	*ts* Mutant percentage
1,250	76	10	13.2
425	117	7	6.0
200	681	31	4.6

[a] Total number of clones isolated was 874, and the total number of *ts* mutants obtained was 48.

Higher concentrations of 5-FU were required with the New Jersey strain, however, to produce effects comparable to those previously obtained with the Indiana strain (12, 13). The reason is not clear but may be a consequence of the inferior yields of infectious virus obtained with the New Jersey strain. Most of the mutants should be single-step mutations, however, since the majority were isolated at 200 μg of 5-FU per ml at which the expectation of multisite mutations is 0.1% (assuming a Poisson distribution).

Classification of the mutants into complementation groups. Of the 48 mutants, 47 have been classified into 6 groups by complementation tests. The groups are designated A, B, C, D, E, and F and contain 17, 21, 4, 1, 3, and 2 mutants, respectively; the mutants within each group have been prefixed with the group letter and numbered serially. One mutant failed to complement consistently and could not be allocated to a group. This mutant was isolated at the highest mutagen concentration and may represent a multisite mutant. Complementation is reciprocal; i.e., mutants in any one group complement mutants in any other group. This is illustrated in Table 2, which gives mean complementation indexes from three replicate experiments. The complementation indexes are lower than those obtained with the Indiana *ts* mutants (12, 13), but all are statistically significant.

Evidence for a nonoverlapping pattern of complementation. Significant complementation has not been observed in mixed infections of mutants from the same group as far as has been tested. Five mutants from group A, four from group B, and four from group C have been tested in three experiments (Table 3). In each experiment, an additional mutant from a different group was

included to indicate the level of complementation observed in intergroup infections. There is no significant complementation in any combination involving mutants of the same group: the pattern is thus a nonoverlapping one, as observed with the Indiana *ts* mutants (13).

Interstrain complementation test. The results of complementation tests involving mutants of different serotype have all been negative. Table 4 presents data from one experiment in which mutants from each of the six New Jersey and the five Indiana complementation groups were tested in all combinations. In this particular experiment, the input multiplicity had been increased to approximately 100 PFU/cell. The complementation indexes in interstrain combinations were close to unity. Because of this failure to obtain interstrain complementation, it has not been possible to establish genetic homologies between the groups by this method.

TABLE 2. *New Jersey M strain; intergroup complementation indexes*[a]

Mutant	A *ts* A1	B *ts* B1	C *ts* C1	D *ts* D1	E *ts* E1	F *ts* F1
A *ts* A1		95	15	3.8	50	71
B *ts* B1			203	7.2	139	4.3
C *ts* C1				9.9	27	60
D *ts* D1					7.3	9.3
E *ts* E1						24
F *ts* F1						

[a] Values in the table are mean values obtained from three replicate experiments, and all indicate significant complementation. Groups are designated A through F, and mutants within each group are numbered serially.

Ribonucleic acid phenotype. The ability of some of these New Jersey strain mutants to induce actinomycin-resistant ribonucleic acid (RNA) synthesis under restrictive conditions has been examined to allow comparison with the Indiana mutants. In the presence of 2.5 μg of actinomycin D per ml, cumulative RNA synthesis in uninfected cells was reduced to 0.62% of the normal level by 4 hr and to 0.37% by 12 hr.

The data presented in Table 5 are the maximum levels of incorporation of ^{3}H-uridine in mutant infected cells up to 12 hr after adsorption. Mutants classified in the same complementation group have the same RNA phenotype, with the exception of group E. Mutants in groups A, B, and F were unable to induce significant amounts of actinomycin-resistant RNA synthesis, and infection with mutants in groups A and B frequently resulted in a depression of residual RNA

TABLE 3. *New Jersey M strain of VSV: the result of three intragroup complementation experiments*[a]

Group	Mutant	*ts* A1	*ts* A2	*ts* A3	*ts* A4	*ts* A5	*ts* B1
A	*ts* A1		1.2	0.6	0.8	0.9	>33
A	*ts* A2			1.1	1.0	1.4	10
A	*ts* A3				1.4	1.5	>33
A	*ts* A4					1.1	6.4
A	*ts* A5						3.4
B	*ts* B1						
		ts B2	*ts* B3	*ts* B4	*ts* B5	*ts* A1	
B	*ts* B2		0.93	0.37	0.45	11	
B	*ts* B3			0.83	0.91	3.5	
B	*ts* B4				0.59	43	
B	*ts* B5					9	
A	*ts* A1						
		ts C1	*ts* C2	*ts* C3	*ts* C4	*ts* B1	
C	*ts* C1		1.1	0.6	0.7	>20	
C	*ts* C2			0.6	1.3	17	
C	*ts* C3				1.2	>20	
C	*ts* C4					4.3	
B	*ts* B1						

[a] One mutant from a heterologous group was included in each experiment for comparison. In these experiments, indexes of 3.4 and above indicate significant complementation; all other values are not significant.

TABLE 4. *Interstrain complementation test including for comparison the intrastrain complementation indexes obtained in the same experiment*[a]

Mutant	New Jersey, M strain						Indiana, C strain				
	ts A1	*ts* B1	*ts* C1	*ts* D1	*ts* E1	*ts* F1	*ts* 11	*ts* 22	*ts* 31	*ts* 41	*ts* 0/45
New Jersey, M strain											
A *ts* A1		36	379	42	19	98	1.9	1.3	0.74	1.9	1.9
B *ts* B1			305	3.4	13	3.6	0.21	0.24	0.11	0.17	0.46
C *ts* C1				13	11	60	0.43	0.26	0.17	0.30	0.43
D *ts* D1					4.4	11	1.9	0.22	0.34	0.18	1.20
E *ts* E1						33	0.45	0.23	0.41	0.29	0.24
F *ts* F1							0.72	0.52	0.78	1.9	1.2
Indiana, C strain											
I *ts* 11								124	1,000	66	ND[b]
II *ts* 22									123	330	ND
III *ts* 31										81	ND
IV *ts* 41											ND
V *ts* 0/45[c]											

[a] Complementation indexes of 3.4 and above are significant; all other values are not.
[b] Not determined.
[c] Isolated by Anne Flamand (6) from the Orsay strain of VSV Indiana.

TABLE 5. *Ability to induce actinomycin-resistant RNA synthesis at 39 C*[a]

Group	Mutants tested	Level of incorporation of ^{3}H-uridine as per cent of the wild-type level	RNA phenotype
A	*ts* A1, *ts* A6, *ts* A9	<0.51 – <1.67	–
	ts A7	1.06	–
	ts A8	1.93	–
B	*ts* B1, *ts* B4, *ts* B5, *ts* B7, *ts* B8	<0.51 – <4.76	–
	ts B2	1.40	–
	ts B3	4.76	(–)
C	*ts* C1, *ts* C2, *ts* C3, *ts* C4	44.5 – 159.2	+
D	*ts* D1	45.7 – 193.2	+
E	*ts* E1 (two clones)	<0.40	–
	ts E2 (two clones)	51.5 – 88.7	+
	ts E3 (one clone)	0.90	–
F	*ts* F1	0.64	–
	ts F2	3.80	(–)

[a] Values in column 3 are the maximum levels of incorporation of ^{3}H-uridine in mutant-infected cells up to 12 hr after adsorption. The symbol < indicates that incorporation was less than in uninfected cells.

synthesis. Mutants in groups C and D induced actinomycin-resistant RNA synthesis ranging between 45 and 193% of the wild-type value. Group E is anomalous in that two of the three mutants in this group are RNA-negative and the other is RNA-positive.

DISCUSSION

Evidence has been presented to support the genetic classification of 48 *ts* mutants of New Jersey serotype into 6 complementation groups, which show no overlap. Table 6 summarizes the genetic properties of *ts* mutants of both the Indiana and the New Jersey serotypes of VSV. Although genetic homologies cannot be established directly by complementation test, some comparison is possible. The largest groups in both serotypes contain mutants unable to induce RNA synthesis at the restrictive temperature. Complementation groups A, B, and F of the New Jersey serotype resemble groups I and IV of the Indiana serotype in this respect. Likewise, complementation groups C and D (New Jersey serotype) resemble groups III and V (Indiana serotype), since all mutants in these groups are able to induce actinomycin-resistant RNA synthesis at 39 C. The New Jersey group E can be equated provisionally with the Indiana group II, since these groups are atypical in that they contain mutants of both RNA-negative and RNA-positive phenotypes.

Cooper (3) described heterotypic exclusion in VSV-infected chick embryo cells after superinfection as early as 12 min after primary infection.

TABLE 6. *The ts mutants of VSV*

Indiana serotype					New Jersey serotype		
Complementation group	Induced[a]		Spontaneous[b]		Complementation group	5-FU-induced	
	No.	RNA phenotype	No.	RNA phenotype		No.	RNA phenotype
I	177	−	58	−	A	17	−
II	2	−/+	2	+	B	21	−
III	3	+	4	+	C	4	+
IV	22	−	4	−	D	1	+
V	0	0	3	+	E	3	−/+
Unallocated	6	0	0	0	F	2	−
					Unallocated	1	−

[a] Data from Pringle (12, 13). The majority of these mutants were 5-FU-induced.
[b] Isolated and characterized by Anne Flamand (6) and shown to be homologous with the 5-FU-induced Indiana *ts* mutants by Flamand and Pringle (7).

Exclusion (interference) was not observed when the primary and superinfecting viruses belonged to the same serotype. We are investigating the possibility that heterotypic exclusion may explain the failure to obtain interstrain complementation, even though in our experiments infection with the heterologous strains was simultaneous.

The failure to obtain significant complementation in mixed infection with mutants of different serotype is interesting, since phenotypic mixing may occur between VSV and unrelated enveloped viruses such as parainfluenza virus SV5 (2), fowl plaque virus, and murine leukemia virus (J. Závada, *personal communication*). The apparent specificity of the complementation reaction contrasts with the tolerance in assembly of the viral envelope.

The occurrence of mutants with RNA-negative phenotypes in four of the six complementation groups implies that four viral genes are concerned with RNA synthesis, assuming that each complementation group can be equated to a viral cistron. Alternatively, *ts* mutations affecting the virion-associated polymerase (1) might produce RNA-negative phenotypes when the transcription function of the enzyme is affected and RNA-positive phenotypes if this enzyme is an essential factor in assembly of the mature virion. Further work is in progress to define the nature of the functions affected by these mutations.

Recombination between mutants in different complementation groups (but not between mutants in the same group) was reported for the Indiana mutants (12), and similar results have been obtained with the New Jersey mutants. Subsequent work with the Indiana mutants has shown that presumed wild-type recombinants invariably yield the parental mutants by segregation (Pringle, *unpublished data*). Consequently, the phenomenon cannot be considered real genetic recombination. The precise explanation of all of the observations is not clear, but complementation is implicated. This is shown clearly by the observation that recombination is absent in heterotypic crosses, where occurrence of complementation can be excluded.

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