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Defective Particles in BHK Cells Infected with Temperature-Sensitive Mutants of Vesicular Stomatitis Virus

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Defective particles were the major product after undiluted passage of certain temperature-sensitive (*ts*) mutants of the Indiana C strain of vesicular stomatitis virus in BHK-21 cells at the permissive temperature (31 C). Essentially homogeneous preparations of defective particles were obtained with the wild-type and individual *ts* mutants. The defective particles associated with some of the *ts* mutants, however, were morphologically and physically distinguishable from wild type and from each other. All varieties of defective particle interfered with the multiplication of mutant and wild-type virus at the permissive temperature at early times of infection but failed to complement virions of different complementation groups at the restrictive temperature (39 C) at any time during infection.

The role of defective particles in virus multiplication is poorly understood in spite of their relatively frequent occurrence (8). Their ability to interfere specifically with the multiplication of the homologous virion suggests that they may be important factors in the regulation of virus synthesis. Regulation could be achieved by the preferential expression of certain parts of the viral genome, which would be incorporated in the defective particle. The observation that the nucleic acid of defective particles is smaller than the virion nucleic acid would be consistent with this interpretation.

Although many animal viruses produce defective particles, the T (or truncated) particles of vesicular stomatitis virus (VSV) have been extensively studied with respect to their biological and physical properties (2, 3, 5, 6, 8–11).

The recent availability of temperature-sensitive (*ts*) mutants in our laboratory (16) stimulated a new approach to the study of the defective interfering T particles and resulted in the discovery of an unexpected phenotypic effect of these mutants at the permissive temperature. Our initial findings are the subject of this communication.

MATERIALS AND METHODS

Preparation of defective T particles from ts mutants and wild-type virus. The *ts* mutants were originally derived from the Indiana C strain of VSV and were propagated in BHK-21 clone 13 cells (13). Their origin and classification into four complementation groups have been described previously (16, 17). All of the *ts* mutants and the wild type (ts^+) were propagated at the permissive temperature of 31 C, whereas the wild type was also grown at 37 and 39 C, temperatures which are restrictive for the mutants. Defective T particles were obtained by three undiluted passages in monolayer cultures of 4×10^8 cells in rotating Burrler bottles (7) containing 80 ml of Eagle's medium supplemented with 10% calf serum. When cell destruction was complete, the contents of the Burrler bottles were frozen and thawed, and the cell debris was removed by centrifugation at 3,000 × *g* for 10 min. The supernatant fluid was then centrifuged at 40,000 × *g* for 90 min. The pellets were suspended overnight at 4 C in tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.2; 0.12 M Tris-acetate, 0.06 M sodium acetate, 0.003 M ethylenediaminetetraacetic acid), and the particles were purified on a sucrose gradient [0 to 40% (w/v)] sucrose in Tris buffer by centrifugation in a Beckman SW 25 rotor at 20,000 rev/min for 90 min. The T-particle zone was removed by side puncture of the centrifuge tube, and the sucrose was removed by dialysis against Tris buffer.

Electron microscopy. The dimensions of the T particles of the various *ts* mutants were measured from negatively stained preparations photographed at × 40,000 magnification in a Siemens Elmiskop IA electron microscope and further magnified × 4 photographically. Ammonium molybdate (2%) was used as the negative stain. Measurements of *ts* 12 T particles are from preparations pretreated with 2% glutaraldehyde in Tris buffer, as unfixed preparations

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showed severe distortion of the outer membrane. Standard deviations are based on measurements of at least 40 particles. All measurements exclude the particle fringe.

Preparation of radioactive T particles and their RNA. The T particles and their RNA were prepared from infected Burrler bottles to which ^{3}H-labeled uridine or ^{32}P-orthophosphate (200 μCi per bottle) was added immediately after virus adsorption for 30 min at 31 C. Both radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks, England. In the case of labeling with ^{32}P, a phosphate-free Eagle's medium containing 10% calf serum was used. The T particles were isolated and purified as described above. The ribonucleic acid (RNA) was isolated by disrupting the T particle with sodium dodecyl sulfate (1%) followed immediately by the addition of phenol-cresol (12). After vigorous shaking for 5 to 10 min on a Vortex homogenizer, the phenol-cresol was extracted with ether and the latter was blown off in a stream of nitrogen.

Determination of sedimentation coefficients. Solutions of purified T particles (0.3 to 1.0 mg/ml) in 0.12 M Tris buffer (*p*H 7.2) were centrifuged in the Beckman model E analytical ultracentrifuge at either 17,250 rev/min (large T particles) or at 20,410 rev/min (small T particle). Photographs were taken at 2- or 4-min intervals with the schlieren optical system. The sedimentation coefficients were calculated in the usual manner; no measurements were made for possible concentration dependency corrections.

Sedimentation coefficients of radioactively labeled RNA were measured by centrifugation in 5 to 20% sucrose gradients in Tris buffer with ^{32}P-labeled T7 phage deoxyribonucleic acid (DNA; 32*S*) as a marker (19). The latter was a gift from D. A. Ritchie. The location of the RNA was established by collecting drops on glass-fiber filters which, after evaporation at 50 C, were suspended in a toluene-2,5-diphenyloxazole (PPO)-1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) liquid scintillator and counted in an Intertechnique liquid scintillation counter.

Polyacrylamide gel electrophoresis. The electrophoresis was performed as described in reference 1. The gels were frozen in dry ice and cut into 1-mm slices (The Mickle Laboratory Engineering Co. Gel-Slicer, Gomshall Surrey, England). The slices were placed on glass-fiber filters soaked with 10% NH_4OH and kept overnight in a closed chamber saturated with 10% NH_4OH. The membranes were then dried at 50 C, dropped into vials containing 10 ml of toluene-PPO-POPOP liquid scintillation fluid, and counted in the Intertechnique liquid scintillation counter (model ABAC SL40).

Interference. The ability of the mutant T particle to interfere with the replication of the VSV virion was examined and compared with that of the wild-type T particle. In these experiments, BHK cells were exposed to wild-type or *ts* mutant virion at a multiplicity of infection of 1 or 10 plaque-forming units (PFU)/cell and subsequently superinfected with the various T-particle preparations. These preparations of purified T particles contained approximately 10^{11} particles/ml (determined with the electron microscope) and were used at dilutions of 1:10, 1:100, and 1:1,000, corresponding to a multiplicity of infection of 500, 50, and 5 particles per cell. The experiments were performed at 31 and at 39 C in the case of combinations of wild-type virion and *ts* mutant T particles. Monolayers of 5×10^6 BHK cells in 30-ml bottles were infected with 1 or 10 PFU/cell of purified virion and absorbed at 31 C. The superinfecting T particles were added after 20 min and allowed to adsorb at 31 C for a further 30 min. The monolayers were washed with three changes of medium and then incubated at either 31 or 39 C totally immersed in a temperature-controlled water bath. Incubation was terminated after 8 hr, and the whole culture was rapidly frozen. The samples were stored at −20 C until total infection was assayed by plaque counting.

Complementation. Complementation between different *ts* mutants of VSV can be detected efficiently by comparing the yield obtained from mixed infections at the restrictive temperature with the sum of the yields from the self infections, corrected for input multiplicity (16, 17). The possibility of complementation between virions and T particles of different *ts* mutants was investigated in a like manner. Monolayers of 5×10^6 BHK-21 cells in 30-ml bottles were infected with approximately 10 PFU/cell of an appropriate purified virion preparation. The monolayers were held at room temperature for 30 min for adsorption, before total immersion in a precision water bath at 39 C. Pairs of bottles were superinfected at 0, 60, 120, and 180 min after adsorption with defective T particles (500 particles/cell) from a complementing *ts* mutant and immediately returned to 39 C. At 240 min after adsorption, all of the monolayers were washed with three changes of warm medium and again incubated at 39 C totally immersed. At 10 hr, the cultures were rapidly frozen and stored at −20 C until total infectivity was assayed by plaque counting. This gives the mixed infection yield. The self-infection yields were obtained from a virion only and a T particle only control series which underwent the same manipulations simultaneously, substituting buffer solution for the omitted T particle or virion as appropriate. Complementation is indicated if the mixed infection yield is significantly greater than the sum of the yields of two control series.

RESULTS

Physical properties of VSV T particles and their RNA. In the course of purifying the T particles of different *ts* mutants, it was observed that the position of the T-particle zone in sucrose gradients relative to the virion zone exhibited some variation, suggesting differences in T-particle size. Electron micrographs of the characteristic wild-type (ts^+) virion and T particle, together with T particles of mutants *ts* 11, *ts* 12, and *ts* 31, are shown in Fig. 1. Estimates of the dimensions and sedimentation coefficients of the T particles are given in Table 1. The sedimentation coefficients are average values, since in all cases the schlieren peaks, although well-defined through-

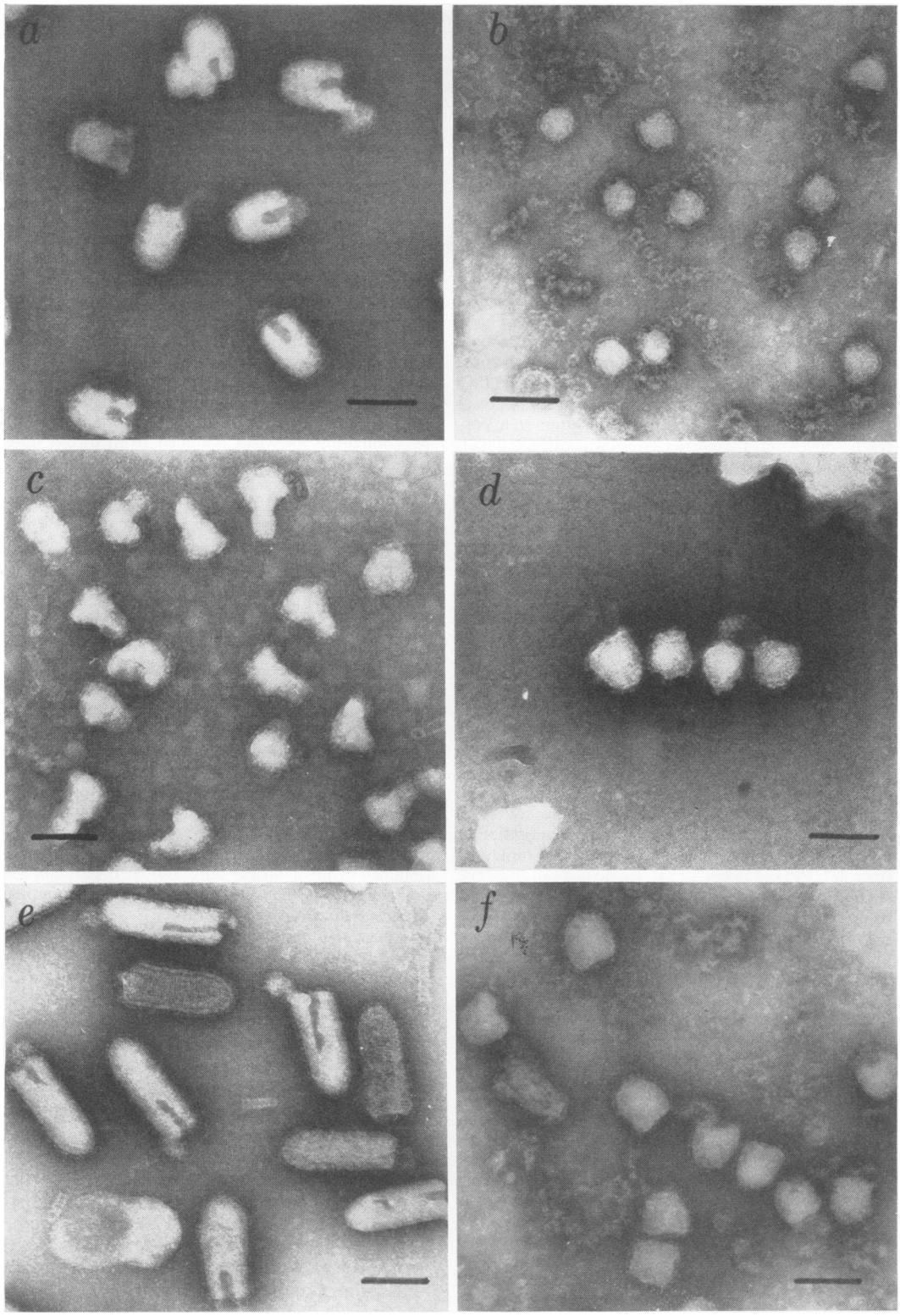

FIG. 1. *Electron micrographs of negatively stained preparations of T and B particles. The staining and fixing procedure was as described in Materials and Methods. (a) ts 11 T particle; (b) ts 31 T particle; (c) ts 12 T particle unfixed; (d) ts 12 T particle fixed; (e) wild-type virion (B particle); (f) wild-type T particle. Bar = 100 nm. The ts 41 T particle and the ts 22 T particle are not shown because of their identical appearance with the wild-type and ts 11 T particles, respectively.*

TABLE 1. *Dimensions of the T particles of various ts mutants and the sedimentation coefficients of the T particles and their RNA*

Complementation group	Mutant	Length (nm)	Width (nm)	Sedimentation coefficient (S)	
				Particle	RNA
I	*ts* 11 T.	98.0±6	73.0±4	410	28
I	*ts* 12 T		68.0±4	280	
II	*ts* 22 T	99.0±4	69.0±4	435	
III	*ts* 31 T		50.0±3	150	15
	ts$^+$ T	79.0±5	72.0±4	330	19
	ts$^+$ B	172.0±7	68.0±4	610	

out the entire run, exhibited some broadening probably due to slight heterogeneity. The sedimentation coefficient obtained with the wild-type T particle is in agreement with the original estimate (330S) reported by Bradish, Brooksby, and Dillon (2) for the T particle of this strain. The physical properties of the T particles of the wild type were not affected by the temperature of multiplication (31, 37, or 39 C) and are similar to those reported in the literature (2, 3, 5, 6, 8–11). Furthermore, identical results were obtained with three other preparations of wild-type virus derived from single-plaque isolates on different occasions.

The longer T particles of *ts* 11 (complementation group I) and the very short T particles of *ts* 31 (complementation group III) were also reproducibly prepared from two separate plaque isolates in each case. The T particles of mutant *ts* 12 (complementation group I) were unstable, and the measurements were obtained from preparations prefixed with 2% gluteraldehyde. Therefore, the differences between the physical properties of the *ts* 12 T particle and the wild type, although slight, are probably real. No distinction could be made, however, between the longer T particles of mutants *ts* 11 and *ts* 22 (complementation group II). The properties of these T particles and of their RNA are similar to those of the long T particle isolated from a heat-resistant variant of an Indiana strain by Petric and Prevec (14). The defective T particles associated with mutant *ts* 41 (complementation group IV) are apparently identical to the wild type and these are not illustrated.

RNA from *ts* 31 T particle was difficult to separate from wild-type T-particle RNA when co-centrifuged in a 5 to 20% sucrose gradient. This was probably due to some heterogeneity of the two preparations and their relative proximity on the gradients. The sedimentation coefficient of *ts* 31 T-particle RNA was consistently lower when determined in separate centrifugation runs relaitve to marker RNA (Table 1). Also *ts* 31 T-particle RNA and wild-type T-particle RNA had different electrophoretic mobilities in 2.4% polyacrylamide gel electrophoresis. The relative positions of the T-particle RNA species are shown in Fig. 2a (sucrose gradient) and 2b (gel). The shoulder on the left side of the *ts* 31 T-particle RNA in Fig. 2b is probably due to some contamination with higher-molecular-weight RNA from cores.

Interference. All of the T particles were found to have interfering activity, and the results obtained were substantially similar whether the virion input was 1 or 10 PFU/cell. Table 2 contains the data for the morphologically extreme particles obtained from mutants *ts* 11 and *ts* 31 at a virion input of 10 PFU/cell. Pronounced interference was observed at both 31 and 39 C and was dependent on the concentration of the superinfecting T particle. Interference was more pronounced at 39 C, due in part to greater resolution achieved as a result of the temperature sensitivity of any *ts* mutant virion contaminating the T-particle preparations. Additional control experiments were performed in which wild-type infected cells (10 PFU/cell) were superinfected with *ts* 31 virion at a multiplicity of 500 particles per cell. No interference was observed, confirming that T-particle-mediated interference is a specific effect and not a result of high multiplicity of infection.

Failure to obtain genetic complementation. Preliminary attempts to rescue *ts* mutant virions by co-infection with T-particle preparations had given negative results (17). These experiments have been extended to ensure that optimal conditions were obtained for the synthesis of T-particle RNA and for the expression of any function encoded in it. In these experiments, the T particles (all of which had been shown to be biologically active in the sense that they were capable of inducing auto-interference) were added at various intervals after infection. Table 3 summarizes data obtained with *ts* 11 and *ts* 31, mutants presumed to affect "early" and "late" functions, respectively (C. R. Pringle, W. H. Wunner, and I. B. Duncan, *in preparation*), and therefore a favorable combination. However, no significant complementation was observed. The reciprocal experiment in which *ts* 11 virions were added at various intervals to cells preinfected with *ts* 31 T particles also yielded no evidence of complementation. Other combinations of mutants involving all of the complementation groups (*ts* 12 virion plus *ts* 31 particle; *ts* 22 virion plus *ts* 11, *ts* 31, and *ts* 41 T particles; *ts* 31 virion and *ts* 22 T particle; *ts* 41 virion plus

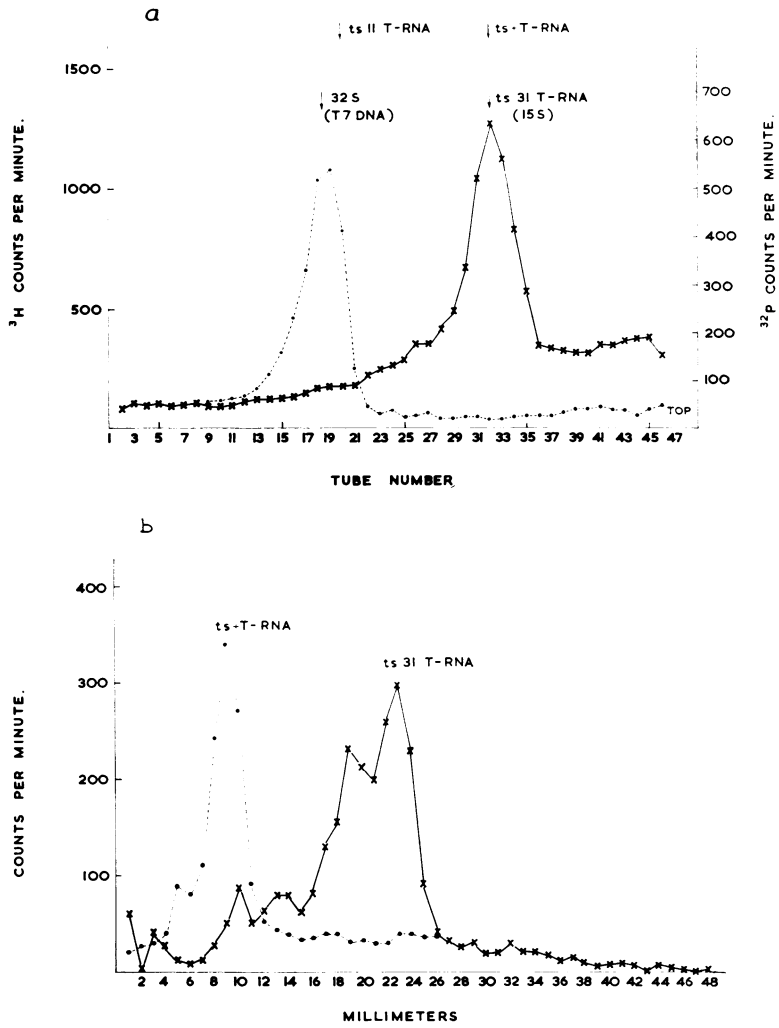

FIG. 2. *(a) Relative position of ts 31 T-particle RNA in a 5 to 20% (w/v) sucrose gradient in 0.12 M NaCl and 0.05 M Tris-acetate buffer (pH 7.2) after 150 min of centrifugation in a Beckman SW 20 rotor at 41,000 rev/min. Phage T 7 DNA, labeled with ^{32}P, was used as a marker (32S). Arrows indicate the relative positions of the wild-type T-particle RNA and ts 11 T-particle RNA centrifuged on separate gradients. (b) Relative position of ts 31 T-particle RNA in 2.4% polyacrylamide gel after electrophoresis at 4 ma for 3 hr in E buffer (1). Wild-type T-particle RNA, labeled with ^{32}P, was co-electrophoresed with ^{3}H-uridine-labeled ts 31 T-particle RNA to illustrate the difference in size.*

ts 31 T particle; and *ts* 11, *ts* 12, *ts* 111, *ts* 31, *ts* 33, and *ts* 41 virions plus wild-type T particles) were tested in like manner, but no evidence of complementation was obtained.

DISCUSSION

The results presented in this communication suggest that the type of T particle found in VSV-infected cells may in some way be a function of the viral genome. The data do not exclude the possibility, however, that the various T particles found in *ts* mutant infections are all present in very small quantities in the wild-type infection and that the mutant virion influenced only the relative frequencies of their occurrence. The detection of a low level of T-particle heterogeneity would be extremely difficult. For example, in their studies of viral RNA synthesis, Stampfer et al. (18) reported that the RNA from infected cells has a bimodal distribution in the region 15 and 19*S*. This would effectively mask the presence of small amounts of the *ts* 31 T particle

15S RNA. Similarly, small amounts of the long *ts* 11 T-particle 28S RNA would be obscured by the 28S RNA component found by these authors. We have attempted to detect these particles in the wild-type infection by electron microscopic examination of fractions from sucrose gradients. However, the presence of ribonucleoprotein cores with physicochemical properties similar to the small *ts* 31 particle obscured the possible presence of small quantities of this particle, and the occurrence of partially damaged B particles interfered with the search for small amounts of the long *ts* 11 T particles.

TABLE 2. *Interfering ability of wild-type ts 11 and ts 31 T particles*

Superinfecting T particles	Per cent yield at 39 C[a]	Per cent yield at 31 C[a]		
	ts+ virion	*ts*+ virion	*ts* 11 virion	*ts* 31 virion
ts+				
1:10	0.3	3.1	3.0	3.5
ts 11				
1:10	0.4	5.5	5.7	2.0
1:100	1.7	21.0	25.1	22.0
1:1000	29.3	70.9	66.6	88.0
ts 31				
1:10	2.1	2.3	7.6	3.4
1:100	10.7	34.1	20.0	43.2
1:100	30.8	82.6	79.9	76.2

[a] Interference was measured as $(yV + T - yT) \times 100/yV$, where $yV + T$ is the yield obtained from cells infected with both virion and T particle, yT is the yield from cells infected with T particle alone, and yV is the yield from cells infected with virion alone.

The possibility that the T particles were being selected from an initially heterogeneous T-particle population via single-plaque isolations was eliminated by the reproducible production of the characteristic T particle by single-plaque isolates of the same mutant. Moreover, the properties of the characteristic T particle associated with the wild-type virion which had been propagated from single-plaque isolates were similar to those reported in several laboratories. Indeed, there have been no reports of the presence of T particles in wild-type infections with physical properties similar to those associated with some of these *ts*- mutants.

The investigation of a relationship between defective T particles derived from *ts* mutants belonging to the same complementation group yielded negative results. The data in Table 1 show that *ts* 11 and *ts* 12, both belonging to complementation group I, produced different defective T particles, whereas the T particle of *ts* 41 (complementation group IV) was indistinguishable from the wild type. However, mutant *ts* 33, belonging to group III, did give rise to the small T particle obtained with *ts* 31 (also group III). There is thus no simple correlation between the type of defective T particle produced by a mutant and its complementation group.

The ability of all of the defective T particles isolated from *ts* mutants to interfere with the replication of wild-type virion was in marked contrast to the failure of defective T particles obtained from serologically different strains to interfere with the heterologous particle (15). This can be attributed to the direct mutational origin of the *ts* mutants from the parental wild type. The ability of these defective T particles to

TABLE 3. *Absence of genetic complementation between defective and infective particles of complementing ts mutants*[a]

Conditions of infection	Time of addition of superinfecting T particles (min after adsorption)[b]			
	0	60	120	180
ts 11 virion alone (10 PFU/cell)	3.7×10^3	4×10^3	6.5×10^3	10^4
ts 31 T particle (500 particles/cell) plus *ts* 11 virion (10 PFU/cell)	8×10^3	10^3	2.1×10^4	1.6×10^4
ts 31 T particle (500 particles/cell) alone	7×10^1	4×10^1	1.5×10^1	$<10^1$
ts 31 virion alone (10 PFU/cell)	2.6×10^3	2.7×10^4	1.4×10^4	3.5×10^3
ts 11 T particle (500 particles/cell) plus *ts* 31 virion (10 PFU/cell)	6×10^2	10^3	1.2×10^3	2.1×10^3
ts 11 T particle (500 particles/cell) alone	$<10^1$	$<10^1$	$<10^1$	$<10^1$

[a] Values are the mean yields from duplicate cultures at 10 hr and 39 C.

[b] First three lines of data refer to *ts* 31 T particles. Last three lines refer to *ts* 11 T particles.

interfere with the heterotypic New Jersey strain is now being tested.

Although we expected the long *ts* 11 and *ts* 22 T particles to have a greater probability than the short T particles to contain those parts of the viral genome coding for the defective functions, the complementation experiments were all negative. These results may have been due to the absence of the pertinent part of the genome in all of the combinations used. Alternatively, the RNA in defective T particles may correspond to the strand which is not replicated and whose genetic information is not translated. In this connection, we are investigating the multiplication of T particles in cells co-infected with wild-type virion and mutant T particles at the non-permissive temperature.

The long T particle isolated by Petric and Prevec (14) was reported to interfere with the heterologous virion (15). However, the multiplication of these T particles by the heterologous virion would be difficult to examine because of the possible contamination of the T particle with homologous virion. The availability of the *ts* 11 and *ts* 22 long T particles allows a critical test of this possibility, because any contaminating virion can be made temperature-sensitive by incubation at 39 C.

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