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# Infectious Center Assay for Complementation and Recombination Between Mutants of Reovirus

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An infectious center assay has been developed to measure recombination and complementation in L cells mixedly infected with *ts* mutants of reovirus. The mutants studied so far fall into complementation groups that correspond to the recombination groups previously defined by other laboratories (B. N. Fields, 1971; B. N. Fields and W. K. Joklik, 1969).

Genetic recombination between pairs of *ts* mutants of reovirus occurs with a relatively high frequency or not at all (1, 2). The results of pairwise crosses between some 70 such mutants has permitted their classification into seven groups designated A to G. No recombination could be detected between the mutants of a given group but recombination between the groups gave frequencies of 3 to 8% $ts^+$ recombinants. Since the genome of this virus is comprised of 10 distinct segments of double-stranded RNA (dsRNA) each class of mutants is considered to be associated with one or other single genomic segment and recombination is thought to occur through reassortment of whole segments of the genomes (1, 2).

On the other hand complementation between mutants in this viral system is very inefficient. Complementation indexes up to 5 have been measured between mutants (2, 7) but with the inherent errors in the standard complementation assay these levels are on the borderline of significance. Another type of assay, which measured the increased viral RNA synthesis in cells coinfected with two *ts* mutants at 39 C over that found in cultures infected separately with the mutants (4), suggested that complementation occurred but the test is tedious to perform and is confined to $dsRNA^-$ mutants. We have recently shown that when monolayers of L cells are coinfected at the nonpermissive temperature with defective reovirions, which lack the largest ($L_1$) segment of the viral genome, and certain classes of *ts* mutants there is marked increase in the number of plaques compared with monolayers infected separately with each of the viral populations (7). This test showed that complementation can occur between defective virions and classes A, B, D, F, and G *ts* mutants; it permitted the assignation of the class C mutation to the $L_1$ segment and the E mutation to a *cis*-function (6, 7). Nevertheless, it is important to classify the *ts* mutants into complementing groups and to determine whether or not these groups correspond to the classification based on recombination frequencies. The complementation test with defective virus could not do this, but it did suggest that complementation between *ts* mutants of reovirus could be measured by an infectious center assay and the present paper describes such a test. An infectious center method has been used previously to detect complementation between *ts* mutants of influenza virus (3).

The growth of cells, purification of virus and its assay, and the nomenclature used for the various strains have been described (6, 7). The wild-type strain from which the *ts* mutants of Fields and Joklik were derived (2) has been designated $R_2$ (6). Isolation of defective virions from the wild-type strain $R_1$ has been described (6) and these are referred to as $R_1$d ($L_1$). Purified viral populations were used for all infections carried out in this work and the relative efficiencies of plating (EOP) at 39 and 31 C for most of the strains used here have been described (7). Recently W. K. Joklik kindly sent us three additional mutants and the relevant data for these strains are shown in Table 1 of the present paper.

The infectious center assay has been described in detail (8). In summary, confluent monolayers of cells were infected with *ts* mutants at a multiplicity of infection of 10 PFU/cell for each mutant or defective virions at an MOI of 200 particles/cell (8). In coinfections with two mutants each virus preparation was diluted and the required amount of each virus was added directly to the plate with the minimum of time between additions. Adsorption of virus was for 1 h at room temperature, the plates were then washed three times with minimal essential medium, and the cells were suspended with trypsin. A known number of these

infected cells was mixed with $3 \times 10^6$ to $4 \times 10^6$ uninfected L cells from a suspension culture placed in a 60-mm plastic dish and incubated at the required temperature for 3 h to permit the cells to attach. Agar overlay was then added, the incubation continued under conditions to be specified and the plaques were finally scored after staining the cells with neutral red.

Table 2 shows the results of one experiment set up to determine whether complementation could be measured between several mutants by the infectious center technique. The actual number of plaques found on each plate is entered in this table to show the scatter in the results. When 50 or 100 infected cells were plated at 31 C, 75 to 80% of them uniformly gave rise to plaques whether the cells were infected with one mutant or coinfected with two mutants and this is taken as the plating efficiency of infected cells. At 39 C a very much smaller fraction of the infected cells gave plaques as expected. Several sets of infectious center assays had been set up for each cross, each set containing a different number of infected cells to ensure that sufficient plaques would be counted regardless of the efficiency of the cross. For each infection shown in Table 2 the percentage of plated cells giving plaques at 39 C was calculated and a complementation level (CL) was defined. A CL of unity means that the fraction of mixedly infected cells giving plaques at 39 C equals the sum of the fractions infected by the two mutants separately and thus there was no complementation. A CL greater than unity denotes that complementation occurred between the two mutants. From Table 2 it is clear that there was no complementation between $R_2$C (447) and $R_1$d ($L_1$) as we found previously (7). The other three crosses gave ratios of 2 or more showing that complementation had occurred in these cases. In fact complementation had already been shown between $R_2$A (201) and $R_1$d ($L_1$) by a different technique (7).

The results of Table 2 encouraged us to extend this infectious center method to crosses between a variety of mutants. The experiments were carried out exactly as described for Table 2 and the complementation results are summa-

TABLE 1. *EOP of purified reovirus mutants*

| Mutants[a] | PFU/$OD_{260}$[b] assayed at | | Efficiency of plating 39 C/31 C | Particles/PFU assayed at 31 C[c] |
|---|---|---|---|---|
| | 31 C | 39 C | | |
| $R_2$A (340) | $4.2 \times 10^{10}$ | $4.6 \times 10^6$ | $1.1 \times 10^{-4}$ | 50 |
| $R_2$A (376) | $3.0 \times 10^{10}$ | $6.0 \times 10^6$ | $2.0 \times 10^{-4}$ | 70 |
| $R_2$B (405) | $3.5 \times 10^{10}$ | $3.5 \times 10^6$ | $1.0 \times 10^{-4}$ | 60 |

[a] All mutants were cloned and clones grown to large stocks from which virus was purified.
[b] $OD_{260}$, optical density at 260 nm.
[c] Calculated from the ratio $2.1 \times 10^{12}$ particles/$OD_{260}$ (5).

TABLE 2. *Complementation between mutants determined by infectious center assay*

| Infection[a] | Plates incubated (C) | Infected cells/plate | | | | | | Infected cells (%) giving plaques at 39 C[c] | Complementation level[d] |
|---|---|---|---|---|---|---|---|---|---|
| | | 50 | 100 | 1,000 | 5,000 | 10,000 | 30,000 | | |
| | | No. of plaques/plate[b] | | | | | | | |
| $R_2$A(201) × $R_2$B(353) | 31 | 38,43 | 67,86 | | | | | | |
| | 39 | | 1,2 | 8,14 | 41,50 | 76,84 | | 0.86 | 2.7 |
| $R_2$A(201) × $R_2$C(447) | 31 | 31,41 | 72,80 | | | | | | |
| | 39 | | 1,1 | 4,8 | 25,41 | 52,71 | | 0.63 | 2.4 |
| $R_2$A(201) × $R_1$d($L_1$) | 31 | 35,43 | 70,83 | | | | | | |
| | 39 | | 1,0 | 3,6 | 16,25 | 28,50 | | 0.40 | 2.0 |
| $R_2$C(447) × $R_1$d($L_1$) | 31 | 40,41 | 66,90 | | | | | | |
| | 39 | | 0,0 | 1,2 | 4,5 | 6,7 | 15,29 | 0.07 | 0.88 |
| $R_2$A(201) | 31 | 38,49 | 78,80 | | | | | | |
| | 39 | | 0,0 | 1,3 | 8.13 | 17,22 | 48,67 | 0.19 | |
| $R_2$B(352) | 31 | 42,50 | 81,85 | | | | | | |
| | 39 | | 0,0 | 1,2 | 4,7 | 10,16 | 30,46 | 0.13 | |
| $R_2$C(447) | 31 | 36,41 | 65,78 | | | | | | |
| | 39 | | 0,0 | 1,1 | 2,5 | 5,9 | 16,27 | 0.07 | |
| $R_1$d($L_1$) | 31 | 0,0 | 0,0 | | | | | | |
| | 39 | | 0,0 | 0,0 | 0,0 | 0,2 | 2,4 | 0.01 | |

[a] The MOI for each mutant was 10 PFU/cell or 250 particles of $R_1{}^3$d ($L_1$) cell (8).
[b] Each column gives the number of plaques scored on each of duplicate plates.
[c] Total number of plaques scored (sum of all the columns)/total number of infected cells plated (sum of all the columns × 100).
[d] Complementation level is defined as: % of infected cells giving plaques in a cross (X × Y)/% of X-infected cells giving plaques + % of Y-infected cells giving plaques.

rized in the last two columns of Table 3. In addition, for each mixed infection the percentage of infected cells giving $ts^+$ recombinants was also determined and the results are shown in the third column of Table 3. This test for recombination has been described (8) but, in summary, 100 mixedly infected cells were plated in an infectious center assay and the plates were placed at 31 C for 30 h to permit the *ts* mutants to grow and any incidental recombination to occur. The plates were then raised to 39 C. Plaques that subsequently develop have been shown to originate from $ts^+$ recombinants (8). In this test the percentage of infected cells giving plaques at 39 C denotes the percentage of cells in which recombination occurred.

In the first five rows of Table 3 are shown the results of mixed infections between three class A mutants, between two class B mutants, and between two class D mutants and in each no recombination was found. That is, when the number of plaques from the cross is reduced by the number of plaques given by cells infected with each mutant separately the result is close to zero. For the same crosses the CL was close to unity showing that no complementation had occurred. These results set a base line against which recombination and CL determined in interclass crosses can be assessed. Mutants of all known classes were then crossed against the prototype class A mutant, $R_2$A (201), in the recombination and complementation tests (Table 3). In addition B × C and C × D crosses were done. In each cross virtually all mixedly infected cells gave rise to recombinants and, moreover, the CL was well above the base line of unity observed in the intraclass crosses. The CL for two crosses, $R_2$A (201) × $R_2$B (352) and $R_2$A (201) × $R_2$C (447) were similar in the two separate experiments (Tables 2, 3).

TABLE 3. *Recombination and complementation between ts mutants of reovirus by the infectious center assay*

| Infection[a] | Infected cells (%) giving plaques at 31 C[b] | Infected cells (%) showing recombination[c] | Infected cells (%) giving plaques at 39 C[d] | Complementation level[e] |
|---|---|---|---|---|
| A(201) × A(340) | 75 | 2 | 0.38 | 0.93 |
| A(201) × A(376) | 75 | 2 | 0.36 | 0.88 |
| A(340) × A(376) | 73 | 2 | 0.39 | 0.95 |
| B(352) × B(404) | 75 | 2 | 0.25 | 0.81 |
| D(357) × D(585) | 73 | 2 | 0.35 | 0.63 |
| A(201) × B(352) | 77 | 75 | 0.95 | 3.1 |
| A(201) × B(405) | 75 | 77 | 1.2 | 2.9 |
| A(201) × C(447) | 73 | 77 | 0.76 | 2.7 |
| A(201) × D(357) | 76 | 77 | 0.88 | 1.9 |
| A(201) × D(585) | 73 | 76 | 0.88 | 1.8 |
| A(201) × E(320) | 72 | 69 | 0.85 | 2.3 |
| A(201) × F(556) | 71 | 72 | 1.3 | 4.2 |
| A(201) × G(453) | 71 | 72 | 1.1 | 3.7 |
| B(352) × C(447) | 76 | 73 | 0.68 | 4.0 |
| C(447) × D(585) | 71 | 74 | 0.96 | 3.1 |
| A(201) | 76 | 1 | 0.20 | |
| A(340) | 72 | 0 | 0.21 | |
| A(376) | 74 | 1 | 0.19 | |
| B(352) | 74 | 1 | 0.10 | |
| B(405) | 76 | 2 | 0.19 | |
| C(447) | 71 | 1 | 0.07 | |
| D(357) | 77 | 1 | 0.22 | |
| D(585) | 75 | 1 | 0.25 | |
| E(320) | 73 | 1 | 0.15 | |
| F(556) | 74 | 1 | 0.10 | |
| G(453) | 75 | 0 | 0.10 | |

[a] All of the strains used were $R_2$ and this prefix has been omitted from the Table. The MOI for each mutant was 10 PFU/cell.
[b] Plaques derived from 200 infected cells.
[c] Plaques derived from 200 infected cells plated in the infectious center assay, kept at 31 C for 30 h and then raised to 39 C until the plaques developed (9).
[d] Plaques derived from 32,200 or 92,200 infected cells as shown in Table 2.
[e] Complementation level as defined in the legend to Table 2.

To determine the nature of the viral population formed through complementation of reovirus mutants, plaques were picked at random from the 39 C infectious center assays of several crosses. Twenty-four plaques were taken from an A × B cross, 10 each from the C × D and B × C crosses, the resulting viral populations were plaque titered at 39 and 31 C, and the relative EOPs were calculated. Compared with an EOP of 0.8 for $ts^+$ virus (7) the EOPs of the viral samples varied from 0.3 to $10^{-3}$ for each of the three crosses. Of the total of 44 plaques picked, five had an EOP greater than 0.1 and it was inferred that these contained between 10 to 30% of $ts^+$ virus: the remaining plaque samples were predominantly but not exclusively *ts*. This inference was examined in the following way. Virus from one plaque formed by an infectious center at 39 C in the C × D cross had an EOP of 0.3, that from another plaque had $8 \times 10^{-2}$, and the distribution of $ts^+$ and *ts* virus in each sample was determined. Both samples were titered at 31 C, 10 plaques were picked from each assay and titered at 31 and 39 C. For the sample with EOP 0.3, four out of 10 plaques were $ts^+$ and the remainder were *ts*. For the sample with EOP $8 \times 10^{-2}$, all 10 plaques were *ts*. Thus the EOP of virus from the 39 C plaque of an infectious center gives an estimate of the relative amounts of $ts^+$ and *ts* viruses in the plaque.

These results are interpreted to mean that complementation is the primary genetic interaction between reovirus mutants that leads a mixedly infected cell to form a plaque at 39 C. If the two mutants had first to undergo recombination before replication all plaques should contain largely if not exclusively $ts^+$ virus. For unknown reasons complementation is very in-

efficient in this system and occurs in less than 1% of mixedly infected cells. Once an infectious center initiates a plaque at 39 C, however, $ts^+$ recombinants would be expected to arise and the ratio of $ts^+/ts$ virus in the plaque, the EOP of the virus, is a reflection of whether recombination occurs early or late during development of the plaque. We conclude that complementation between these *ts* mutants of reovirus can be readily measured by the infectious center assay and that the complementation groups so far determined here correspond to the groups established through the measurement of recombination frequencies (1, 2).

The infectious center assay provides a rather simple means for detecting complementation between *ts* mutants of reovirus. It involves the single operation of plating infected cells compared with the numerous operations and plaque titrations required for the standard complementation test (7) and is thus more sensitive than the standard test. The test might well be extended to use with other virus systems where complementation is inefficient. One of the important features of the method is that recombination can also be determined in the same mixedly infected culture used for the complementation assay by the simple expedient of measuring infectious centers under a different set of temperature conditions. Although recombination frequencies cannot be measured by this method it is clear that in the reovirus system recombination occurs in virtually all mixedly infected cells or it does not occur at all. The method therefore provides an efficient short-cut to the conventional methods for classifying mutants into recombination and complementation groups.

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