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Qualitative Complementation Test for Temperature-Sensitive Mutants of Herpes Simplex Virus

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A simple, rapid, qualitative method for classifying temperature-sensitive mutants of herpes simplex virus into functional complementation groups has been developed. The positive reaction observed in this test reflects the ability of mutant pairs to interact by both complementation and recombination.

For the complete genetic analysis of a virus, mutants with defects in all viral genes must be available for study. The herpesvirus genome is large and encodes approximately 100 genes; consequently, the task of saturating the genetic map is considerable.

Studies with temperature-sensitive (ts) mutants of herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) conducted in this laboratory have resulted in the identification of 15 cistrons of HSV-1 (9) and 7 cistrons of HSV-2 (5). In other studies with HSV ts mutants, Brown et al. (1) have identified 8, and Hughes and Munyon (Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S322, p. 267) have identified 7 cistrons of HSV-1; Timbury (13) and Halliburton and Timbury (manuscript in preparation) have identified 13, and Koment and Rapp (6) have identified 2 cistrons of HSV-2. Whether any of the complementation groups identified in the various studies are the same has not yet been determined. In view of its size, therefore, it is clear that the number of cistrons identified to date accounts for only a small portion of the total coding capacity of the HSV genome.

Efforts to identify additional HSV cistrons should include combined tests with members of all existing complementation groups as well as newly isolated mutants not yet subjected to complementation analysis (7, 11, 14). Although conventional methods of quantitative complementation are definitive in assigning mutants to new and existing complementation groups, they are both laborious and time-consuming.

To simplify the identification of new HSV cistrons, a qualitative complementation test has been developed. The present report describes the test and demonstrates its usefulness for the rapid identification of new HSV cistrons.

MATERIALS AND METHODS

Viruses. The isolation and preliminary characterization of the HSV-1 and HSV-2 ts mutants used in this study have been described previously (5, 8-10). Two new ts mutants of HSV-2 strain 186 were isolated after UV mutagenesis as described previously for ts mutants of HSV-1 (9). These mutants, designated ts49 and ts314, were isolated from wild-type virus suspensions treated with UV light for 60 s and 30 s, respectively. Both mutants revert with low frequency and exhibit multiplicity-dependent leak. Plating efficiencies of these mutants (PFU/ml, 38 C/34 C) are 2×10^{-4} and 4.6×10^{-4}, respectively. Based on analytical cesium chloride density gradient centrifugation of lysates of mutant-infected cells maintained at both 34 and 38 C (9), ts314 is DNA^- and ts49 is DNA^+.

Cells and cell culture. Monolayer cultures of serially propagated human embryonic lung fibroblasts were used for the preparation of virus stocks. Vero cell monolayers were used for virus titration and for complementation tests. Both human embryonic lung and Vero cells were grown in Eagle medium supplemented with 10% fetal bovine serum and 0.075% $NaHCO_3$ for cultures in closed vessels and 0.225% $NaHCO_3$ for cultures in open vessels in a 5% CO_2 atmosphere. Tris-phosphate buffer at pH 7.4 was used for washing monolayers.

Virus stocks and assays. Virus stocks were prepared by infecting human embryonic lung cells in 100-mm petri dishes at multiplicities of 0.001 to 0.01 PFU/cell. Infected cells were incubated at 34 C for 4 to 6 days. When complete cytopathic effect was evident, cells were scraped from dishes into the medium with a rubber policeman. The cell suspension was sonically treated for 45 s in a Raytheon sonic oscillator at 10 kc/s and clarified by low-speed centrifugation, and supernatant fluids were stored in aliquots at −90 C. Virus infectivity was assayed on Vero cell monolayers by a plaque method utilizing a 2% methyl cellulose overlay (3). Tris containing 1% fetal bovine serum was used as virus diluent.

Complementation tests. Standard quantitative complementation tests between pairs of ts mutants

were performed as previously described (9). A simplified qualitative complementation test is illustrated in Fig. 1. Three- to 4-day-old fully confluent monolayers of Vero cells in 60-mm petri dishes were used for all tests. Three dilutions of each test mutant were prepared to contain 2×10^6, 4×10^5, and 8×10^4 PFU/ml. Equal volumes (0.5 ml) of single virus suspensions at the same dilution were then mixed. Suspensions of each virus alone at each of the three dilutions served as controls. Vero cell monolayers were washed once with Tris, leaving a small volume (0.1 to 0.2 ml) of buffer on the monolayer to prevent cells from drying. Sterile filter paper disks (Whatman filter paper no. 4) with diameters of 6 mm were saturated with virus suspension and drained before being placed on the monolayer. Each disk contained about 0.01 ml of solution when saturated with virus suspension. Four disks were placed on each 60-mm petri dish: duplicate test disks saturated with mixed virus suspension and two control disks each saturated with one virus of the test pair (Fig. 1). Virus was allowed to adsorb to monolayers for 45 min at 37 C in a CO_2 incubator. Disks were then removed with sterile forceps, and 8 ml of Eagle medium containing 5% fetal bovine serum and 2% methyl cellulose was added to each plate. Plates were incubated at 39 C for HSV-1 and 38 C for HSV-2 ts mutants. After 4 days of incubation, 5 ml of neutral red solution (1:20,000 in Tris) was added to each plate and monolayers were observed for areas of clearing on the following day.

Progeny virus tests. Analysis of progeny from mixed infections between HSV-1 ts mutants was conducted to determine whether the yield was composed of ts and/or ts^+ virus. For progeny analysis, no neutral red was added to monolayers and clear areas were visualized by indirect light. Well-isolated plaques and clear, lysed areas of monolayers were picked after 4 days of incubation at 39 C. Plaque material was suspended in 0.8 ml of virus diluent and frozen at −90 C. Virus suspensions were then thawed, sonically treated, clarified by low-speed centrifugation, and assayed on Vero cell monolayers at 39 and 34 C.

RESULTS

Results of qualitative complementation tests. Three characteristic reactions were observed at sites on monolayers infected with mixed suspensions of mutants (Fig. 2). (i) Negative reactions were characterized by intact monolayers; i.e., no evidence of lysis or clearing was observed (Fig. 2A). Mutant pairs that exhibited this reaction were said to produce negative spots. (ii) The most frequently observed positive reaction is illustrated in Fig. 2B. The monolayer beneath the site of infection was totally lysed, and large plaques were evident in the vicinity of the inoculation site (Fig. 2B-1). (iii) The least frequently observed positive reaction is illustrated in Fig. 2C. The monolayer

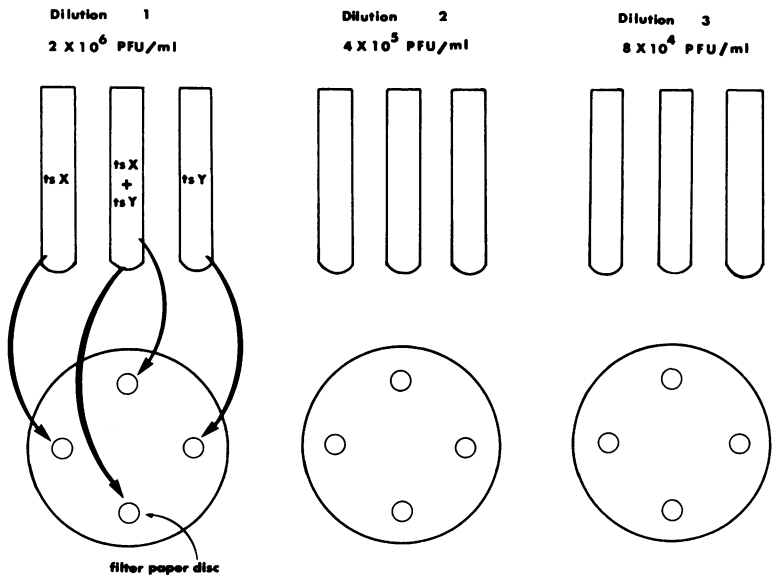

FIG. 1. *Qualitative complementatoion test. Three fivefold dilutions of each mutant to be tested were prepared to contain 2×10^6, 4×10^5, and 8×10^4 PFU/ml. Equal volumes (0.5 ml) of two test mutants at the same dilution were mixed. Filter paper disks were saturated with each virus suspension, drained, and placed on Vero cell monolayers. Disks containing each test mutant alone were also placed on monolayers as controls. After incubation for 45 min at 37 C in a CO_2 incubator, filter paper disks were removed and a methylcellulose overlay was added. Plates were incubated at the nonpermissive temperature for 3 or 4 days, neutral red was added, and reactions were read the following day.*

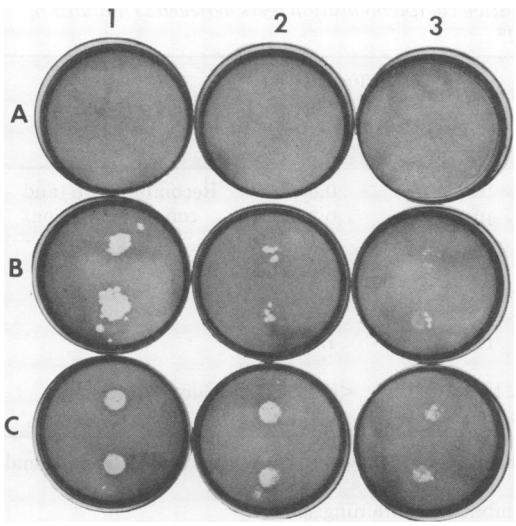

FIG. 2. *Characteristic results of spot complementation tests with three pairs of HSV-1 ts mutants. Virus dilutions were as follows: (1) 2 × 10⁶ PFU/ml; (2) 4 × 10⁵ PFU/ml; and (3) 8 × 10⁴ PFU/ml. (A) tsB21 + tsB2, no reaction; (B) tsB21 + tsE6, positive reaction (ts and ts+ virus in yield); and (C) tsE5 + tsM19, positive reaction (ts virus only in yield).*

beneath the site of infection characteristically exhibited either complete lysis or a mottled appearance. Occasionally, tiny plaques were observed in the vicinity of the inoculation site. Positive reactions were less prominent at higher virus dilutions, demonstrating that the magnitude of the reaction was multiplicity dependent.

Although monolayers infected with suspensions of single mutants were usually intact at all dilutions tested (Fig. 2A, B, and C, horizontal positions on monolayers as illustrated in Fig. 1), they occasionally exhibited minor mottling due to leak at the highest virus concentration tested.

Progeny analysis of virus produced in qualitative tests. To determine the temperature-sensitive phenotype of the progeny produced in mixed infections, virus was isolated from individual plaques and from the lysed monolayer (spot) directly beneath the inoculation disk. Progeny virus was tested from mixed infections with five HSV-1 ts mutant pairs which exhibited reactions of the type illustrated in Fig. 2B. Virus was also isolated from spots produced by two mutant pairs which exhibited the kind of reaction illustrated in Fig. 2C. Virus was assayed on Vero cell monolayers at 34 and 39 C. Representative results of these tests are shown in Table 1. When progeny produced in the first type of reaction were tested, plating efficiencies (39 C/34 C) of virus from isolated plaques ranged from 0.5 to 1.2 PFU/ml and from spots, 1.0 to 1.3 PFU/ml. Thus, progeny virus plated nearly as well at 39 C as at 34 C, demonstrating that it was phenotypically ts$^+$. Since single mutant controls were negative, this virus was generated by recombination and not reversion. Furthermore, since this type of reaction was characteristic of nearly all positive tests, recombination had occurred between most mutant pairs.

Progeny virus isolated from spots produced in the second type of positive reaction (Fig. 2C) produced plaques at 34 C but not at 39 C, indicating that this virus was phenotypically ts and was generated by complementation.

Comparison of results of qualitative and quantitative complementation tests with ts mutants of HSV-1 and HSV-2. Fifteen ts mutants of HSV-1 representing 15 cistrons (A through O; 9) and 8 HSV-2 mutants representing 7 cistrons (A through G; 10) were tested for their ability to interact in the qualitative complementation test. Results of these tests were compared with previously determined results of quantitative complementation and recombination tests (Table 2). Since recombinant virus was produced in most cases, recombination frequencies are also shown (10). Two mutants, tsE5 and tsE6, in the same complementation group as determined by negative results in quantitative tests were negative in the qualitative test as well. In tests with mutants in two other complementation groups, the results of qualitative and quantitative tests also agreed well (i.e., tsE5 and tsE6 × tsC4, and tsE5 and tsE6 × tsO22). In tests of mutants representing all 15 cistrons, the qualitative test was negative, whereas the quantitative test had been positive in only one case (tsC4 × tsO22). Since more definitive quantitative tests had previously demonstrated that the two mutants were defective in different cistrons, this reaction was thus a "false-negative" result.

In qualitative tests of HSV-2 ts mutants, only tsA1 and tsA8 exhibited a negative reaction (Table 2). These mutants are in the same complementation group as demonstrated by quantitative tests, and they recombine poorly. All other qualitative tests were between mutant pairs previously shown to represent different complementation groups by quantitative tests, and all pairs gave positive results in the qualitative test. The great majority of positive spots produced by HSV-2 ts mutant pairs were also of the "recombinant" type. In fact, the magnitude of positive reactions produced by both HSV-1 and HSV-2 mutant pairs was a better reflection of the efficiency of recombination (10) than the efficiency of complementation (5, 9).

TABLE 1. *Analysis of progeny from representative qualitative complementation tests between ts mutants of HSV-1*[a]

Mutant pair	Plaque or spot no.	Virus yield (PFU/ml)		Efficiency of plating (PFU/ml, 39 C/34 C)	Type of interaction between mutant pair
		34 C	39 C		
tsA16 + tsB2	1[b]	8×10^3	5×10^3	0.6	Recombination (and complementation)
	2	1×10^4	7×10^3	0.7	
	3	4×10^3	2×10^3	0.5	
	4	1×10^4	9×10^3	0.9	
	5	6×10^2	7×10^2	1.2	
	S_1[c]	4×10^4	5×10^4	1.3	
	S_2	2×10^2	2×10^2	1.0	
tsE5 + tsM19	S_1	2×10^3	$<10^1$	<0.005	Complementation
	S_2	1×10^2	$<10^1$	<0.1	

[a] Similar results were obtained in tests of four additional recombinant-containing spots and one additional recombinant-negative (complementation-initiated) spot.
[b] Individual plaques were isolated in the vicinity of recombinant-containing spots.
[c] Total virus in spots (S) was isolated and tested.

TABLE 2. *Comparison of representative qualitative complementation data with results of quantitative complementation and recombination tests*

HSV-1							HSV-2						
Complementation group	Mutant[a]	Test[b]	tsC4⁻	tsE5⁺	tsE6⁺	tsO22⁺	Complementation group	Mutant[a]	Test[b]	tsA1⁻	tsA8⁻	tsB5⁻	tsD6⁺
C	tsC4⁻	QC	NA[c]	+	+	−	A	tsA1⁻	QC	NA	−	+	+
		CI		1100	750	52			CI		0.3	530	17
		RF		0.8	1.5	3.3			RF		<0.12	4.5	8.3
								tsA8⁻	QC		NA	+	+
									CI			520	130
									RF			6.8	4.4
E	tsE5⁺	QC		NA	−	+	B	tsB5⁻	QC			NA	+
		CI			0.5	13			CI				180
		RF			<0.001	14.0			RF				12
	tsE6⁺	QC			NA	+							
		CI				20							
		RF				12.4							

[a] Viral DNA phenotypes of mutants at the nonpermissive temperature; +, DNA positive; −, DNA negative.
[b] QC, Results of qualitative complementation tests; −, negative spot; +, positive spot. CI, Complementation indexes from quantitative tests. Values ≥2 are considered to be positive (9). RF, Recombination frequencies (10).
[c] NA, Not applicable.

A summary of the results from all qualitative and quantitative tests performed with HSV-1 and HSV-2 ts mutants is shown in Table 3. Of 105 HSV-1 mutant pairs tested by both methods, 99 were positive in the qualitative test and 100 were positive in the quantitative test; thus, false-negative results were observed in only one instance. No false-negative results were observed in tests of 28 HSV-2 ts mutant pairs. False-positive reactions have not been observed in from three to five replicate tests of over 150 mutant pairs.

Identification of new HSV cistrons. To evaluate the qualitative test as a means of identifying new HSV cistrons, two recently isolated UV-induced ts mutants of HSV-2 were

tested by both qualitative and quantitative tests (Table 4). Mutant ts314 complemented mutants representing six of seven complementation groups in the qualitative test and seven of seven groups in the quantitative test. Repeat tests of ts314 with tsA1 and tsA8, which were negative in the qualitative test, were positive in the more sensitive quantitative test. Therefore, by combined qualitative and quantitative complementation tests, ts314 was shown to represent a new HSV-2 cistron. This cistron has been designated H, and the mutant ts314 has been renamed tsH10. Mutant ts49, on the other hand, failed to complement the group D mutant in both kinds of tests and was, therefore, assigned to that group; ts49 has been designated tsD11.

DISCUSSION

This paper describes a rapid, simple qualitative complementation test for ts mutants of herpes simplex virus. Two qualitative complementation tests have been described recently for ts mutants of simian virus 40 (2, 12). Since recombination occurs rarely in the SV40 system, the positive reaction in both tests is based upon the ability of mutant pairs to generate progeny virus by complementation. A spot complementation for ts mutants of bacteriophage T4D was described in 1964 by Edgar et al. (4). Recombination occurs readily in the T4 system, and the authors not surprisingly observed the presence of recombinant virus in mixed yields of spot complementation tests. In part, it is a misnomer to term the test described in this report a complementation test since the primary event observed was recombination. This is inevitable with herpes simplex viruses, however, since (i) recombination is frequent with these viruses, (ii) as devised, the test measures the yield after multiple rounds of replication resulting from complementation which favors recombination, and (iii) ts^+ recombinant virus usually outgrows ts mutant virus. Although the majority of progeny in mixed infections represented recombinant virus, the less frequently observed result (Fig. 2C) probably reflects the inability of mutants in this mixed infection to undergo multiple rounds of replication. Hence, in this case true complementation was the consequence. The good correlation between the qualitative and quantitative tests probably reflects the fact that mutants in the same complementation group recombine inefficiently.

In initial attempts to demonstrate qualitative complementation between HSV ts mutants, we observed that the use of thin agar overlays inhibited the diffusion of virus to the underlying monolayer and inoculum spread freely over the surface of the agar. In contrast, the use of virus-saturated filter paper disks insured direct contact between cells and virus and resulted in a highly localized infection. Disks were removed after adsorption since the most prominent reaction was evident immediately beneath the disk. This was especially true in the case of minimally positive reactions.

Preliminary tests also indicated that the clearest evidence for a positive reaction in the qualitative test was obtained when both mu-

TABLE 3. *Comparison of results from qualitative and quantitative complementation tests*

HSV serotype	Complementation test	No. of mutant pairs	No. of tests positive	No. of false-negative results in qualitative test
HSV-1	Qualitative	105	99	1
	Quantitative	105	100	
HSV-2	Qualitative	28	27	0
	Quantitative	28	27	

TABLE 4. *Results of qualitative and quantitative complementation between two newly isolated HSV-2 ts mutants and eight established HSV-2 ts mutants*

Mutant[a]	Test[b]	Complementation between ts mutant pairs							
		tsA1	tsA8	tsB5	tsC2	tsD6	tsE7	tsF3	tsG4
ts314	QC	−	−	+	+	+	+	+	+
	CI	23	23	5,333	588	1,233	1,400	1,017	108
ts49	QC	+	+	+	+	−	+	+	+
	CI	6,700	209	231	122	0.5	2,421	4,483	141

[a] ts314 and ts49 are newly isolated ts mutants induced with UV irradiation.

[b] QC, Results of qualitative complementation tests; −, negative spot; +, positive spot. CI, Complementation indexes from quantitative tests (9). Values ≥ 2 are considered to be positive.

tants in a pair were equally diluted to contain 4 $\times$ 10^5 to 2 $\times$ 10^6 PFU/ml. Although concentrations of 8 $\times$ 10^4 PFU/ml produced reactions consistent with those observed at higher concentrations, they were more difficult to interpret. Thus, the test was applicable over a 0.7- to 1.5-log range of virus titers. Concentrations below 10^4 PFU/ml always gave negative results in mixed infections—even in tests of mutant pairs shown to complement in quantitative tests.

The qualitative test was demonstrated to be slightly less sensitive than the quantitative test since false-negative results were occasionally observed. The occasional appearance of false-negative reactions emphasizes the necessity for all negative results in qualitative tests to be tested by more definitive quantitative complementation tests. False-positive reactions, on the other hand, have never been observed in the qualitative test.

The age of the Vero monolayers was found to influence the results of qualitative tests. Subconfluent monolayers exhibited extensive leak and nonspecific damage, even when inoculated with single mutant control suspensions, thus making results difficult to interpret. Consequently, fully confluent 3- to 4-day-old monolayers were used.

Results were least ambiguous if tests were evaluated after 4 or 5 days of incubation (4 days was optimal for HSV-2 and 5 days was optimal for HSV-1). After longer periods of incubation (as long as 7 days), the interpretation of results remained unchanged.

Mutants that exhibit moderate levels of leak and reversion are often the best mutants to be used in genetic and biochemical studies since those which fail to leak or revert may contain more than one mutation. One advantage of the qualitative complementation test is that it is applicable to mutants with low and moderate levels of leak and/or reversion. Mutants with leak or reversion levels equal to or less than the lowest virus concentration tested, i.e., 10^4 PFU/ml, would be suitable for qualitative complementation tests. Furthermore, for very leaky mutants the highest concentration of each mutant that fails to exhibit leak can be determined readily before testing.

The results presented in this report demonstrate that the qualitative complementation test offers a rapid and sensitive means for assigning mutants of HSV-1 and HSV-2 to new and existing complementation groups and that patterns of complementation obtained by this method and the more laborious quantitative method agree well. We estimate that the time saved through the use of the qualitative test is such that 1 year of quantitative tests can now be completed in 1 or 2 months. The test is currently being used for screening new ts mutants of HSV and for comparative complementation studies between groups of HSV-1 and HSV-2 ts mutants isolated in different laboratories.

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